表１　３歳児健診事業対象者における発達チェックリストの結果

よく認められると答えた％を示す

大項目	小項目	%
育てやすかった面	おとなしかった	15.3
	よく寝た	52.4
	反応が少なかった	0.0
	ニコニコ笑う	45.2
	泣いてもすぐに泣きやんだ	27.4
	知らない人でも割とすぐなついた	29.8
育てにくかった面	ぐずりやすかった	18.5
	寝つきが悪かった	16.1
	音などに敏感だった	21.8
	寝つく時間や睡眠時間が一定でなかった	13.7
	目を合わせなかった	0.8
	知らない人になれるのに，とても時間がかかった	19.4
	肌触りにとても敏感で自分の好きなものしかうけいれなかった	2.4
気質	活発である	76.6
	おっとりしている	6.5
	ニコニコしている	37.1
	かんしゃくをすぐに起こす	25.8
	気むずかしい	13.7
	寝る時間や起きる時間，食事の時間はほぼ一定である	61.3
	決まったスケジュール以外のことをするのを嫌がる	0.8
	知らない人やもの，場所にもすぐなれる	42.7
	知らない人やもの，場所になかなか慣れず時間がかかる	21.8
	気が散りやすくてひとつの遊びに集中できない	13.7
言語面	言葉の置き換えがある	40.3
	発音の不明瞭さがある	31.5
	意味がわからない音や叫び声をだしたりする	8.9
	テレビのコマーシャルなどの決まり文句やセリフを一言一句覚えていたりする	75.0
	聞いたことをすぐにまねする	60.5

	会話というよりも一方的に話をする	6.5
	呼びかけても，返事がなく，聞こえていないのでは？と思われるようなことがある	4.0
行動面	歩きはじめた頃は転びやすかったり，突然走り出したりした	16.1
	ちょろちょろしている	43.5
	ケガをしやすい	7.3
	ぼーっとしている	1.6
	高いところを好む	31.5
	高いところを嫌う	1.6
	お気に入りのビデオなどがありそれを何度もみたがる	72.6
	そのお気に入りのビデオなどの特定の場面を何度も巻き戻し・再生・止めたりする	8.1
	ひとつのことに没頭しやすい	11.3
	おままごとなどの想像したりする遊びは好まない	0.8
	ブロックなどの組み立てるものはあまり好きではない	0.8
	水遊びが好きである	78.2
	音がたくさんでるおもちゃを好む	29.8
	光ってきらきらするものを好む	25.0
	人の話が聞けない	8.1
	ウワの空になりやすい	2.4
	同じ失敗を何度も繰り返す	5.6
	なんども聞き返す	8.9
	人がやるのを見てから取り組む	30.6
	文字・数字・マークをよく覚えている	33.9
	カレンダーや列車など非常に興味をしめすものがある	23.4
	お決まりのスケジュールや遊び方があり，それが変わるととても不機嫌になる	4.0
	どれもあてはまらない	24.2
運動面	不器用である	8.1
	バランス感覚がよくない	1.6
	あまり動かない(じっとしていることが多い)	0.8
	お誕生日のローソクが上手に吹き消せない	0.0
	力の加減が出来ない	4.8
	筋力が弱い	6.5
	動作が変わっていたり，かたかったり，どこかぎこちなかったりする	1.6

	同じ動きを何度も繰り返す	0.0
	どれもあてはまらない	75.8
対人面	初対面の人にもなれなれしい，物怖じしない	19.4
	緊張しやすい	21.8
	初めての人に弱い	43.5
	男の人が苦手	7.3
	他の子どもを怖がる	1.6
	友だちに興味がない	0.8
	べたべたと人を触りたがる	4.8
	同じ人でも違う場所で会うとわからない	1.6
	人がそのもので遊んでいても，目にはいったものだけにとらわれてしまい，つい奪い取ってしまうことがある	15.3
	友人をフルネームで呼ぶ	7.3
	一人を好む	2.4
	ちょくちょく遊ぶ相手が変わる	1.6
	手をつなぎたがらない	2.4
	よく知った大人にも抱っこされたがらない	4.0
	遊びなどの場面で，自分の順番がなかなか待てない	13.7
	どれにもあてはまらない	19.4
感覚面	においに敏感	22.6
	特定の手触りのものを好む	4.3
	特定の音を嫌う	4.0
	手などか汚れる遊びを嫌い，汚れたらすぐに洗いたがる	8.1
	ケガに対する反応があまりない(痛がっていないように見える)	1.6
	自分のスペースがとても必要である	6.5
	靴下を必ず脱ぐ	44.4
	同じ服を着たがる	14.5
	なにかと口に入れたがる	6.5
	決まった物しか食べたがらない	4.8
	ウンチをおしえない，おむつがとれていない	17.7
	水やガラスなど反射するものにとても心を奪われる	7.3
	どれにもあてはまらない	21.0

表2　通常 3 歳児でよく認められる発達指標

育てやすかった面	よく寝た
	ニコニコ笑う
気質	活発である
	寝る時間や起きる時間，食事の時間はほぼ一定である
言語面	テレビのコマーシャルなどの決まり文句やセリフを一言一句覚えていたりする
	聞いたことをすぐにまねする
行動面	お気に入りのビデオなどがありそれを何度もみたがる
	水遊びが好きである
運動面	特に心配なところがない
感覚面	靴下を必ず脱ぐ

表3　ADHD 傾向と PDD 傾向を示す項目

ADHD 傾向を示す因子	
気質	気が散りやすくてひとつの遊びに集中できない
言語面	意味がわからない音や叫び声をだしたりする
行動面	ちょろちょろしている
	人の話が聞けない
対人面	人がそのもので遊んでいても，目にはいったものだけにとらわれてしまい，つい奪い取ってしまうことがある
	遊びなどの場面で，自分の順番がなかなか待てない
PDD 傾向を示す因子	
育てやすかった面	おとなしかった
気質	知らない人やもの，場所になかなか慣れず時間がかかる
運動面	不器用である
対人面	初めての人に弱い

表 4　母親のストレス尺度の比較

	平均値	全健診群 (n=124)	健診 ADHD 群 (n=10)	健診 PDD 群 (n=11)	non-ADHD. PDD 群 (n=103)	子育て支援センター (n=287)
この子の育て方	9.56	8.06	10.70 *	6.18	8.00	9.24
この子の家庭内の問題行動	5.54	4.99	6.00 *	4.00	5.00	5.51
この子の家庭外の問題行動	7.87	7.20	9.40 *	5.73	7.15	7.57
夫婦の育児方針	6.2	5.71	7.00 *	5.55	5.60	6.17
この子と母親とのかかわり	7.35	5.96	7.90 *	5.73	5.79	6.89
普通児との比較	10.7	5.91	8.40	5.36	5.71	8.95
将来への不安	12.29	7.10	8.70	6.82	6.97	10.58
家庭生活	7.14	7.11	7.30 *	7.00	7.10	7.17
夫婦の調和	5.17	5.09	5.30 **	5.00	5.07	5.32
母親自身の健康	6.98	6.53	8.20 *	6.45	6.37	7.24
母親自身の不安・悩み	9.51	5.41	6.50	5.55	5.28	6.86
母親自身の自由の制限	6.95	5.98	7.60 *	6.64 ***	5.75	6.33
しんせき関係	5.42	4.35	4.89	5.00	4.24	5.13
きょうだいの養育への制限	8.41	6.90	6.80	6.88	6.91	7.52
この子とのきょうだい関係上の問題点	6.08	4.75	5.10	5.00	4.69	5.49
祖父母とこの子とのかかわり	5.35	4.88	5.80 *	5.36 **	4.73	5.48
老親と夫婦とのかかわり	6.38	5.63	6.30 ***	6.20 ***	5.50	6.06
保育園・通園施設への不満	5.11	4.59	5.00	5.13 *	4.51	4.74

＊平均値・子育て支援センターより高値　　＊＊平均値より高値　　＊＊＊子育て支援センターより高値

表5　3歳児健診で最低限確認しておく項目

育てやすかった面	おとなしかった	PDD 因子
	よく寝た	健康因子
	ニコニコ笑う	健康因子
気質	活発である	健康因子
	寝る時間や起きる時間，食事の時間はほぼ一定である	健康因子
	知らない人やもの，場所になかなか慣れず時間がかかる	PDD 因子
	気が散りやすくてひとつの遊びに集中できない	ADHD 因子
言語面	意味がわからない音や叫び声をだしたりする	ADHD 因子
	テレビのコマーシャルなどの決まり文句やセリフを一言一句覚えていたりする	健康因子
	聞いたことをすぐにまねする	健康因子
行動面	ちょろちょろしている	ADHD 因子
	お気に入りのビデオなどがありそれを何度もみたがる	健康因子
	水遊びが好きである	健康因子
	人の話が聞けない	ADHD 因子
運動面	不器用である	PDD 因子
	特に心配なところがない	健康因子
対人面	初めての人に弱い	PDD 因子
	人がそのもので遊んでいても，目にはいったものだけにとらわれてしまい，つい奪い取ってしまうことがある	ADHD 因子
	遊びなどの場面で，自分の順番がなかなか待てない	ADHD 因子
感覚面	靴下を必ず脱ぐ	健康因子

表6　3歳児健診で最低限確認しておく項目と健診場面の留意点

項目	内容	健康因子	ADHD 因子	PDD 因子
育てやすかった面	おとなしかった	■	■	
	よく寝た		■	■
	ニコニコ笑う		■	■
気質	活発である		■	■
	寝る時間や起きる時間，食事の時間はほぼ一定である		■	■
	知らない人やもの，場所になかなか慣れず時間がかかる	■	■	
	気が散りやすくてひとつの遊びに集中できない	■		■
言語面	意味がわからない音や叫び声をだしたりする	■		■
	テレビのコマーシャルなどの決まり文句やセリフを一言一句覚えていたりする		■	■
	聞いたことをすぐにまねする		■	■
行動面	ちょろちょろしている	■		■
	お気に入りのビデオなどがありそれを何度もみたがる		■	■
	水遊びが好きである		■	■
	人の話が聞けない	■		■
運動面	不器用である	■	■	
	特に心配なところがない		■	■
対人面	初めての人に弱い	■	■	
	人がそのもので遊んでいても，目にはいったものだけにとらわれてしまい，つい奪い取ってしまうことがある	■		■
	遊びなどの場面で，自分の順番がなかなか待てない	■		■
感覚面	靴下を必ず脱ぐ		■	■

ADHD 因子は，4 つ以上あれば要注意であり，以下の項目に留意して対応する

1) 母親のメンタルヘルスを丁寧に危機，子育てに疲れていないか，チェックしつつ以下の事柄を勧める
   (ア) 休息は必要である
   (イ) 自分自身の時間を作る，確保する
   (ウ) 無理をしない
   (エ) なにかあれば，いつでもすぐに相談できる
2) 子育ての大変さを労い，相談にのれることを告げる
3) 親を責めないように配慮して，子どもにある心配な問題行動を尋ねる
4) 夫婦間の育児方針にずれが生じていないか尋ねる
5) 両家の親の動向，気持ちをさりげなく尋ねておく

PDD 因子は 3 つ以上あれば要注意であり，以下の項目に留意して対応する

1) 子育ての手応えの有無を尋ねる
2) 子どもの小さな，ささやかな変化，成長を増幅して伝え，ともに喜ぶ
3) 日々の生活で自由時間の確保について尋ねておく
4) 両家の親と育児方針をめぐってずれがないか，尋ねておく
5) 育児について，いつでも相談にのることを伝えておく
6) 保健師のほうで，この因子の強い親子に油断せず，十分な配慮を行う

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者氏名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Morinobu, S. Fujimaki, K. Kawano, K. Tanaka, K. Takahashi, J. Ohkawa, M. Yamawaki, S. Kato, N.	Influence of immobilization on stress on the expression and phosphatase activity of protein phosphatase 2A in the rat brain.	Biological Psychiatry	56	1060-1066	2003
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Ⅲ. 研究成果の刊行物・別刷

Influence of Immobilization Stress on the Expression and Phosphatase Activity of Protein Phosphatase 2A in the Rat Brain

Shigeru Morinobu, Koichiro Fujimaki, Ki-ichiro Kawano, Kazuhide Tanaka, Jun Takahashi, Masako Ohkawa, Shigeto Yamawaki, and Nobumasa Kato

Background: *Protein phosphatase 2A (PP2A) is a major kinase phosphatase that plays an important role in regulating the activities of protein kinase cascades. It has been revealed that stress changes neuronal gene expression by activating these cascades. We examined the expression of the catalytic subunit C and serine and threonine phosphatase activity of PP2A in the rat frontal cortex and hippocampus following various immobilization stress paradigms.*

Methods: *Immunoblot and immunohistochemical analyses were performed to examine the expression of PP2A. The level of phosphatase activity of PP2A was determined as the amount of free phosphate generated from a synthetic phosphopeptide.*

Results: *Immunoblot analysis revealed no significant change in the level of PP2A immunoreactivity in response to either a single or repeated stress. Immunohistochemical analysis revealed that neither a single nor repeated stress changed PP2A immunoreactivity in the hippocampus; however, the levels of serine and threonine phosphatase activity in the frontal cortex and hippocampus were significantly upregulated in response to a single or repeated stress.*

Conclusions: *These results demonstrated that both a single and repeated immobilization stress upregulated the activity of PP2A in the rat brain, suggesting that PP2A may be involved, at least in part, in the downregulation of protein kinase activation induced by stress.* Biol Psychiatry 2003;54:1060–1066 © *2003 Society of Biological Psychiatry*

Key Words: Immobilization stress, protein phosphatase 2A, serine, threonine, protein kinase, long-term potentiation, memory

Introduction

Various neuropharmacology studies examining the influence of stress on intracellular signal transduction pathways have been undertaken to elucidate the molecular mechanisms responsible for the pathogenesis of stress-related mental disorders, such as major depression and posttraumatic stress disorder (PTSD). These studies showed that extra cellular stimuli induce changes in the phosphorylation of transcription factors through the activation of protein kinases, which subsequently affect the expression of neuronal genes (Duman 1995; Sabban and Kvetnansky 2001). For example, it is reported that aversive stimuli, such as the forced swim test (Pliakas et al 2001) or restraint stress (Barrot et al 2002), induce the activation of signal transduction in the nucleus accumbens mediated by cyclic adenosine monophosphate (cAMP)–responsive element binding protein (CREB), and the overexpression of CREB reduces the behavioral responses to stimuli. Thus, it is conceivable that an increase in the activity of protein kinases such as protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase (CaMK) may play an important role in the pathogenesis of stress-related mental disorders.

In contrast, serine and threonine protein phosphatases are responsible for the dephosphorylation of protein kinases that are tightly associated with changes in gene expression (Goldberg 1999; Millward et al 1999). One of the protein phosphatases that is conserved from yeast to mammalians is protein phosphatase 2A (PP2A). Two types of PP2A have been identified: the PP2A core enzyme is composed of a regulatory subunit A and a catalytic subunit C, whereas the PP2A holoenzyme is formed by the binding of a cellular regulatory B subunit to the AC core enzyme. The subunit B affects the activity and substrate selectivity of PP2A (Garcia et al 2000; Goldberg 1999; Millward et al 1999). Recent studies indicate that PP2A also plays pivotal roles in the regulation of cell growth, gene expression, and development (Goldberg 1999). For example, the application of okadaic acid or

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calyculin A, inhibitors of PP1 and PP2A, influences the synaptic strength, as determined by measuring the synaptic efficacy in hippocampal long-term depression (LTD) (Kang-Park et al 2003; Mulkey et al 1993; Schnabel et al 2001). It has been suggested that PP2A is associated with gene transcription mediated by the signaling complex of CaMK IV and CREB or of p70 S6 kinase (Westphal et al 1998, 1999). Taken together, it appears that PP2A may play a fundamental role in stress-induced gene expression; however, it is not known whether stress changes the expression level or activity of PP2A.

Although it is clear that stress induces various dysfunctions in neural circuits, including the frontal cortex, hypothalamus, hippocampus, amygdala, and nucleus accumbens, increasing evidence from recent neuroimaging and histologic studies of stress-related mental disorders indicates that anatomic abnormalities of the frontal cortex, such as reductions in neuronal and glial density, and atrophy of the hippocampus were induced by stress (Bremner et al 1995; Rajkowska et al 2001; Uno et al 1989). In addition, we recently reported that a single immobilization stress upregulated the serine and threonine phosphatase activity of protein phosphatase 2B (calcineurin) in the rat frontal cortex and hippocampus (Takahashi et al 2001). In this context, we were interested in studying whether stress similarly affects the expression of PP2A or the level of serine and threonine phosphatase activity of PP2A in these brain regions. Because the subunit C was reported to have a catalytic function in PP2A activity (Goldberg 1999; Milward et al 1999), we examined the influence of a single as well as repeated immobilization stress on the expression of the subunit C and the activity of PP2A in the rat frontal cortex and hippocampus.

Methods and Materials

Animals and Treatment Paradigms

Male Sprague–Dawley rats weighing 200 g (Japan Charles-River, Yokohama, Japan) were group housed and maintained on a 12-hour light–dark cycle with food and water freely available. In the acute stress study, rats were subjected to a single session of immobilization stress for 5, 15, 45, or 90 min and then immediately sacrificed. In the chronic stress study, rats were subjected to repeated immobilization stress for a period of 45 min on 7 consecutive days and sacrificed immediately after the last stress. To investigate the influence of repeated stress on basal PP2A expression, rats were subjected to repeated stress on 6 consecutive days and sacrificed 24 hours after the last stress. In addition, some rats were sacrificed 30 min after a single, 45-min session of immobilization stress. Other rats were sacrificed 30 min after the last immobilization stress of repeated stress for 7 days. Rats were handled daily for 7 days before experiments. Rats were immobilized by placing each rat in a clear polyfilm disposable bag (Restraint Cones; Harvard Apparatus, South Natick, Massachusetts). All immobilization experiments were performed between 9 and 11 AM. All animal experiments were performed using procedures approved by the Shiga University Medical Science Animal Care Committee and Research Facilities for Laboratory Animal Science at Hiroshima University School of Medicine.

Immunoblotting

Immunoblot analysis for catalytic subunit C of PP2A was performed using the methods of Sim et al (1994) and Strack et al (1997) with minor modifications. In brief, the rat hippocampus was washed and homogenized by sonication in protein extraction buffer containing 1 × phosphate buffered saline (PBS), 1 mmol/L ethylenediamine tetraacetate (EDTA), 1% Nonidet P-40 (NP-40), .5% sodium deoxycholate, .1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, and 10 μg/mL aprotinin. The homogenates were centrifuged at 15,000 *g* at 4°C for 10 min.

Aliquots of the cellular extracts (containing 40 μg of protein) were then applied to 12% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated three times for a duration of 20 min each with blocking buffer, consisting of 5% nonfat dry milk in Tris-Tween buffered saline [TTBS; 10 mmol/L Tris(hydroxymethyl)aminomethane, pH 7.5, 100 mmol/L NaCl, .1% Tween 20], at room temperature, followed by incubation for 1 hour at room temperature with the primary antibody (i.e., antigoat PP2A antibody; 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, California). This antibody recognizes an epitope that maps at the carboxy terminus of the protein phosphatase 2A catalytic subunit. The membranes were washed in TTBS once for 15 min and twice at room temperature for 10 min and then incubated with horseradish peroxidase–conjugated antigoat immunoglobulin-G antibody (1:1000 dilution; Cell Signaling, Beverly, Massachusetts) as the secondary antibody. The blots were developed using the enhanced chemiluminescence (ECL) Western Blotting Detection System (Amersham Pharmacia Biotech, Buckinghamshire, UK). The density of the immunoreactive bands was quantified using a Macintosh-based ATTO Image analysis program (version 4.0; ATTO, Tokyo, Japan). The protein concentrations in the samples were determined using a BCA Protein Assay Kit (Pierce, Rockford, Illinois) and were verified to be in the linear range for the assay. An aliquot of pooled "standard" rat hippocampus was electrophoresed on one lane of each gel. Data were normalized against the rat hippocampus standard to minimize the between-blot variability.

Immunohistochemistry

Coronal sections of frozen rat brains (20 μm) were cut on a cryostat. Tissue sections were mounted on RNase-free Probon slides (Fischer, Pittsburgh, Pennsylvania) and were postfixed in PBS containing 4% paraformaldehyde for 5 min. The coronal sections were incubated with a polyclonal antigoat catalytic subunit of PP2A antibody (dilution 1:500; Santa Cruz Biotechnology). Staining was detected using the avidin–biotin–peroxi-

dase system (DAKO Liquid DAB+ Substrate-ChromogenSolution; Dako Corporation, Carpinteria, California). The immunohistochemical signal was detected using a digital video image analyzer (Nikon ACT-1 Ver 2.00, Tokyo, Japan).

Serine and Threonine Phosphatase Assay

The level of serine and threonine phosphatase activity of protein phosphatase 2A was quantitated as the amount of free phosphate derived from the synthetic phosphopeptide RRA(pT)VA (Promega, Madison, Wisconsin) by measuring the absorbance of molybdate-malachite green-phosphate complex as previously described (Cieslik et al 1998; Sidhu and Omiecinski 1997; Tian et al 1998), with a minor modification. Briefly, within 1 min after decapitation, the removed sections of frontal cortex and hippocampus were homogenized on ice using 1 mL of storage buffer containing 50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L ethylene glycol bis-tetraacetic acid (EDTA), .1% β-mercaptoethanol, .1 mmol/L leupeptin, and 75 μmol/L pepstatin A. After centrifugation (100,000 *g* at 4°C for 1 hour), the supernatant solution was applied to a Sephadex G-25 resin column and was centrifuged at 600 *g* at 4°C for 5 min, yielding the sample lysate in storage buffer.

The sample lysate (5 μL) was added to the reaction premix containing 100 μmol/L RRA(pT)VA in 5 μL of phosphate-free water, 10 μL PPTase-2A 5 × buffer (250 mmol/L imidazole, pH 7.2, 1 mmol/L EGTA, .1% β-mercaptoethanol, 500 μg/mL bovine serum albumin) and 30 μL of storage buffer in the well of a 96-well plate. After incubation for 30 min, 50 μL of Molybdate Dye/Additive mixture was added to stop the reaction. The optical densities of the samples were obtained 30 min later using a micro plate reader with a 630-nm filter. The level of serine and threonine phosphatase activity in each sample was calculated using a standard curve plotting free phosphate that had been generated by a phosphate standard solution. After the calculation, the level of phosphatase activity was divided by the protein content in each sample as measured by a Micro BCA assay (Pierce). All experiments were performed in duplicate.

Data Analyses

Immunoreactive bands were quantified with a Macintosh-based ATTO Image analysis program. The results were subjected to statistical analysis. The results of experiments containing groups of three or more rats were subjected to one-way analysis of variance (Fisher's protected least significant difference [PLSD] test for post hoc comparison) with a significance level of $p < .05$. The results of experiments containing groups of two rats were subjected to the Mann–Whitney *U*-test, with a significance level of $p < .05$.

Results

The influence of a single immobilization stress on the level of the C subunit of PP2A in the frontal cortex and hippocampus was examined by Western blot analysis. The level of PP2A immunoreactivity was measured at various

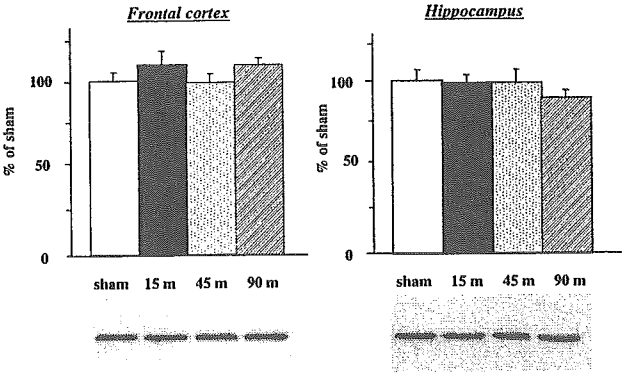

Figure 1. Influence of a single immobilization stress on the expression level of the catalytic subunit C of protein phosphatase 2A (PP2A) in rat brain regions. Representative immunoblots of the catalytic subunit C of PP2A in the rat frontal cortex and hippocampus after a single immobilization stress for 15, 45, or 90 min, are shown. Each bar represents the mean ± SEM for six rats.

times following the initiation of the immobilization stress. Each of the single immobilization stress paradigms used in this study (15, 45, or 90 min) did not result in a change in the level of PP2A immunoreactivity in either the rat frontal cortex or hippocampus (Figure 1). Similarly, the level of PP2A immunoreactivity in the rat frontal cortex and hippocampus did not change immediately after the rats were subjected to repeated immobilization stress for 7 days (Figure 2). The basal level of PP2A immunoreactivity in the rat frontal cortex and hippocampus also did not change after the rats were subjected to repeated stress for

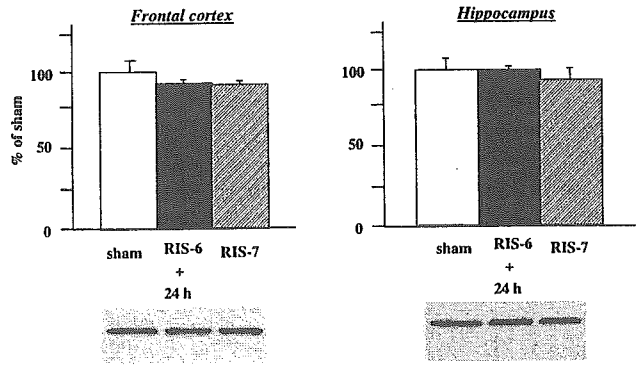

Figure 2. Influence of repeated immobilization stress on the expression level of the catalytic subunit C of protein phosphatase 2A (PP2A) in rat brain regions. Representative immunoblots of the catalytic subunit C of PP2A in the rat frontal cortex and hippocampus are shown. RIS-6 + 24 hours, 24 hours after the last stress of repeated stress (45 min/day) for 6 days; RIS-7, immediately after the last immobilization stress of repeated stress (45 min/day) for 7 days. Each bar represents the mean ± SEM for six rats. RIS, repeated immobilization stress.

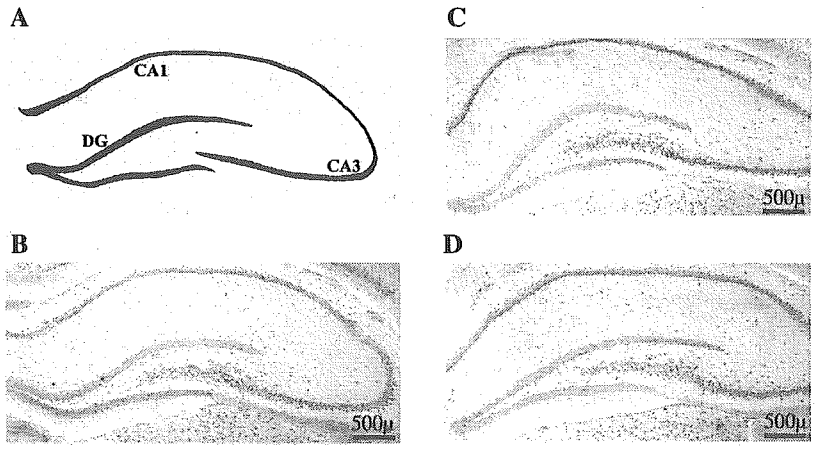

Figure 3. Immunohistochemical analysis of the influence of a single or repeated immobilization stress on protein phosphatase 2A (PP2A) expression in the rat hippocampus. (**A**) Schematic illustration of hippocampal slice. (**B**) Sham treatment. (**C**) Immediately after single immobilization stress for 45 min. (**D**) Immediately after the last immobilization of repeated immobilization stress (45 min/day) for 7 days. DG, dentate gyrus; CA, cornu anmonis.

6 days and sacrificed 24 hours after the last stress (Figure 2).

To identify the hippocampal cell layers where PP2A was expressed and regulated, we examined the influence of a single or repeated immobilization stress on the subunit C of PP2A expression in the hippocampus by immunohistochemical analysis. Our results show high PP2A expression in the CA1 and CA3 pyramidal cell layers and in the dentate gyrus granule cell layer in a sham-treated rat (Figure 3). Neither the single nor repeated immobilization stress changed the level of PP2A immunoreactivity in these hippocampal regions (Figure 3).

Because the activated form of PP2A dephosphorylates various substrate proteins and consequently regulates intracellular signal transduction, it is also important to determine whether immobilization stress influences the activity of PP2A in the brain. Therefore, we examined the level of PP2A activity using a chemically synthesized phosphopeptide that is compatible with serine and threonine phosphatase. In response to a single immobilization stress, time course analysis indicated that whereas an immobilization stress of 45 min or longer significantly increased PP2A activity in the frontal cortex, an immobilization stress of 15 min or longer significantly increased PP2A activity in the hippocampus (Figure 4). In contrast, no significant increase in PP2A activity was found 30 min after a single 45-min immobilization stress in either the frontal cortex or hippocampus (Figure 5). In rats that were subjected to repeated immobilization stress of 45 min per day for 7 days and sacrificed immediately after the last immobilization stress, there were significant increases in PP2A activity in the frontal cortex and hippocampus in comparison with the respective values in the sham-treated rats (Figure 6); however, in rats that were subjected to repeated immobilization stress and sacrificed 30 min after the last immobilization stress, there were no significant differences in the level of PP2A activity in these brain regions in comparison with those in the sham-treated rats (Figure 7).

Discussion

The results of this study demonstrate that both a single and repeated immobilization stress increase the serine and threonine phosphatase activity of PP2A in the rat frontal cortex and hippocampus in the absence of a change in the level of the subunit C of PP2A. Although the precise mechanism of the upregulation of PP2A activity in response to stress is unknown, several possibilities exist. One possible means of upregulation is through the activation of the sphingomyelin signaling pathway. Tumor necrosis factor (TNF) and interleukin-1 (IL-1) have been reported to increase sphingomyelin hydrolysis and to upregulate the level of ceramide, the latter of which has been shown to regulate the activity of PP2A (Dobrowsky

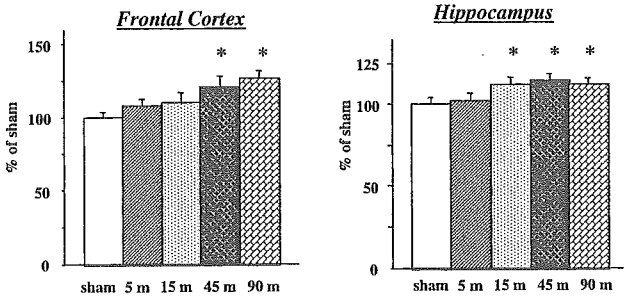

Figure 4. Influence of a single immobilization stress for 5, 15, 45, or 90 min on the level of serine and threonine phosphatase activity of protein phosphatase 2A (PP2A) in the rat frontal cortex and hippocampus. Data are expressed as the percentage of the sham level. Each bar represents the mean ± SEM of 10 rats. $*p < .05$ compared with sham group (one-way analysis of variance with Fisher's PLSD test). PLSD, protected least significant difference.

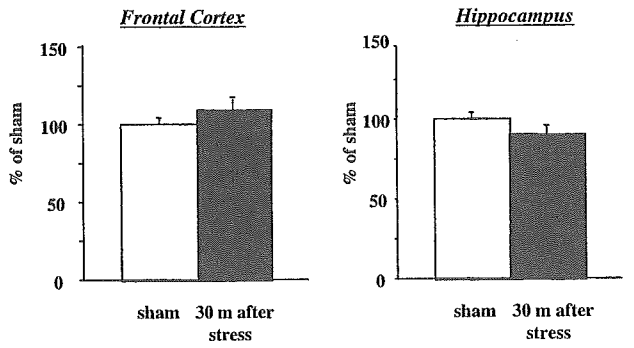

Figure 5. Levels of serine and threonine phosphatase activity of protein phosphatase 2A (PP2A) in the rat frontal cortex and hippocampus 30 min after a single restraint stress of 45 min. Data are expressed as the percentage of the sham level. Each bar represents the mean ± SEM of six rats.

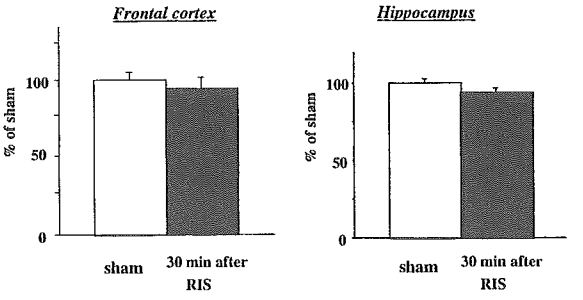

Figure 7. Levels of serine and threonine phosphatase activity of protein phosphatase 2A (PP2A) in the rat frontal cortex and hippocampus 30 min after the last immobilization stress of repeated stress (45 min/day) for 7 days. Data are expressed as the percentage of the sham levels. Each bar represents the mean ± SEM of six rats. RIS, repeated immobilization stress.

et al 1993; Law and Rossie 1995; Wolff et al 1994). Because a single immobilization stress increases the TNF-α level by activating glutamate receptors in the rat brain (Madrigal et al 2002), it is conceivable that the increase in the ceramide level mediated by TNF may upregulate the activity of PP2A. In addition, a single immobilization stress has also been reported to upregulate IL-1 activity in the rat hypothalamus (Shintani et al 1995). Therefore, it is also plausible that the enhanced activity of IL-1 in response to stress is associated with the upregulation of PP2A activity in the rat brain. In contrast, stimulation of N-methyl-D-aspartate (NMDA) receptors was reported to lead to the dissociation of PP2A from the NR3A (subunit of NMDA receptor) and the reduction of PP2A activity in the cerebrocortical neurons from mice at embryonic day 16 (Chan and Sucher 2001). It is conceivable that an immobilization stress increases the synaptic level of glutamate and subsequently stimulates NMDA receptors; however, because the levels of NR3R expression in the brain was lower in the adult rats compared with those in the neonatal rats (Chan and Sucher 2001), it is unlikely that the stress-induced activation of NMDA receptors dominantly regulates PP2A activity in the adult rat brain.

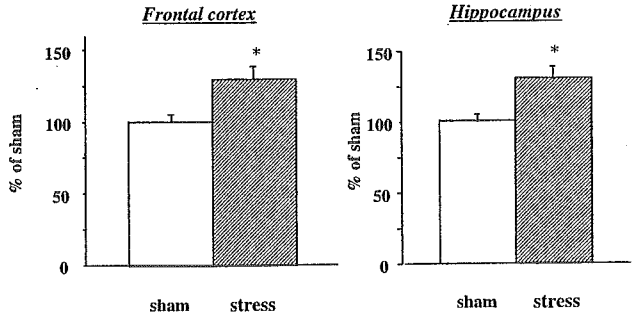

Figure 6. Influence of repeated immobilization stress on the level of serine and threonine phosphatase activity of protein phosphatase 2A (PP2A) in the rat frontal cortex and hippocampus immediately after the last immobilization stress of repeated immobilization stress (45 min/day) for 7 days. Data are expressed as the percentage of the sham level. Each bar represents the mean ± SEM of 11 rats. *$p < .05$ compared with sham group (Mann–Whitney U-test).

Furthermore, it is well known that the increase in the synaptic level of noradrenaline during immobilization stress in turn increases the level of cAMP and subsequently activates PKA via the stimulation of β-adrenoceptors (Duman 1995). Nagase et al (1997) reported that PKA phosphorylated a 72-kDa delta/B′′ subunit of PP2A and stimulated the activity of PP2A in the rat brain. Interestingly, it was recently reported in NRK cells that cAMP-mediated PP2A activation could occur without the activation of PKA (Feschenko et al 2002). Thus, it is likely that enhancement of cAMP-dependent signal transduction is also involved in the upregulation of PP2A activity in response to stress.

It has been reported that PP2A plays an important role in the dephosphorylation of various phosphoproteins such as autophosphorylated CaM kinases (CaMK; Barnes et al 1995; Ishida et al 1998; Park and Soderling 1995; Strack et al 1997; Westphal et al 1998). In addition, Fukunaga et al (2000) demonstrated that the reduced activity of PP2A induced by the activation of CaMK II autophosphorylation was important for maintenance of stable phosphorylation of synaptic proteins involved in the induction of long-term potentiation (LTP). Furthermore, recent studies examining the molecular mechanism of memory indicated that CREB phosphorylation mediated by CaMK IV phosphorylation was required for fear memory and for the consolidation of long-term memory (Kang et al 2001; Wei et al 2002). In this context, it is postulated that the upregulation of PP2A activity in response to stress may enhance the dephosphor-

ylation of autophosphorylated CaM kinases, which may subsequently impair the induction of LTP. Based on clinical studies of stress-related mental disorders, it is well known that disturbances in memory function, such as psychogenic amnesia, are often seen in patients with PTSD and major depression (Friedman and Yehuda 1995). Taken together, our results suggest that the upregulation of PP2A activity during stress may, at least in part, play a role in the impairment of memory in patients with PTSD and major depression.

On the other hand, the elevated intracellular Ca^{2+} level that results from the stimulation of glutamate receptors, L-type Ca^{2+} channels, and inositol 1,4,5-trisphosphate receptors in response to stress, leads to the autophosphorylation of CaM kinases and to subsequent changes in neuronal gene expression through CREB phosphorylation. Moreover, extracellular stimuli result in the phosphorylation of extracellular signal-regulated kinase (ERK) through the activation of mitogen-activated protein (MAP) kinase and ERK kinase (Anderson et al 1990; Gomez and Cohen 1991), which induces changes in neuronal gene expression via the activation of ribosomal S6 kinase (Westphal et al 1999). In addition, PP2A is involved in the regulation of gene expression because PP2A dephosphorylates and inactivates CaM kinases and MAP-kinase. Thus, it is conceivable that the upregulation of PP2A activity observed in this study inhibits stress-induced neuronal gene expression; however, it has yet to be determined whether the upregulation of PP2A activity in response to stress is associated with adaptive or maladaptive responses. Thus, the effect of pretreatment with a PP2A inhibitor, such as okadaic acid, on the reaction to stress in rats should be studied using behavioral and histochemical analyses.

The results of this study indicate that a single as well as repeated immobilization stress upregulates PP2A activity in the rat frontal cortex and hippocampus. Although the detailed pathway of PP2A-mediated signal transduction in viruses and parasites has been determined (Garcia et al 2000), the detailed pathway in neurons has not been elucidated. Therefore, if the actions of PP2A in neurons are found to be the same as those in nonneuronal cells, such as regulation of the MAP kinase pathway, PP2A might be involved, at least in part, in the regulation of the neuronal response to stress in the rat brain.

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