神経生理学的検査からみる発達

MEGを用いた小児の発達の解析

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MEG：magnetoencephalogram（脳磁図）

＊1
中心・側頭部に棘波焦点を有する良性小児てんかん．

BRE：benign rolandic epilepsy

良性Rolandてんかんとは

- 良性Rolandてんかん[*1]（BRE）は❶の特徴を有する小児期特有のてんかん症候群である．
- この症候群は年齢依存性に脳波異常や発作の発現，消失がみられ，とくに感覚運動皮質の発達上の変容が基盤にあると考えられる．
- この特異な症候群の脳波変化と発達特性の解析はてんかんと発達を考察する際に重要な知見を提供する．
- 「良性」と冠されることから無治療経過観察も可能な場合があるが，その定義は以下のような臨床脳波学的定義であり，不均一な症候群と考えられる．

睡眠とRDの関係

RD：rolandic discharges

REM：rapid eye movement

- 終夜脳波の観察によると，Roland発射（RD，❷）は覚醒時では認めないこともあるが，non-REM睡眠で増加，REM睡眠で激減する（❸）．
- とくにREM期から浅睡眠（睡眠第Ⅰ，Ⅱ段階）へ向かう時期で急増し，深睡眠（睡眠第Ⅲ～Ⅳ段階）からREM期に向かう時期に急減する．
- REM期は脳幹コリン作動系の活性化とセロトニン作動系，ノルアドレナリン作動系の抑制により形成される．
- これに対しnon-REM期には脳幹コリン作動系は抑制される．
- RDの出現頻度と脳幹コリン作動系の活性は逆相関するといえる．

RDの電流源

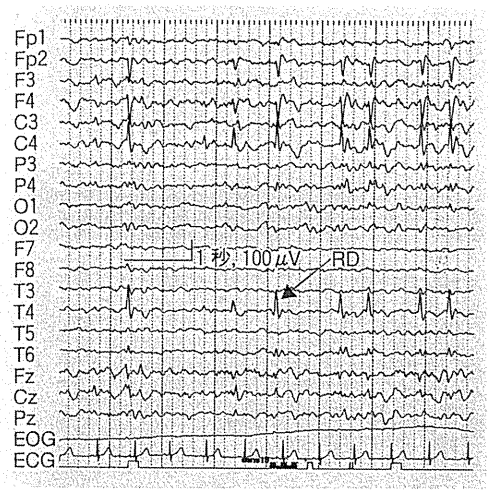
- MEGによる検索では，典型的BREのRD電流源は中心溝周囲の口腔顔面の一次感覚運動皮質にある[2]（❹）．
- この局在はSylvius発作の起始部と考えられる[*2]．

＊2
このことは口唇刺激による誘発磁場とRDの頂点の局在解析から初めて確かめられた[3]．

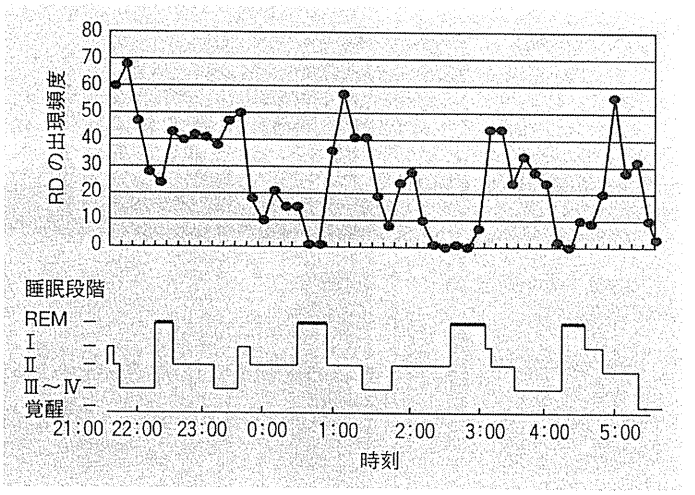
❶ 良性Rolandてんかんの特徴

発症年齢	3～12歳（平均7歳）．
典型的発作型	寝がけや起きがけの片側口腔顔面の運動発作，唾液分泌過剰，発語停止（これらをSylvius発作と呼ぶ）などの単純部分発作，上肢の運動発作，および二次性全般化．
脳波	中心側頭部のRD（❷）．右中心側頭部に高振幅陰性鋭波を認めるが，時に小さい陽性棘波が先行する．また前頭部には高振幅陰性鋭波に対応した陽性鋭波を認める．頭頂部にまで及ぶことや律動化や全般化することもある．
経過	発作コントロールは良好で，10歳前後での発作の消失が先行し，その後RDは遅くとも14～15歳までには消失する．急性期に空間認知の異常，注意集中困難，学習障害などを認めることがある．
病理	中枢神経系に器質的異常を認めず．
原因	不明であるが，患者の同胞の脳波検査の解析からRD自体が常染色体優性遺伝で伝わり，その中のごく一部（10%以下）が発作を発現することが示唆され，遺伝的要因が強いと考えられる[1]．男児優位であることも遺伝的要因が単純ではないことを示唆している．

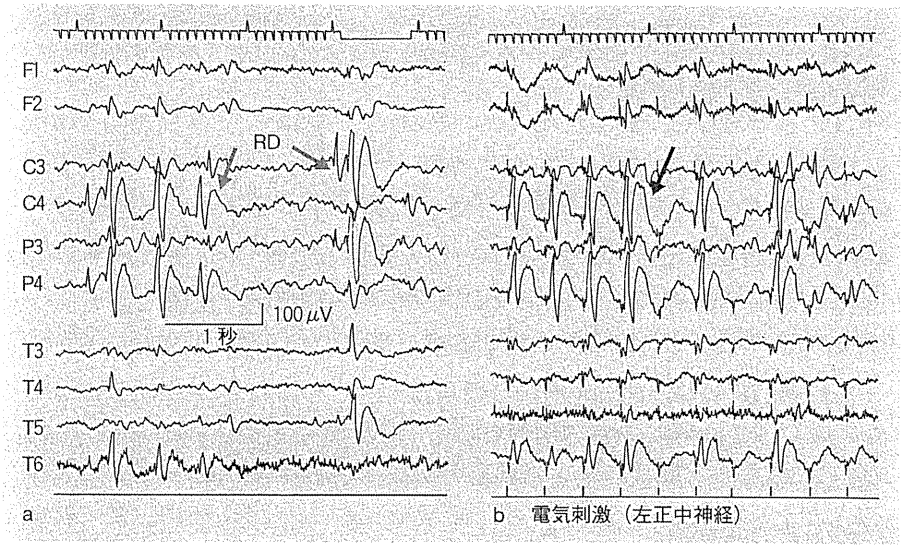
❷ BRE 7歳男児の睡眠脳波



❸ 睡眠段階とRDの出現頻度

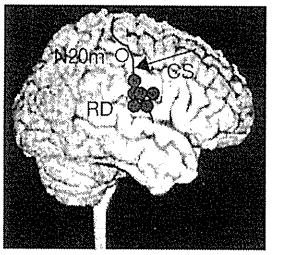


❺ 左正中神経の電気刺激でC4，P4に出現したRD（a）と類似した波形（b. ⟶）



a：通常の脳波でのRD（⟶），b：左正中神経刺激でのRD類似波形（C4，P4）

❹ N20mとRDの位置



N20m（○）は中心溝（CS）後方の一次感覚皮質の場所を示し，RDの電流源はその下方，口腔顔面の一次感覚運動皮質に存在する（●）．

CS：central sulcus

- 症例によっては，手の一次感覚運動皮質や二次感覚皮質に局在が広く分布して認められる．

巨大誘発電位を有する症例

- ときにBREの患者で正中神経の電気刺激で反対側頭頂部にRDと類似の波形（巨大SEP*3）を認める[4]（❺b）が，形態および頂点間潜時からこれらは同一の起源をもつと考えられる．
- この巨大誘発電位の起源や変化を探ることでRDの起源や発達特性に接近することができる*4．
- これらの患者でRDおよび正中神経刺激による反応を，脳波脳磁図同時記録したものが❻である．
- 自発脳波およびMEGにおけるRDと体性感覚誘発電位および磁場（SEP，SEF*3）は形態，頂点間潜時ともに対応していることがわかる．

*3
巨大SEP
体性感覚誘発電位（SEP）は正中神経の電気刺激で頭皮上脳波を加算平均して得られる電位．同様にして得られる磁場を体性感覚誘発磁場（SEF）という．皮質の過興奮性により反応が巨大化したものを巨大（giant）SEP（SEF）という．正中神経刺激での巨大SEPは手の感覚処理過程にかかわる皮質の過興奮性を示唆する．

SEP：somatosensory evoked potentials

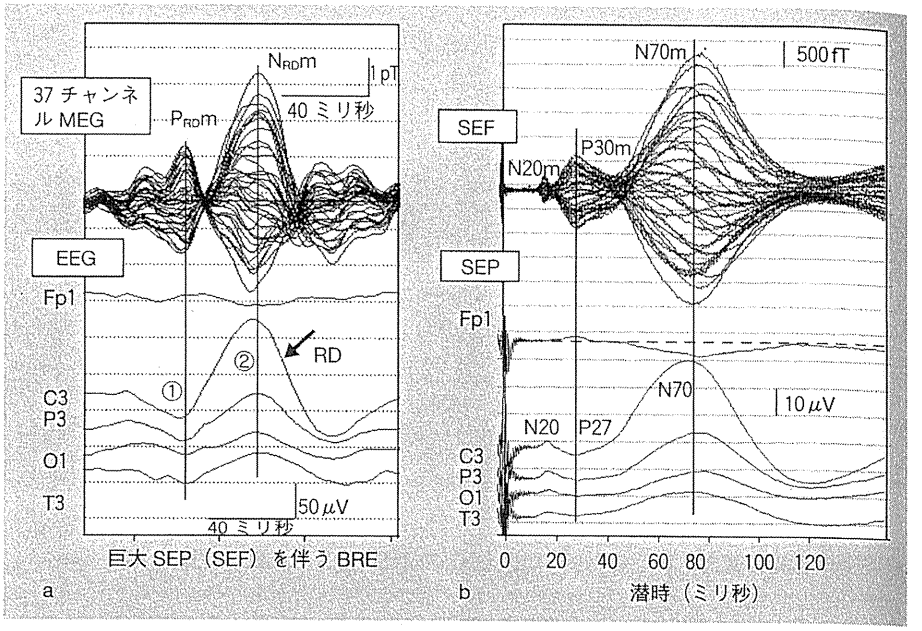
SEF：somatosensory evoked fields

*4
すでに1970年代からDe MarcoがTESとSESEの解析を行い，先駆的な業績をあげている[5]．

TES：tactile evoked spikes

SESE：somatosensory evoked spikes epilepsy

❻ RD および体性感覚誘発反応（正中神経）の脳波脳磁図同時記録



a：左側 RD の MEG と EEG，b：右正中神経刺激による SEF と SEP.

EEG：electroencephalogram（脳波）

＊5
P30m
m：magnetic

＊6
Lafora 病，ガラクトシアリドーシス，セロイドリポフスチン病など．

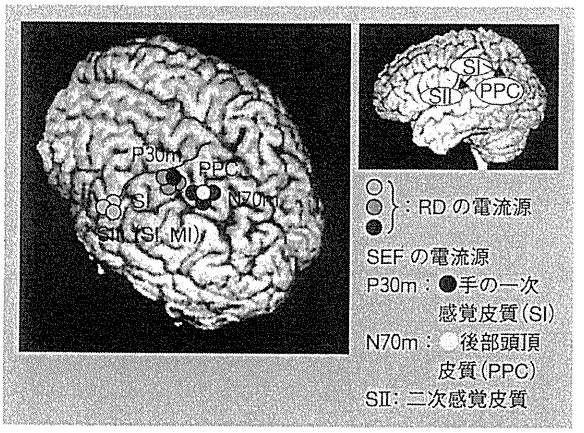
PPC：posterior parietal cortex

＊7
一部の症例では N70m 電流源は二次感覚皮質（SII）に局在した．

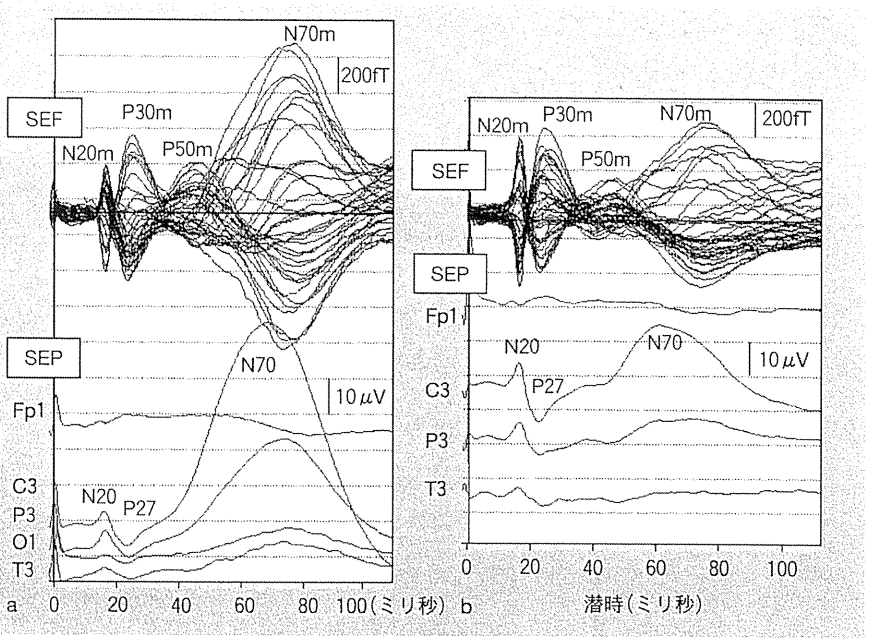
＊8
ascending sequential maturation.

- RD の初期陽性波（❻①）は P30（P30m[*5]）に対応し，陰性鋭波（❻②）は巨大中潜時成分 N70（N70m）に対応する．
- 皮質反射性ミオクローヌスを有する進行性ミオクローヌスてんかん[*6] の患者では巨大 SEP が出現するが，巨大化する成分は SEP における初期（P25-N33）成分であり，BRE で巨大化する中潜時成分とは異なる．
- それぞれの頂点の電流源を求めてみると❼のようになり，P30m は手の一次感覚皮質（SI），N70m は後部頭頂皮質（PPC，おそらく Brodmann の area 5, 7）に局在し，RD の初期陽性波と陰性鋭波の電流源は P30m，N70m の電流源に重畳した．
- RD と正中神経電気刺激による SEP，SEF は，波形，空間的分布，頂点の時間的関係において対応することがわかる．
- 正中神経刺激による巨大 SEP の有無で BRE を 2 群に分けると，巨大 SEP を有する群は P30m，N70m の電流源強度が有意に大きいものであった．
- これに対し，初期皮質反応である N20m は電流源強度に有意差はなかった．
- このことから，RD の発生の要因は視床から手の一次感覚皮質（SI）へ到達以降の感覚情報処理過程（P30m-N70m の巨大化），とくに中潜時成分の巨大化にあると思われる[*7]．
- 高次な機能ほど成熟に時間を要する[*8] とすると，SII，PPC における反応の巨大化は感覚処理過程における SII，PPC の成熟遅延と思われる．
- 遅れた成熟がくるころに，けいれん源性は終息する．
- ❽に示すように，発作のあった 9 歳時に比較す

❼ 巨大 SEP を有する BRE の RD 電流源と SEF 電流源



❽ 巨大 SEP を有する BRE の 9 歳(a)および 11 歳(b)の SEP(SEF)右正中神経刺激



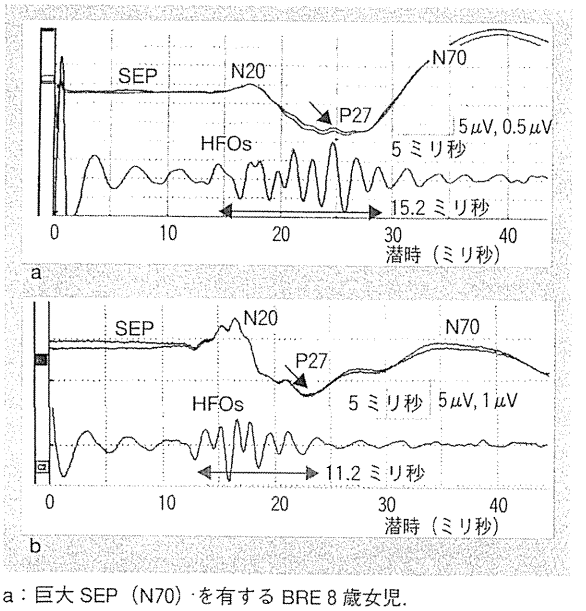
ると，発作消失後の 11 歳時での N70（N70m）は振幅，電流源強度がともに低下した*9.

- BRE の少なくとも一部は，高次の感覚処理過程に関連する皮質の過興奮性が発作および RD 出現に関与する.
- RD を有する者のうち 9 割以上は臨床的に発作を起こさないが，その理由は不明である.

SEP における高周波振動（HFO）

- 通常の正中神経刺激による SEP の記録に 400～800 Hz のフィルター処理を行うと，初期成分 N20，P27（N20m，P30m にほぼ等価）に重畳した約 600 Hz の高周波成分がとらえられる.
- 一般に BRE と他の小児期発症てんかん症候群で比較すると，BRE のほうが 600 Hz HFO の持続が長いことが知られている[6].
- 先述の巨大 SEP（巨大 N70）を有する BRE では，それをもたない BRE に比してさらに振幅が大きく潜時の長い 600 Hz HFO を認める（❾）.
- 後者では N20 にほぼ重畳するが，前者では P27 にまで及ぶ.
- 同様の 600 Hz HFO*10 持続の増加は Parkinson 病や一部の進行性ミオクローヌスてんかんで認められる.
- この高周波の生理学的意義については諸説ある

❾ 通常の正中神経刺激 SEP とフィルター処理（400～800 Hz）して得られた高周波振動（HFOs）



a：巨大 SEP（N70）を有する BRE 8 歳女児.
b：巨大 SEP（N70）をもたない BRE 8 歳男児.

*9
図示していないが脳波上の RD の振幅，出現頻度も 11 歳で減少した.

HFO：high frequency oscillation

*10
600 Hz HFO は前半（N20 に重畳）と後半（P27 に重畳）で生理学的意義が異なるが，前半は基底核機能とも関連し，後半はとくに GABA 作動系の burst を反映する.

GABA：γ-aminobutyric acid（γ-アミノ酪酸）

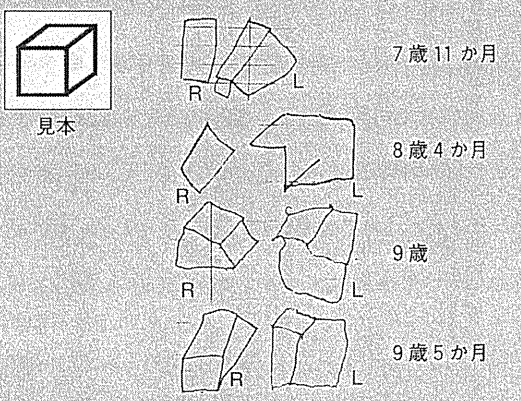
が，橋本は体性感覚3b野の4層に存在するGABA作動性抑制性介在ニューロンの活動を反映していると推定している[7]．

- GABA作動性抑制性介在ニューロンは覚醒とともにアセチルコリンやセロトニンによって賦活され，下流のグルタミン酸作動性錐体細胞の抑制を増強する．
- この600 Hz HFOは覚醒時に出現するが，non-REM睡眠中は消失する[8]．REM期にはその振幅は減衰するが，覚醒時同様認める[8]．
- 興味深いのは❸に示したようにRDはREM期や覚醒時にほぼ消失するがnon-REM期に増加し，600 Hz高周波振動とは相反した振舞いをみせることである．
- 巨大N70に関しても睡眠中の振幅は増大する．
- 以上から，正常において睡眠段階を規定するコリン作動系やセロトニン作動系のGABA系を介した錐体細胞のフィードフォワード抑制や脱抑制に未熟性があり，600 Hz高周波振動の持続延長とRDの相反性の基盤となっている可能性がある．
- 今後RDと睡眠，高周波振動，高次機能のさらに詳細な検討が必要であろう．

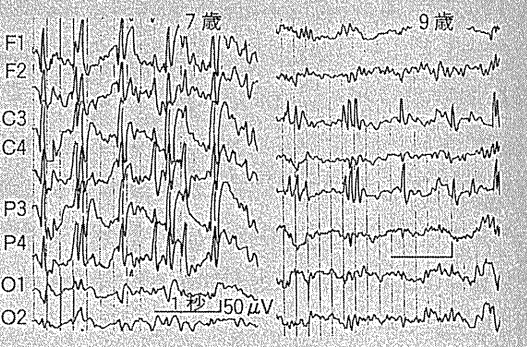
症例1 BRE

11歳，男児．右利き．6歳で典型的Sylvius発作が始まり，月に2回以上となったためバルプロ酸（VPA）の服用を開始．7歳でのWISC-Rの結果：V-IQ 101，P-IQ 112，F-IQ 107であったが，block designやobject assemblyは低得点であった．立体描写は❿のように7～8歳では拙劣であり，臨床的にはごく軽度の注意集中困難や多動を認めたが，とくに治療は要さず．⓫に示すように脳波の改善とともに立体描写は改善し（❿），ADHD様の行動も消失した．左右の半球から出現していたRDが左のみに減少した時期と臨床症状の改善が符合する．右半球のRDと空間認知の異常を示唆する報告がある[9]が，本例も右頭頂葉の機能不全が立体描写に象徴される構成機能を阻害していた可能性がある．

❿ 症例1における立体描写の推移



⓫ 症例1の脳波の変化



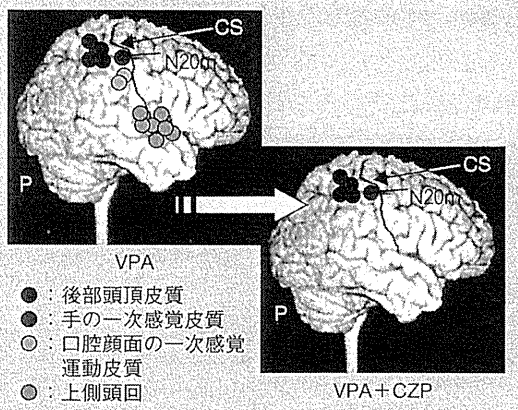
7歳時（a）両側から律動的に出ていたRDが9歳（b）になり左半球に限局した．

VPA：valproic acid
WISC-R：Wechsler intelligence scale for children-revised
ADHD：attention deficit hyperactivity disorder（注意欠陥・多動性障害）

症例 2　口舌失行を伴う Roland てんかん

10 歳，男児[10]．3 歳で典型的 Sylvius 発作が始まり，週 2 回以上となったため，カルバマゼピンの服用を開始．無効であったためバルプロ酸（VPA）に変更．発作頻度は 2 か月に 1 回に減少．その後発作はほぼ消失したが，9 歳時の神経学的所見としては口舌失行，四肢協調運動障害，流涎，不明瞭な発音を認めた．MEG による RD 電流源の解析では後部頭頂皮質，手の一次感覚皮質，口腔顔面の一次感覚運動皮質，上側頭回に広く分布する電流源を認めた（⑫）．また，ひらがなを読む事象関連磁場の解析では，右口腔顔面の一次感覚運動皮質に持続した活性を認めた（⑬）．頻発する RD と臨床症状の関連を考慮し，RD の減少をねらってクロナゼパム（CZP）を加えたところ，流涎の消失，発音の改善，口舌失行の改善を認めた．CZP 投与後の RD 電流源は後部頭頂皮質のみとなり（⑫），RD 頻度も減少した．本例は多彩な高次機能の問題を示す非典型的な Roland てんかんだが，発作以外の症状にも RD 自体が関連したと考えられ，抗てんかん薬の使用が奏効した．RD 電流源の分布と強度により Roland てんかんの症状は多彩になると考えられる．CZP 投与により RD の減少をみたことは RD の出現に GABA 作動系を介した制御が関与していることを示唆する．

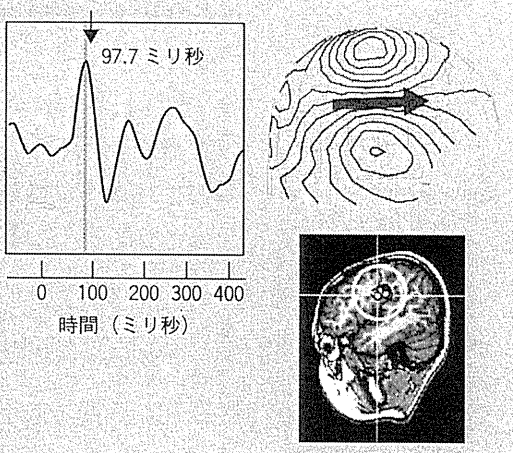
⑫ 症例 2 の CZP 追加前後の MEG 所見



広く分布していた RD 電流源が CZP 追加後，後部頭頂皮質のみに限局した．

CZP：clonazepam

⑬ ひらがなを読む事象関連磁場



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Segawa Dopa Responsive Dystonia

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Definition and Classification

Autosomal dominant (AD) GTP cyclohydrolase I (GCH-I) deficiency or Segawa disease is a dopa responsive dystonia (DRD) caused by heterozygous mutation of GCH-I gene, located on chromosome 14q22.1–q22.2. This disease was initially reported by Segawa as hereditary progressive dystonia with marked diurnal fluctuation (HPD). Prior to identification of the causative gene of HPD, this disease was called as Segawa syndrome with recessive type, which is clarified later as recessive tyrosine hydroxylase (TH) deficiency. In the 1990s, this was called as DRD. Recently, it is classified in DYT5 with recessive TH deficiency.

AD GCH-I deficiency is an autosomal dominantly inherited, generalized, postural dystonia with female predominance. The hallmarks of this disease included onset in childhood, diurnal fluctuation of symptoms, and marked and sustained response to levodopa. But some patients show action dystonia, ballistic dystonic movements, associated with postural dystonia from late childhood. AD GCH-I deficiency is characterized by the age dependency of the symptoms and clinical courses, and adult onset patients may start with tremor but without generalized dystonia or diurnal fluctuation. Other neurological conditions include psychiatric disorders, autism, depression, and migraine; furthermore, subjects with compound heterozygotes show hypotonia, failure in locomotion, and delay in mental and motor development in infancy.

Neuropathological studies revealed no degenerative changes. Neurohistochemistry shows decrease of the TH protein at the terminal of the nigrostriatal (NS)–dopamine (DA) neuron with predominance in the ventral area, which causes postural dystonia through the descending pathways of the basal ganglia. However, clinical, neurophysiological, and neuroimaging studies suggest the involvement of the NS–DA neurons projecting to the subthalamic nucleus (STN) with D_1 receptor for tremor and action dystonia. With lesions at the terminal, this disease shows diurnal fluctuation and age-related variation of symptoms.

Clinical Signs and Symptoms

Clinical symptoms are characterized by age dependency both in the initial signs and the clinical course. The ages of onset are childhood from 1 to 11 years, mostly ~6 years. The tremor disappears by passive stretching of the muscle, that is, it does not appear as cogwheel rigidity. However, there are patients who have onset in adulthood and some in ages older than fifties.

Childhood onset cases start with postural dystonia, dystonic posture of one of the lower extremities, mostly with pes equinovarus. There are patients who start with dystonia of one upper extremity at ages a little bit later. However, some patients show action dystonia, rigorous dystonic movements, besides postural dystonia. These appear later than postural dystonia from ~8 years. These are observed in neck and shoulder commonly as action retrocollis. Occurogyric crisis may associate with this. The occurrence of action dystonia is depending on the family. Postural tremor appears later in upper extremities with asymmetry, mostly after 10 years.

Adult onset patients start with hand tremor. They may show gait disturbance due to generalized mild rigidity. Some adult onset patients start with writer's cramp. Patients with onset after 50 years show tremor and generalized rigidity with bradykinesia similar to parkinsonism. These adult onset patients do not show the postural dystonia expanding to generalized dystonia. Asymmetry is a characteristic feature and is observed in dystonia, rigid hypertonus, and tremor irrespective of ages at onset.

Dystonia shows marked diurnal fluctuation, that is, aggravates toward the evening and recovers markedly or nearly completely in the morning after sleep. The fluctuation is also observed in tremor. But these fluctuations are mild or not apparent in adult onset patients.

In patients with onset in early childhood, the body length fails to grow normally with the onset of dystonia and becomes short stature in the late teen ages. This is not observed in patients with onset after adolescence. Locomotion or interlimb coordination is preserved and psychomental activities are not affected even in advanced stages.

Clinical courses are also characterized by age dependency. The postural dystonia of the lower extremity occurring in childhood expands to all limbs and develops to be generalized dystonia by the middle of the teen age and its grade progressively aggravates toward the early twenties. However, the

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progression attenuates with age, and once the age has crossed 30 years, symptoms become stational. With the attenuation of the progression, diurnal fluctuation decreases its grade and becomes not apparent in the stational stage. However, tremor, with onset in early teen, expands all limbs and trunk with age and this progression is observed until 30 years. Writer's cramp or torticollis, that is, focal or segmental dystonia, may appear in adulthood.

Neurological Examinations

Muscle stretch reflexes demonstrate a rigid hypertonus, but there is no plastic rigidity, and repeated testing will produce fluctuations in the tonus. The tremor is a high-frequency postural tremor (8–10 Hz), but a parkinsonian, resting tremor is not observed. However, adult onset patients may show resting tremor of lower frequency. These clinical signs show asymmetry, but the pattern of involvement of the sternocleidomustoideus (SCM) differs between rigidity and tremor. That is, the side predominantly affected in the SCM is contralational to that of extremities in rigidity, while it is ipsilateral in tremor. However, in adult onset cases, the side of predominance of the rigid hypertonus is ipsilateral between the SCM and the muscles of extremities. Bradykinesia or postural instability appears with advancing symptoms of dystonia. However, freezing phenomena or the *marche a petit pas* of Parkinson's disease (PD) are not seen, and locomotion is preserved throughout the course of illness. The tendon reflexes are brisk and ankle clonus may be observed, but the plantar reflexes are flexor. Although some patients exhibit sustained dorsiflexion of the toe, this is 'striatal toe sign', not elicited by plantar stimulation, and is associated with basal ganglia involvement. There are neither cerebellar signs nor sensory disturbances. Psychomental activities are preserved normally.

Clinical Variation

Besides the classical type with generalized postural dystonia, there is a type associated with action dystonia. This variation is depending on the family and suggests existence of two types, that is, postural and action dystonia type. Ages at onset also show another phenotypical variation, that is, adult onset patients show tremor, gait disturbance, focal dystonia, or parkinsonism but without generalized dystonia and diurnal fluctuation. There are patients with paroxysmal dystonia, dystonic cramp, or oculogyric crisis. These symptoms are tended to be observed in patients with action dystonia. There are also intrafamilial variations, that is, there are families with anticipation in the ages at onset, while others show identical features or marked phenotypical variation irrelevant to the generation. Some patients show autistic features, depressive state, or migraine, and others, particularly in patients with compound heterozygotes, show hypotonia, failure in locomotion, and delay in mental and motor development. These symptoms are caused by serotonergic deficiency. These association are expected because GCH-I deficiency affects the 5-hydroxytryptophan (5-HTP), the coenzyme for synthesizing serotonin (5-HT) and observed in patients with marked deficiency of GCH-I. Thus, the heterogeneity of symptoms in this disorder appears to be related to age at onset, pathophysiological differences among families, and the grade of the involvement of the serotonergic neurons.

Treatment and Prognosis

In most cases, a dose of 20 mg kg^{-1} day^{-1} of plain levodopa without decarboxylase inhibitor alleviates the symptoms completely. Some patients starting treatment with plain levodopa before 10 years tend to decrease the response after ~13 years. Older onset subjects do not always respond to plain levodopa. To these patients, levodopa with decarboxylase inhibitor with doses of 4–5 mg kg^{-1} day^{-1} alleviates the symptoms completely. In a few patients, choreic movements develop by a rapid increase of dosage or by administration of a higher dose of levodopa in the initial stage of treatment. In patients with action dystonia, action retrocollis and oculogyric crisis may be aggregated by initial doses. In patients with compound heterozygote, aggravation of dystonia by initial dosage is prominent. In these patients, the unfavorable symptoms disappear with decrease of the doses. After titration to an optimal dosage by starting with smaller doses and by slowly increasing it, levodopa shows sustained and favorable effects without side effects. Levodopa is effective in almost all patients without any relation to the age of onset and the longevity of the clinical courses, and improves short stature, if administered before puberty.

Anticholinergic drugs may have a marked and prolonged effect, but do not afford a complete relief, either clinically or polysomnographically. It does not improve the tremor. Amantadine has proven beneficial for levodopa-related chorea. Tetrahydrobiopterin (BH_4) monotherapy is not favorable, but there are a few patients who show complete remission after administration of BH_4 in addition to levodopa. In a patient with compound heterozygote, administration of BH_4 is necessary for complete recovery.

Investigations

Biochemical Studies

Cerebrospinal fluid (CSF) examinations reveal low levels of homovanillic acid (HVA). But characteristic features are marked decrease (20% of normal levels) of both biopterin and neopterin levels. Moderate reduction of these pteridine metabolites is also observed (about 30–50% of normal levels) in CSF of asymptomatic carriers.

The activity of GCH-I in the mononuclear blood cells of patients is less than 20% of those in normal individuals, while asymptomatic carriers reached 30–40% of normal levels. Phenylalanine loading tests in both child and adult patients reveal a 6-hour increase in phenylalanine levels. In addition, the phenylalanine-to-tyrosine ratios remain at elevated levels during the postloading period, while biopterin levels decline. However, this test tends to show false negative results.

Neuroimaging Studies

Magnetic resonance imaging (MRI) and computed tomography (CT) scans of the brain show no abnormalities, while positron emission tomography (PET) scanning demonstrates normal or low normal [18F] dopa uptake levels. [11C] raclopride PET shows normal activity in symptomatic subjects. [11C] *N*-spiperone PET reveals mild increase in receptor binding. There is no increase in receptor binding in follow-up PET analysis after 7 months of levodopa therapy. Childhood onset patients with 30-year untreated courses and a 59-year-old patient with onset at 58 years showed no abnormalities either in [18F] dopa or [11C] *N*-spiperone PET scan. [123I] β-CIT SPECT scanning is normal in this disease.

Neurophysiological Studies

Polysomnographies (PSGs) reveal abnormalities in the phasic components of sleep. These changes include a decrease in the number of gross movements (GMs) and twitch movements (TMs) and abnormality in the pattern of occurrence of GMs against sleep stages.

These phasic components of sleep are modulated by the basal ganglia and the NS–DA neuron; of them, the numbers of TMs during rapid eye movement (REM) sleep reflect NS–DA neuronal activity. Sleep structure, percent sleep stages, and other parameters modulated by the brain stem aminergic neurons are preserved normally.

Normally, the number of REM-associated TMs decline with age and show decremental nocturnal variation with sleep cycle. In AD GCH-I deficiency, these ages and the nocturnal variations of the TMs are preserved, but the amount of TM declines to approximately 20% of normal values. Abnormalities of the patterns of GMs differ between postural dystonia and action dystonia type and that of the latter suggests DA-D_2 receptor supersensitivity.

Evaluation of saccadic eye movements reveals abnormalities in both visually guided and memory guided saccades, and implicates involvement of the both the direct and the indirect pathways. Besides hyperactive nigrocollicular inhibition associated with slowing in both memory and visually guided saccades, disinhibition of the superior colliculi has been postulated by failure in suppression of unnecessary saccade in memory-guided task.

Supracranial magnetic stimulation was normal, showing preservation of the corticospinal tract. Paired pulse magnetic stimulation showed normal short-interval intracortical inhibition of the motor cortex in postural type AD GCH-I deficiency. This suggests that reduction of GABAergic inhibition of the thalamocortical pathway may not contribute to generation of dystonia in postural-type AD GCH-I deficiency.

Brain Pathology and Histochemistry

Neuropathological examination reveals no demonstrable changes in the substantia nigra (SN) except decrease in the melanin, particularly in the ventral tier of the pars compacta. Histochemically, DA content is reduced in the pars compacta in the SN and striatum. Similar to PD, the reduction is greater in the putamen than in the caudate nucleus, and subregionally, more in the rostral caudate and the caudal putamen. In contrast to PD, AD GCH-I deficiency shows a greater DA loss in the ventral subdivision of the rostral caudate than its dorsal counterpart, and the activity and protein content of TH is decreased only in the striatum, while it is within the normal range in the SN.

There are marked reductions of total biopterin (84%) and neopterin (62%) in the putamen, despite normal concentration of aromatic acid decarboxylase, DA transporter, and vesicular monoamine transporter. A postmortem study on an asymptomatic carrier shows modest reduction of TH protein (52%) and DA (44%), despite marked reduction of striatal biopterin (by 82%).

Molecular Biological Studies

The causative gene of AD GCH-I deficiency is the GCH-I gene located on 14q22.1–q22.2. Although more than 100 independent mutations have now been identified in the coding region of GCH-I, the loci of mutation differ across families but are identical in one family. The rate of mutant GCH-I

mRNA production against normal RNA was 28% in a patient but it was 8.3% in the asymptomatic carrier.

Molecular analysis remains unable to determine mutations of the coding region of the gene in approximately 40% of subjects with AD GCH-I deficiency. In some of these subjects, abnormalities in intron genomic deletion, a large gene deletion, an intragenic duplication or inversion of GCH-I, or mutation in as yet undefined regulatory gene modifying enzyme function are suspected.

Pathophysiology

Although the pathogenetic mechanisms for dominant inheritance are unknown, a classic dominant negative effect and destabilizing effect have been considered. The ratio of mutant/wild-type GCH-I mRNA in lymphocytes is higher in an affected individual than an unaffected heterozygote, and varies depending on the locus of the mutation. Furthermore, the ratio differed among affected individuals in some families, depending on the locus of the mutation. Thus, the degree and the pattern of inactivation of normal enzyme by mutant gene differ among the locus of mutation and may cause inter- and intrafamilial variation of the phenotype as well as the rate of penetrance.

In AD GCH-I deficiency, the TH appears to be preferentially affected when compared to tryptophan hydroxylase. This could be explained by the difference in distribution of GCH-I mRNA in DA and 5-HT neurons, the destabilization of the molecule of TH, or impairment of axonal transport. However, the difference of K_m value for TH and tryptophan hydroxylase is most probable. With heterozygotic mutant gene, the BH_4 decreases partially in AD GCH-I deficiency. Thus, TH with higher affinity to BH_4 is affected rather selectively. In molecular conditions with marked decrease of BH_4, both tryptophan hydroxylase and TH are affected, producing symptoms induced by deficiencies of the 5-HT neurons.

Complete and sustained response to levodopa, especially given the absence of morphological changes, further suggests that the lesion in AD GCH-I deficiency is restricted to the NS–DA neurons. TH activity of the NS–DA neurons shows age-related decrement and circadian oscillation in the terminals, but these age- and state-dependent variations are not observed in the SN or the perikaryon of the NS–DA neuron. The onset of symptoms in the first decade of life with diurnal fluctuation and age related the clinical course correlates to the activities of TH in the synaptic terminals of the NS–DA neurons in the caudate. Neurohistochemical studies confirm the decrease of the TH protein and its activities only at the terminal, and the PSGs suggested that the TH activities at the terminal follow the decremental age and nocturnal variation of normal individuals with low levels but without progressive decrement of the activities. These features and the results of PET scan suggest that in AD GCH-I deficiency are not a progressive or degenerative disorder and the NS–DA neurons preserve their fundamental functions.

Study of GCH-I activity in stimulated mononuclear blood cells shows age-dependent, decreasing activity in the first three decades of life. Putaminal biopterin levels increase in postnatal period, reaching a plateau at 1–13 years of age, before declining in adulthood. These results imply that pteridine metabolism has a critical period beginning early in infancy and extending to early childhood and important roles of GCH-I and BH_4 for neuronal development in the first and the second decades of life.

As for the loss of striatal TH protein with normal preservation of it in the SN, following processes are considered. BH_4 may control protein stability rather than expression. Animal experiments revealed stabilization of TH protein by co-expression of GCH-I and loss of TH protein but not of TH mRNA in the brains of BH_4-deficient mice.

In the rostral caudate in particular, the medial/ventral portions, the striosomes/patches or D_1 direct pathways are more numerous, whereas in the dorsal/lateral portions the matrix compartment is more homogenous. Thus, histochemical findings suggest that the DA loss in AD GCH-I deficiency is more prominent in the striosomes/patches compartment, the terminal for the D_1 receptor. Clinically it is suggested that the D_1 direct pathways mature earlier than the D_2 indirect pathways. Dopa-responsive growth arrest seen in children with AD GCH-I deficiency is a reflection of the tuberoinfundibular D_4 receptor involvement. The D_4 receptor belongs to the D_2 receptor family, which, however, matures early among D_2 families. Thus, the DA neuron in which the DA synthesis is modulated by pteridine metabolism might regulate DA receptors that mature early in the developmental course.

Tremor is levodopa responsive but develops independently, from symptoms of dystonia. The side predominance of tremor is identical to dystonic hypertonus in the extremities while it is contralateral in the SCM. These suggest a different pathophysiology of tremor from that of dystonia and postulate the responsible lesion in the downstream of the striatum, the DA neuron innervating to the STN with D_1 receptor. This confirms unresponsiveness of tremor to anticholinergies. For generation of the rhythmic discharge of tremor, the circuits consisted of the STN

and two globus palliduses are considered. In addition, response of the tremor to stereotactic ventrolateral (VL) thalamic nucleus thalamotomy performed in the era before levodopa suggests involvement of the ascending pathway to the VL nucleus of the thalamus. Given that ascending pathways to the thalamus develop later than the descending pathways, increasing age may be a factor for development of tremor. Further support for the D_1 receptor-mediated hypothesis stems from the PET findings revealing preservation of function of D_2 receptors, and suggesting that the striatal indirect pathway does not play a role in the generation of symptoms. The PSG findings observed in patients with action dystonia could be explained by the hypofunction of the STN.

The 5-HT neuron involves in modulation of locomotion and postural tone as well as of behavioral function. The hypotonia and failure in locomotion observed in patients with compound heterozygotes are associated with deficiency of the 5-HT-regulated activities. Preservation of interlimb coordination or locomotion in AD GCH-I deficiency without symptoms of 5-HT deficiency may depend on the preservation of the descending output of the basal ganglia to the pedunculo pontine nucleus (PPN).

Two studies on penetrance estimating the ratio of symptomatic carries revealed just identical results, that is, for females 87% and 87% and for males 38% and 35%. Gender difference of the base levels of GCH-I in the mononuclear blood cells is yet to be confirmed. Thus, marked female predominance might depend on a genetically determined gender difference of the DA neuron.

The pathophysiology of AD GCH-I deficiency is postulated as follows. The decrease of the TH protein or DA in the ventral area of the striatum causes disfacilitation of the D_1 striatal direct pathway, and disinhibits the output projection of the internal segment of the globus pallidus and pars reticulata of the SN, which suppress the reticulospinal tract and the superior colliculus. These may cause postural dystonia with exaggeration of the tendon reflexes without extensor plantar reflexes and abnormalities in voluntary saccades. Whereas, with involvement of the DA neuron innervating to the STN with D_1 receptor, the ascending outputs to the thalamus are disfacilitated and develop tremor and action dystonia. However, action dystonia is observed in infants with recessive sepiapterin reductase deficiency; the projection of the descending output to the brain stem reticular formation could get involved in this process. This process also disinhibits of the superior colliculus and involves the failure in suppression of unnecessary saccade in memory-guided task. Focal and segmental dystonia observed in patients with action dystonia may be caused by dysfunction of the motor cortex due to disinhibition of the thalamocortical pathway. Considering the ipsilateral finging of the predominant side of involvement in SCM and extremities, this pathway may be preferentially involved in adult onset patients. These pathophysiologies are shown in **Figures 1(a)–1(c)**.

Diagnosis

Confirming the diagnosis of AD GCH-I deficiency is usually not difficult in the setting of characteristic clinical symptoms. The estimation of GCH-I activity in peripheral nucleated cells is accurate, but technically complicated. Thus, estimation of neopterin and biopterin levels in CSF is most reliable for diagnosis.

Differential Diagnosis

All children with gait disturbance and limb dystonia with asymmetry should be evaluated for AD GCH-I deficiency. Other conditions with this clinical presentation may include Wilson's disease, Hallervorden–Spatz disease, hereditary spastic paraplegia, and cerebral palsy. AD GCH-I deficiency is often misdiagnosed as hereditary spastic paraplegia. The differentiation of AD GCH-I deficiency from these disorders is usually not difficult with careful clinical examination.

Cases with axial torsion dystonia, including early onset autosomal dominant torsion dystonia (DYT1), can be differentiated clinically from AD GCH-I deficiency by evaluating the side that is predominantly involved between the SCM and muscles of the extremities; the side of the SCM predominantly affected is contralateral to that of the limb muscles in AD GCH-I deficiency, while it is ipsilateral in dystonias with axial torsion. This difference in the side preference reflects involvement of the NS–DA neurons in AD GCH-I deficiency while involvement of the striatum or the pallidum in torsion dystonia. However, it should be taken care that AD GCH-I deficiency and also DYT1 may appear with focal or segmental dystonia without general dystonia in adolescence or adulthood. Besides biochemical and molecular biological studies, levodopa loading test is recommended.

Dopa Responsive Dystonia Other Than AD GCH-I Deficiency

This group includes recessive disorders of pteridine metabolism and recessive TH deficiencies (recessive DYT5). All of the inherited disorders of pteridine

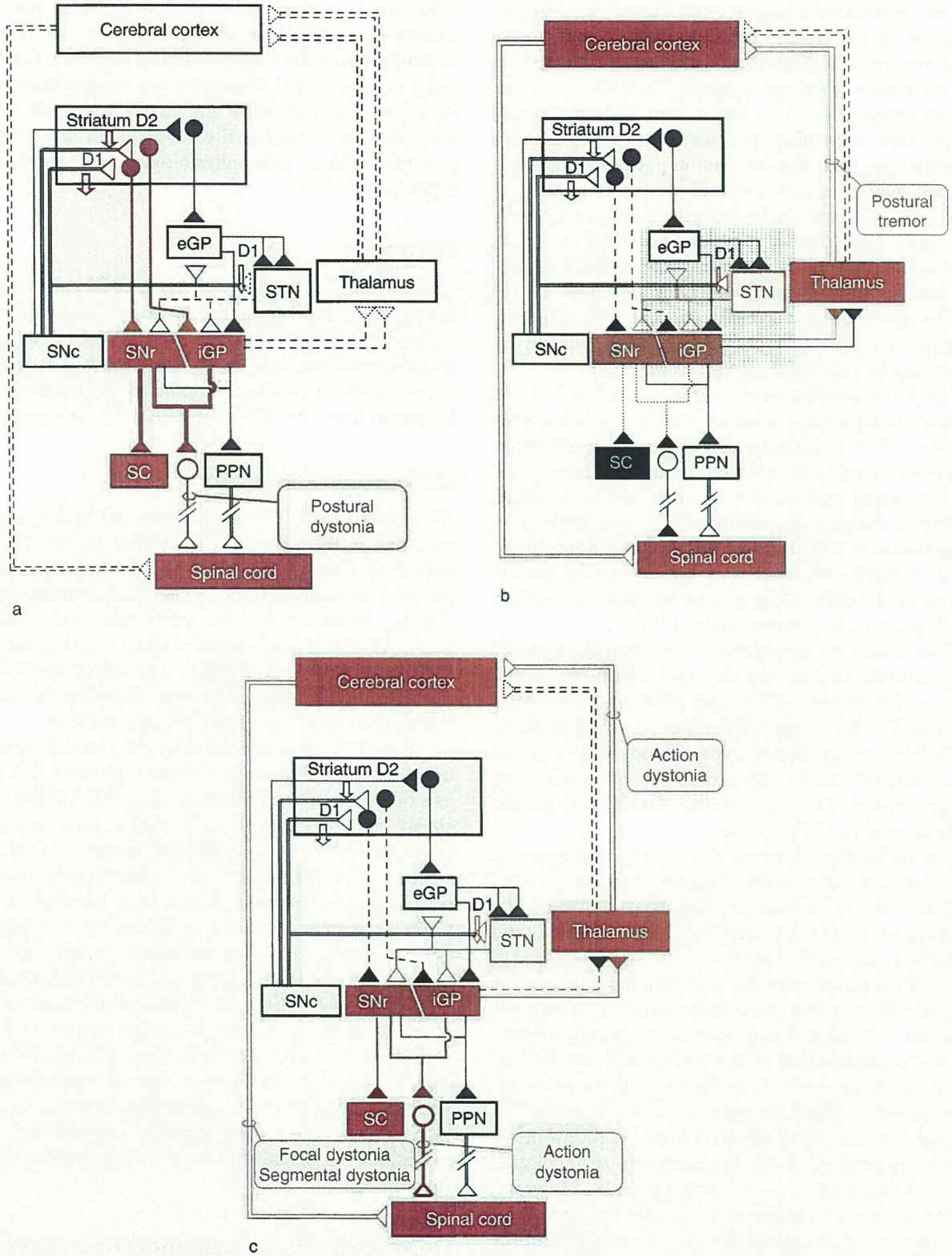

Figure 1 Pathophysiology of SDRD. (a) Neuronal pathways involving in postural dystonia. (b) Neuronal pathways involving in tremor. (c) Neuronal pathways involving in action dystonia and focal or segmental dystonia. eGP, external segment of globus pallidus; iGP, internal segment of globus pallidus; STN, subthalamic nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SC, superior colliculus; PPN, pedunculopontine nucleus. Red lines, pathways involved in pathophysiology; black lines, pathways not involved in pathophysiology; solid line, inhibitory neuron; dashed line, excitatory neuron; closed triangle, inhibitory neuron; open triangle, excitatory neuron; shaded region, the area of the circuit for postural tremor. White letters with red back ground, targeted neurons or neuronal system. Dotted lines and triangles show pathways and terminals involved in SDRD but not involved in the symptoms shown in the figure.

metabolism develop levodopa responsive dystonia caused by decrease of BH_4 in infancy and early childhood as in AD GCH-I deficiency. However, they show postural hypotonia and psychological disturbances caused by deficiency of the 5-HT activities. Recessive TH deficiency shows ptosis, hyperperspiration, and psychomotor disturbances due to noradrenaline (NA) deficiency. However, some show almost identical features as AD GCH-I deficiency and respond well to levodopa. However, the diurnal fluctuation is not marked as AD GCH-I deficiency. For definite diagnosis, estimation of pteridine metabolites and catecholamine metabolites in CSF is necessary.

Dopa Responsive Dystonia Parkinsonism

All of juvenile parkinsonism (JP) appears as dystonia when it occurs in childhood to early teens. Although dystonia of JP responds briskly to levodopa, dyskinesia develops soon after levodopa is started. Among JPs, that caused by parkin gene (PARK2) is a particularly important disease to differentiate from AD GCH-I deficiency when it occurs in early ages.

In addition, some patients with AD GCH-I deficiency develop symptoms later in life (e.g., fifties and sixties). In this population, tremor and gait disturbance are the primary signs, and dystonia is absent or not prominent. In these patients, diurnal fluctuation is not observed and often misdiagnosed as PD. However, the tremor in these cases is mainly postural and their clinical features are milder with minimal progression. For definite diagnosis of PARK2, gene analysis is necessary.

See also: Dopaminergic Agonists and L-DOPA; Dystonia: Classification, Genetics and Therapeutics; Parkinsonian Syndromes; Torsion Dystonia.

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Journal of Human Genetics (2012), 1–2

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COMMENTARY

Commentary on the mutation spectrum of and founder effects affecting the *PTS* gene in East-Asian populations

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Journal of Human Genetics advance online publication, 16 February 2012; doi:10.1038/jhg.2011.153

Tetrahydrobiopterin (BH4) deficiency is a rare disorder affecting phenylalanine metabolism in the liver and neurotransmitters biosynthesis in the brain. In 1975, Smith *et al.*[1] first reported these patients as 'atypical phenylketonuria (PKU)'. Patients with BH4 deficiency appear normal at birth, but experience symptoms such as intellectual disability, progressive problems with development, movement disorders, difficulty swallowing, seizures and behavioral problems. Bartholomé *et al.*[2] reported that the neurological signs in these patients were treatable by the oral administration of the neurotransmitter precursors 3,4-dihydroxyphenylalanine (L-DOPA) and 5-hydroxytryptophan (5-HTP), both of which cross the blood–brain barrier. Shintaku *et al.*[3] recommended that this treatment be started within 2 months of birth to help prevent neurological damage. Therefore, the expression 'BH4 deficiency' should be used rather than the terms 'atypical PKU' or 'malignant hyperphenylalaninemia (HPA)'.[4]

BH4 is an essential cofactor in the enzymatic hydroxylation of three aromatic amino acids (phenylalanine, tyrosine and tryptophan). BH4 is synthesized from guanosine triphosphate (GTP) catalyzed by GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase. In aromatic amino acids hydoxylating system, BH4 is regenerated by pterin-4a-carbinolamine dehydratase and dihydropteridine reductase. (DHPR).[5] They all follow an autosomal-recessive mode of inheritance and the gene mutations of all five enzymes have been reported.[3] The incidence of BH4 deficiency is at 1 in 1 000 000, except that in Taiwanese (much higher than that in Japanese and in Caucasians).[5,6] Liu *et al.*[7] reported that the BH4-deficient HPA was estimated to make up around 30% of the Chinese population in Taiwan suffering from HPA, which is much higher than in Caucasian populations (1.5–2% of HPA). In Taiwan approximately 86% of BH4-deficient HPA in the Chinese population was found to be caused by PTPS deficiency, although it is the most common form of BH4 deficiency in the world.

BH4 deficiency has been diagnosed in patients with HPA by neonatal mass screening based on BH4 oral loading tests, analysis of urinary or serum pteridines and measurement of DHPR activity in the blood from a Guthrie card. BH4 deficiency without treatment causes combined symptoms of HPA and neurotransmitter (dopamine, norepinephrine, epinephrine and serotonin) deficiency, such as red hair, psychomotor retardation and progressive neurological deterioration, as mentioned before. Treatment of BH4 deficiencies consists of BH4 supplementation (2–20 mg kg^{-1} per day) or diet to control the blood phenylalanine concentration and replacement therapy with neurotransmitters precursors (L-DOPA/CarbiDOPA and 5-HTP), and supplements of folinic acid in DHPR deficiency.[5]

In this issue of the *Journal*, Chiu *et al.*[8] investigated mutations in the patients with PTPS (gene symbol: *PTS*) deficiency in East-Asian populations and increased our understanding of the mutation spectrum and founder effects affecting the *PTS* gene in East-Asian populations.[6] The patients were from 176 families (Han Chinese populations: 156 families, Japanese: 6 families, South Korean: 7 families, Thai: 3 families and Filipinos: 4 families) and total of 352 mutations were analyzed. Mutations found in these patients were strongly linked to a microsatellite marker, D11S1347. Among these, five mutations were the most common in East Asia. These mutations were not located in CpG hot spots. These results indicate that each of the common mutations came from a single ancestor. The authors suggested that the founders were ancient Chinese of Mainland China. In contrast, Okinawan people in Japan and Filipinos each showed a unique mutation in *PTS*.[9] This result suggests that these two separated regions had their own founders.

What key concepts and lessons can be derived from this study?

First, the author investigated PTPS-deficiency patients of the Han people in Taiwan, Mainland China and Malaysia in this study. Patients of other countries such as Japan, South Korea and Philippine were also analyzed. The results indicate that mutations of the *PTS* gene in East Asia were within the area of D11S1347, which is important and useful in diagnosing patients with PTPS-deficiency in East Asia.

Second, five common mutations were found in patients of Mainland China on the coast and those of the Han people in other countries. Some of those mutations were found only in East Asia. It is better to collect the information of patients in inland Mainland China, the Mongolian people and other countries to investigate the origin of those mutations.

Third, the author reports that the prevalence rate of HPA in each East-Asian country was lower than that of the Caucasian population. However, the incidence of BH4

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deficiency among HPA in East-Asian countries was higher than that of the rest of the world. It is possible that several founder events occurred in the Han people (or other neighboring peoples) and those mutations spread over other areas along with the immigration of the Han people. It would be of interest to compare mutations of the *PTS* gene in East Asia and in other parts of the world, for it may reveal early human migrations in ancient times. The patients of the two isolated regions (Okinawa islands in Japan and the Philippines) showed other types of mutations in the *PTS* gene.[9] It was suggested that other founder events have occurred in those areas.

In conclusion, this study represents the usefulness of microsatellite marker, D11S1347 to screen of PTPS deficiency in East Asia. These mutations were mainly observed in patients of the Han people. The high prevalence rate of PTPS deficiency in the Han people would explain the high incident rate of this disorder in East Asia.

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Phospho-Ser727 of STAT3 regulates STAT3 activity by enhancing dephosphorylation of phospho-Tyr705 largely through TC45

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Signal transducer and activator of transcription 3 (STAT3) is a latent cytoplasmic transcription factor. It is activated by cytokines, including interleukin-6 (IL-6) through phosphorylation at Tyr705 (pY705), which is required for its dimerization and nuclear translocation. However, the role of Ser727 phosphorylation, occurring during activation, remains poorly understood. Using a combination of HepG2-stat3-knockdown cells reconstituted with various STAT3 mutants and protein kinase inhibitors, we showed that phospho-S727 has an intrinsic mechanism for shortening the duration of STAT3 activity, in turn shortening the duration of *socs3* mRNA expression. Both STAT3WT and STAT3Ser727Asp (S727D) but not STAT3Ser727Ala (S727A) showed rapid dephosphorylation of pY705 after the inhibition of tyrosine kinases. We found that the nuclear TC45 phosphatase is most likely responsible for the phospho-S727-dependent pY705 dephosphorylation because TC45 knockdown caused prolonged pY705 with sustained *socs3* mRNA expression in STAT3WT but not in STAT3S727A, and overexpressed TC45 caused rapid dephosphorylation of pY705 in STAT3WT but not in STAT3S727A. We further showed that phospho-S727 did not affect the interaction of TC45 with STAT3, and that a reported methylation at K140 of STAT3 occurring after phospho-S727 was not involved in the pY705 regulation. These findings indicate that phospho-Ser727 determines the duration of STAT3 activity largely through TC45.

Introduction

Cells have multiple receptors and complex signal transduction mechanisms for receiving and integrating extracellular information to respond properly by initiating the processes for survival, cell proliferation, cell death, differentiation, senescence, and even tumorigenesis. The signal transducer and activator of transcription (STAT) family of proteins are latent transcription factors that are activated in the cytoplasm in response to cytokines and growth factors and participate in various critical cellular processes (Levy & Darnell 2002). Once activated through tyrosine phosphorylation of its critical tyrosine residue in the carboxy-terminal region, STAT protein homo- or heterodimerizes, translocates to the nucleus, and binds to the specific DNA sequences to regulate mRNA expression from by its target genes (Darnell *et al.* 1994; Darnell 1997). Most STAT family members are phosphorylated at one or two serine residues in the carboxyl-terminal transactivation domain in addition to the critical tyrosine phosphorylation (Decker & Kovarik 2000). Although the roles of tyrosine phosphorylation of signal transducer and activator of transcription 3 (STAT3) proteins are well characterized, the roles of serine phosphorylation of STATs have been controversial (Decker & Kovarik 2000).

STAT3 is activated through tyrosine phosphorylation of Tyr705 in response to factors such as the interleukin-6 (IL-6) family cytokines, platelet-derived growth factor, and epidermal growth factor (Hirano *et al.* 1999). It has been shown that Ser727 of STAT3 is phosphorylated by various kinases, including

Communicated by: Shigeo Koyasu
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DOI: 10.1111/j.1365-2443.2011.01575.x

mitogen-activated kinases (MAPK), H7-sensitive kinase, protein kinase Cδ, cyclin-dependent kinase 5 (CDK5), the mammalian target of rapamycin kinase (mTOR), nemo-like kinase (NLK), death-associated protein kinase 3 (DAPK3), and mitogen- and stress-activated kinase 1 (MSK1), depending on the stimulus and cell type used (Boulton *et al.* 1995; Chung *et al.* 1997; Jain *et al.* 1999; Yokogami *et al.* 2000; Abe *et al.* 2001; Wierenga *et al.* 2003; Fu *et al.* 2004; Ohkawara *et al.* 2004; Kojima *et al.* 2005; Sato *et al.* 2005). Although phosphorylation of Ser727 has been suggested to exert positive effects on STAT3-dependent gene activation (Wen *et al.* 1995; Abe *et al.* 2001; Shen *et al.* 2004), most likely through recruiting coactivator proteins (Schuringa *et al.* 2001; Lufei *et al.* 2007; Lee *et al.* 2009), some reports have suggested that phosphorylation of Ser727 represses STAT3 activity (Chung *et al.* 1997). This controversy may reflect the dual roles of phospho-Ser727 or may result from the lack of a proper method for evaluating the role of phospho-Ser727 of STAT3: most works relied on the use of the STAT3Ser727Ala (S727A) mutant to study the role of phosphoSer727 (Wen *et al.* 1995; Abe *et al.* 2001; Shen *et al.* 2004). However, a recent report by Sun *et al.* (2006) showed that the LPMSP motif itself around the Ser727 of STAT3, rather than phospho-Ser727, was important to recruit p300 to STAT3 and the STAT3-bound DNA region, indicating the problem of relying on the use of one mutant of STAT3. Similarly, there are difficulties with the use of protein kinase inhibitors to evaluate the role of Ser727 phosphorylation because of their specificity problems.

STAT3 activity is primarily dependent on the level of phosphorylation at Y705, which is regulated by the activities of tyrosine kinases and tyrosine phosphatases specific to STAT3 (Mertens & Darnell 2007). Several protein tyrosine phosphatases (PTPs), including Src-homology-2 protein phosphatase-1 and 2 (SHP1, SHP2), T-cell PTP (TC-PTP/TC-45), and PTP receptor T (PTPRT), have been shown to regulate the level of pY705 of STAT3 (ten Hoeve *et al.* 2002; Yamamoto *et al.* 2002; Zhang *et al.* 2007; Kim *et al.* 2010). Naturally, cessation of further tyrosine phosphorylation of STAT3 by inhibiting the activities of tyrosine kinases toward STAT3 is another mechanism of regulating the level of STAT3 activity. In the IL-6 receptor system, IL-6 signaling has such negative regulatory systems, one is an inhibitory loop through SHP2 (Schmitz *et al.* 2000) and another through suppressor of cytokine signaling 3 (SOCS3) (Nicholson *et al.* 1999; Yoshimura *et al.* 2007). SHP2, a tyrosine phosphatase with two SH2 domains, has been shown to be recruited to the tyrosine-phosphorylated YSTV motif of gp130 and phosphorylated on its tyrosine residues (Stahl *et al.* 1995). SHP2 not only activates the Ras-MAPK pathway by interacting with Grb2-SOS (Li *et al.* 1994; Fukada *et al.* 1996) but also inactivates both JAK tyrosine kinases, which are associated with the signal transducing subunit gp130 in the IL-6 receptor complex, and STAT3 by dephosphorylating them (Schmitz *et al.* 2000; Lehmann *et al.* 2003). SOCS3 is rapidly induced mainly by STAT3 together with ERK1/2 activity and other transcription factors, and binds to the phosphorylated YSTV-motif of gp130 to inhibit JAK kinase activity, resulting in the cessation of further activation of STAT3 (Schmitz *et al.* 2000; Terstegen *et al.* 2000; Lehmann *et al.* 2003).

Here, we report that phosphorylation of Ser727 of STAT3 intrinsically, not via the negative regulatory loop through SOCS3, regulates the duration of STAT3 activity by promoting dephosphorylation of STAT3 pY705, which shortens the duration of transcriptional activity. We show that the nuclear TC45 phosphatase is most likely responsible for the phosphoSer727-dependent dephosphorylation of pY705.

Results

Duration of STAT3-dependent mRNA expression partly determined by state of Ser727

The role of phosphorylation of Ser727 in STAT3 still remains elusive. To assess the roles of phosphorylation of Ser727, we used HepG2-stat3-knockdown (Stat3KD) cells reconstituted with various STAT3 mutants as described previously (Zhao *et al.* 2004). For this study, we introduced siRNA-resistant STAT3 natural proteins without a tag into HepG2-stat3KD cells by a lentiviral expression system. The expression levels of introduced genes were evaluated either by quantitative real-time PCR (qRT-PCR) for the mRNA levels or immunoblot analysis for the protein levels. The STAT3 protein levels were comparable in STAT3-reconstituted HepG2-STAT3WT cells and STAT3Ser727Ala-reconstituted HepG2-STAT3S727A cells (Fig. 1A). Then we examined the effect of mutation on the levels of STAT3-dependent gene expression. The *socs3* gene was chosen as a STAT3 target gene because the IL-6 induction of *socs3* mRNA expression is mostly dependent on the STAT3 activity in HepG2 cells (data not shown). In HepG2-STAT3WT cells, the *socs3* mRNA level increased rapidly after stimulation with

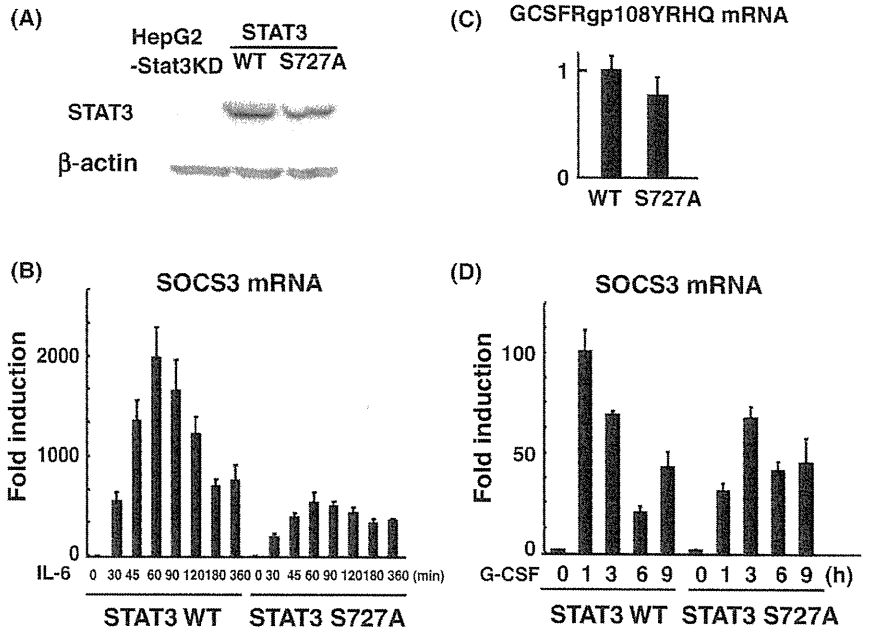

Figure 1 STAT3S727A mutant activates the socs3 gene, one of STAT3 target genes, at a lower level but for longer period than STAT3 wild type. (A) Whole-cell extracts from HepG2-stat3KD, HepG2-STAT3 wild type (WT) and HepG2-STAT3Ser727Ala (S727A) were examined for expression of STAT3 by immunoblotting using an anti-STAT3 Ab. The level of β-actin ensured equal loading. (B) HepG2-STAT3WT cells and HepG2-STAT3S727A cells were stimulated with IL-6 at 20 ng/mL for the indicated times, and total RNAs were extracted. The *socs3* mRNA expression levels in total RNA were measured by qRT-PCR. The level of GAPDH mRNA was used for normalization. The data are averages of three independent experiments; error bars are the standard deviations. (C) HepG2-STAT3WT cells and HepG2-STAT3S727A cells were infected with a lentivirus for the chimeric receptor G-CSFR-gp130 containing the intracytoplasmic gp130 truncated at 108 AA linked with the YRHQ motif (G108YRHQ). The mRNA levels for the chimeric receptor were measured by qRT-PCR. Samples were assayed in triplicate. The average level of G108YRHQ mRNA from HepG2-STAT3WT-G108YRHQ is approximately 15% higher than that from HepG2-STAT3S727A-G108-YRHQ. (D) HepG2-STAT3WT-G108YRHQ cells and HepG2-STAT3S727A-G108YRHQ cells were stimulated with G-CSF at 50 ng/mL for the indicated times. The *socs3* mRNA levels were measured as in (B). The data are averages of three independent experiments. Error bars are the standard deviations.

IL-6, with a peak at 60 min, and it had declined sharply by 180 min. In contrast, the *socs3* mRNA expression in IL-6-stimulated HepG2-STAT3S727A cells was two- to threefold lower than that in HepG2-STAT3WT cells at 30, 60, and 90 min after IL-6 stimulation, but interestingly the *socs3* mRNA level was sustained up to 360 min, suggesting that the intact Ser727 is required not only for the maximal transcription but also for the restricted duration of its transcription. We first focused on the increased duration of STAT3-dependent mRNA expression observed in HepG2-STAT3S727A cells. It has been known that SOCS3 protein, which is rapidly induced in a STAT3-dependent manner with the help of other factors, effectively inhibits further activation of STAT3 by suppressing JAK kinases through binding to the phosphorylated YSTV motif of gp130 (Schmitz *et al.* 2000). To test whether this is the only mechanism responsible for the difference in the duration of STAT3-dependent mRNA expression, we proceeded to test the duration of STAT3 activity under conditions in which the inhibitory effect of SOCS3 was neglected. For this purpose, we introduced a G-CSFR-gp130 chimeric receptor, named G108YRHQ, which contains a short cytoplasmic domain up to 108 amino acid residues linked with a YRHQ motif, one of the YXXQ motifs in gp130, for activation of STAT3 (Abe *et al.* 2001; Kojima *et al.* 2005) into both STAT3 reconstituted cells. The levels of G108YRHQ mRNA in both types of reconstituted cells were roughly comparable (Fig. 1C). The durations of STAT3-dependent mRNA expression were evaluated in these cells. Upon stimulation of HepG2-STAT3S727A-G108YRHQ cells with G-CSF, the level of *socs3* mRNA reached a peak at 3 h which was sustained up to 9 h, showing a kinetic pattern different from that observed in HepG2-STAT3-GYRHQ cells

(Fig. 1D). These findings indicate that some mechanisms intrinsic to the state of Ser727 contribute to the difference in the duration of STAT3-dependent mRNA expression.

STAT3S727A mutant causes prolonged binding of STAT3 and Pol II to the *socs3* gene promoter

The strength and duration of STAT3 transcriptional activity is likely to be determined by multiple factors, including the amount of STAT3 bound to the regulatory regions of the target genes and the constituents of proteins modifying or interacting with STAT3. We then examined the recruitment of STAT3 proteins and RNA polymerase II to the *socs3* gene using a chromatin immunoprecipitation (ChIP) assay. The tested regions corresponded to the promoter region and distal part of the open reading frame (ORF) (Fig. 2A). The *socs3* gene promoter region has two STAT3 binding sites and an activation protein-1 (AP-1) binding site. After IL-6 stimulation, STAT3WT was rapidly recruited to the *socs3* gene promoter with peaks around 15 and 30 min, and the level had declined rapidly by 90 min, and gradually increased again up to 3 h. In contrast, the recruitment of STAT3S727A reached almost a plateau at 30 min, the level was sustained with a small peak at 90 min, and gradually declined until 180 min. It is noted that the amount of recruited STAT3S727A surpassed that of STAT3WT after 90 min of stimulation (Fig. 2B). After the stimulation of HepG2-STAT3WT cells with IL-6, the levels of RNA Pol II bound to the *socs3* promoter and to the distal ORF region showed biphasic patterns similar to that of STAT3, whereas in HepG2-STAT3S727A, the levels of RNA Pol II bound to the *socs3* gene promoter and to the distal ORF region gradually increased and were sustained up to 180 min after stimulation (Fig. 2C). Thus, the kinetic changes in STAT3 recruitment to the *socs3* gene promoter correlated well with the STAT3-dependent *socs3* gene activation, as judged by both the Pol II binding to the *socs3* gene body and the *socs3* mRNA levels shown in Fig. 1B.

Sustained nature of pY705 in STAT3S727A and unphosphorylated STAT3

We thought it possible that prolonged binding of STAT3 may reflect the sustained level of phosphotyrosine of STAT3 at Y705. As expected, STAT3S727A showed phosphorylation at Y705 for a longer period than STAT3WT (Fig. 3A). We next examined the rate of dephosphorylation at Y705 in STAT3WT and STAT3S727A. For this study, we also used HepG2

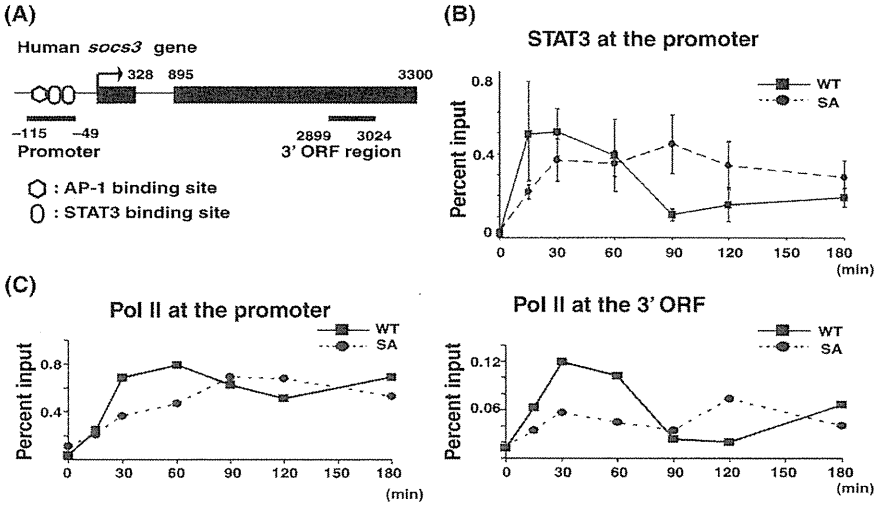

Figure 2 Prolonged binding of STAT3S727A to the *socs3* gene promoter correlates well with prolonged Pol II action. (A) Schematic view of the human *socs3* gene. The two tested regions, corresponding to the *socs3* promoter region (−115 to −49) and the distal part of 3′ ORF (2899–3024), are underlined, and the two exons are depicted as black boxes. (B) HepG2-STAT3WT (WT) and HepG2-STAT3S727A (SA) cells were stimulated with IL6 at 20 ng/mL for the indicated times. ChIP assay was carried out with anti-STAT3. The immunoprecipitated DNAs were quantified by real-time PCR in duplicates with primers specific to the socs3 promoter region (−115 to −49). ChIP results are shown as a percentage of input. The averages of three independent experiments are shown. Error bars are standard deviations. (C) ChIP assays were carried out as in (A), using anti-Pol II and primer pairs specific to the *socs3* promoter (−115 to −49: left panel) and the 3′ end of the *socs3* gene (2899–3024: right panel). ChIP results are shown as a percentage of input. A representative result from two independent experiments is shown.

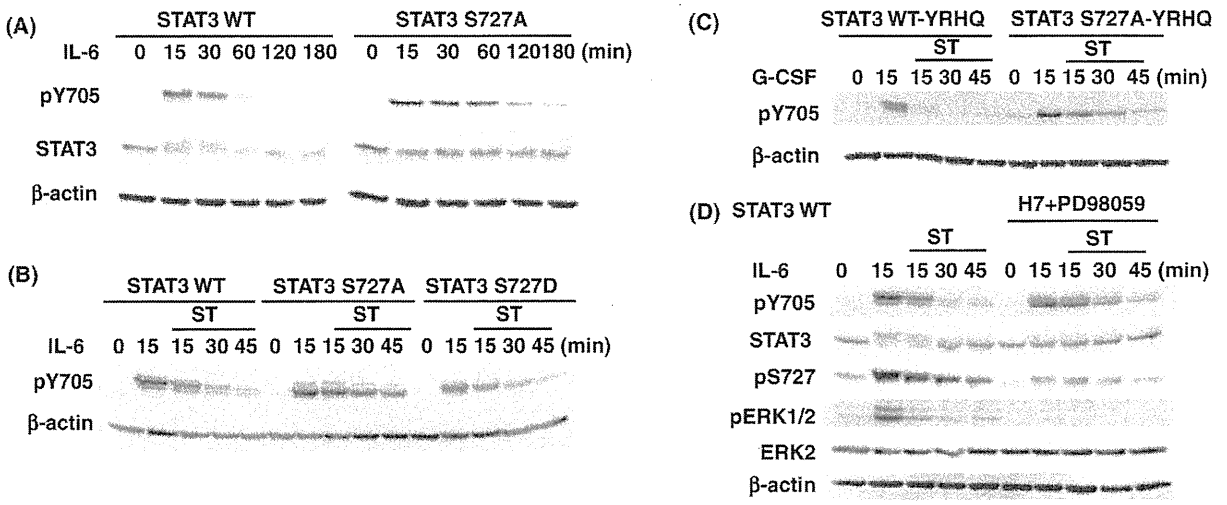

Figure 3 STAT3S727A showed sustained phosphorylation when tyrosine kinase activity was inhibited after stimulation. (A) HepG2-STAT3WT (left panel) and HepG2-STAT3S727A (right panel) cells were stimulated with IL-6 at 20 ng/mL for the indicated times. Immunoblot analysis was carried out on whole-cell extracts (30 μg per lane) using anti-phospho-STAT3Tyr705 (pY705), anti-STAT3 and anti-beta-actin antibody. (B) HepG2-STAT3WT, HepG2-STAT3S727A, and HepG2-STAT3Ser727-Asp (STAT3S727D) cells were stimulated with IL-6 at 20 ng/mL for 15 min, treated with a potent kinase inhibitor, staurosporine (abbreviated as ST: 0.5 μM) for inhibition of tyrosine kinases, and then left for a further 15, 30 and 45 min. pY705 levels of WCEs were examined by immunoblotting. (C) HepG2-STAT3WT-G108YRHQ (left) and HepG2-STAT3S727A-G108-YRHQ (right) cells were stimulated with G-CSF at 50 ng/mL for 15 min, treated with ST (0.5 μM), and then left for a further 15, 30, and 45 min. pY705 levels were monitored periodically as in (B) by immunoblotting. (D) HepG2-STAT3WT cells were pretreated without (left) or with (right) H7 (100 μM) and PD98059 (50 μM) for 30 min, then stimulated with IL-6 at 20 ng/mL for 15 min, followed by ST (0.5 μM) treatment, and then pY705 levels were monitored periodically as indicated by immunoblotting using anti-pY705, anti-STAT3, anti-pS727, anti-phospho-ERK1/2, anti-ERK, and anti-β-actin. The data representative from two or three independent experiments with similar results are shown for this figure.

cells expressing another STAT3 mutant STAT3-Ser727Asp (S727D) that mimics the phosphorylation state at Ser727. To examine the dephosphorylation process, HepG2 cells were stimulated with IL-6 for 15 min, and then treated with a potent kinase inhibitor, staurosporine (ST), to inhibit further phosphorylation (Haspel & Darnell 1999). The pY705 levels were monitored periodically by immunoblot analysis. As shown in Fig. 3B, with inhibition of the responsible tyrosine kinases for STAT3 phosphorylation, both STAT3WT and STAT3S727D showed a rapid decrease in the level of pY705, whereas STAT3S727A showed only minor decreases in the level of pY705 even at 45 min after treatment with ST. The level of pY705 in STAT3S727A slowly decreased thereafter with being apparent after 120 min (please see Fig. 4D). Although it was difficult to estimate the levels of STAT3 proteins especially at early times after stimulation because of the broad shifts of STAT3 proteins, the total STAT3 proteins during the examined period seemed not to change significantly (please see also Fig. 3D). We also tested the possible role of ubiquitin-dependent degradation of STAT3 in the rapid decrease of pY705 in wild-type STAT3. Pretreatment of HepG2 cells with MG132, an inhibitor of proteasome, did not affect either the levels of STAT3 proteins or the levels of pY705 examined under the same conditions as used in the Fig. 3B (Fig. S1 in Supporting Information). Together, these findings indicate that the rapid decrease in the level of pY705 observed in STAT3WT is not because of the changes in the STAT3 protein levels but reflects the level of phosphorylation itself. These data also suggest that the S727A mutant is resistant to the action of some tyrosine phosphatase(s). The efficient dephosphorylation of pY705 in STAT3WT and STAT3S727D suggests that Ser727 phosphorylation may provide a platform for the action of some tyrosine phosphatase(s). The difference in the dephosphorylation of pY705 between STAT3WT and STAT3S727A could be observed in HepG2 cells stimulated with the chimeric receptor activating only the YXXQ-derived pathway (Fig. 3C), which has been shown to cause Ser727 phosphorylation mostly