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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   該当なし。
2. 実用新案登録
   該当なし。
3. その他
   該当なし。

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分担研究報告書

神経発達障害関連分子に着目したバイオマーカー・治療薬の開発

分担研究者；那波　宏之　新潟大学脳研究所　教授

研究要旨

ニューレグリン１は統合失調症関連遺伝子として注目されている。連鎖ゲノムの変異領域は、そのプロモーター部に存在し、その発現を正制御しているとされるが、その病理学的対応は明らかになっていない。今回、統合失調症の神経発達障害仮説に基づき、我々はニューレグリン１蛋白を新生児マウスに投与し、どのような神経回路発達が傷害されるか、それは成熟後までも持続するか、認知行動変化との対応があるか等、その病態機序の解明を試みた。結果、本モデルは前頭葉内側部への永続的なドーパミン神経支配過剰を呈するとともに、ドーパミン過剰伝達によると思われる認知行動変化を示した。これらの事実は、新生児脳虚血等により誘導されたニューレグリン１は、ドーパミン神経発達をかく乱し統合失調症関連の脳機能障害を誘発しうることを実証している。よって本研究はドーパミン関連分子が治療薬標的として、再評価されるべきであることを提起している。

A. 研究目的

統合失調症のリスク因子として、出産障害や妊娠母体ウイルス感染が取りざたされている。これらの現象は、未熟な時期での全身性炎症が、サイトカインを介して脳神経発達を傷害するものと理解されている。数あるサイトカインの中でも、ニューレグリン１（NRG1)とその受容体である ErbB4 は統合失調症の感受性遺伝子として最も良く知られ、この疾患の病因・病態に深く関わっていると考えられている。しかし、その NRG1 に関する神経科学的な病態メカニズムは未だに不明である。「神経発達障害仮説」は現在もっとも注目されている統合失調症の原因仮説の一つである。この仮説に従うと NRG1 の遺伝子多型や胎児の環境因子は共に脳内の NRG1 の発現を調節していると考えられ、その破綻は脳発達を障害し統合失調症のリスクを上昇させていると仮想される。しかし、この仮説を基に NRG1 異常シグナルと統合失調症病態との関係を調べた研究はない。

最近、ほぼ全ての中脳ドーパミン神経が ErbB4 を発現していることが報告された。さらに、ErbB4 の発現は胎生期から出生後の神経発達期に特に高いことが示されている。しかし上記 NRG1 シグナルがどのようにこの神経細胞種の発達に関わっているかは検討されていなかった。そこで本研究では、ドーパミン神経を中心に、神経発達期における過剰 NRG1 シグナルが引き起こす神経発達障害の実態を解明し、統合失調症発症との関連性を検討した。

B. 研究方法

①新生児 NRG1 投与モデルの作製

動物は，C57BL/6N（日本チャールズリバ

ー）生後２日齢マウスを使用した．NRG1蛋白は，大腸菌内で組み替え蛋白として作製した。リフォールデイングの後ニッケルカラムとイオン交換カラムで精製した。この組替えマウス NRG1beta1 を生理食塩水に溶解させ、生後２日目より毎日計９回（生後 10 日目まで），頚部にラット体重 1g 当たり 1.0 マイクログラム皮下投与した。生後 11 日齢、もしくは生後 56－80 日齢で以下の実験に用いた。

②モノアミン測定

脳組織は解剖後、すぐに 0.1M の過塩素酸溶液中でホモジナイズとソニケーションにより、総モノアミンを抽出した。祖抽出液は、電気化学検出器（EICOM、モデル３００）を装着した HPLC（島津製作所）によりＯＤＳカラム（4.6x150mmm）で分離、検出した。移動層は 45mM のクエン酸―50mM 酢酸ナトリウム pH3.6 に 285mg/L のオクタンスルフォン酸ナトリウムと１３％メタノールよりなる。市販のドーパミン代謝物とセロトニン代謝物の標準品ドーパミン（DA)、水酸化フェニル酢酸（DOPAC），ホモヴァレリン酸(HVA)、水酸化インドール酢酸(HIAA)、セロトニン（５－HT）をスタンダードとした。

③メタアンフェタミン投与

ドーパミン放出促進剤（メタンフェタミン：MAP)を用いた薬理学的実験を行った。２ヶ月齢の NRG1 投与マウスを下記、運動量測定装置に１時間放置後、探索性運動が低下したころ、MAP を 0.5～3.0mg/kg の用量で腹腔内投与をし、その後の運動量亢進を１時間にわたって計測した。

④音驚愕反応の測定

小動物驚愕反応測定装置（San Diego Instruments）にて驚愕反応強度およびプレパルスインヒビションを測定した．驚愕反応を誘発する感覚刺激としては，音刺激（120ｄB）を用い，プレパルス刺激として環境騒音レベルより 3, 6, 9, 12 デシベル高い音圧の刺激を与えた．120 dB 単独の時の驚愕反応とプレパルスを組み合わせた時の反応比の減少分をプレパルスインヒビション(PPI)とした。

⑤運動量の測定と社会行動の測定

赤外線によるマウス用の自動運動量測定装置（Med Associates）にてラットの水平・垂直運動量を測定した。１時間後の新規環境になれた時点で、異なるケージ育った同性の標的真数を測定装置中に入れ、モデルマウスの標的マウスに対する社会行動（匂いカギ、追尾、マウンテイング）を計測した。

（倫理面への配慮）

これらの動物実験は、新潟大学動物実験倫理委員会からその実験法についての承認を得て実施した。

C. 実験結果

①新生児マウスへの NRG1 末梢投与の急性効果

新生児マウスへの NRG1 蛋白の末梢投与により中脳 NRG1 受容体（ErbB4）のリン酸化が亢進した。さらにビオチン化 NRG1 投与によって、末梢 NRG1 蛋白が中脳領域に到達していることも確認した。この脳領域では、ドーパミン神経が ErbB4 の主要な発現細胞であるので、末梢投与された NRG1 はこれらドーパミン神経に到達したと推察された。投与直後の脳内ではドーパミン生合成律速酵素であるチロシン水酸化酵素(TH)の発現・リン酸化に上昇が見られた。同時にこのマウス脳内のドーパミン含量に有意な増加が観察された。

②新生児マウスへの NRG1 末梢投与の慢性効果

成長後の NRG1 投与マウスのウエスタンブロットによると、前頭前野内側部で TH 発現量が持続的に上昇していた。TH の免疫染色でも NRG1 投与マウスの同部位におい

て過剰なドーパミン神経終末が観察されるとともにドーパミン放出能も対照群と比較して上昇していることが明らかとなった。この事実を裏付けるように、このNRG1投与マウスはドーパミンの遊離促進剤であるMAP に対して高感受性を示すことが、MAP 誘発運動量測定並びに c-fos 免疫染色によって明らかとなった。

③新生児マウスへのNRG1末梢投与の認知行動学的影響

成熟したNRG1投与マウスを統合失調症に関連する行動テストバッテリーにかけた。NRG1 投与マウスは PPI、潜在学習、社会性行動といった統合失調症患者で障害が見られる脳機能に選択的に障害を示した。また、これらの認知行動異常は抗精神病薬、リスペリドンの慢性投与によって改善した。なお、運動量試験、学習試験及び病理学的解析も平行して実施したが、一般行動や学習に異常はなく、脳構造にも問題が見られないことが確認された。

D. 考察

神経発達期の過剰NRG1投与はドーパミン神経発達の異常な亢進をまねき、前頭前野では成熟後もこの過剰神経支配が持続していた。そのためこのモデル動物は、MAP 投与後より多量のドーパミンを放出するため、より高い MAP 感受性を示したものと考えられた。このNRG1投与マウスのMAP高感受性は、fos の免疫染色でも顕著で、そのドーパミンの過剰支配の脳領域である前頭前野内側部でより高い fos 誘導が見られた。

このようなドーパミン神経機能の亢進は統合失調症の病態で疑われる所見である。NRG1 の活性標的となりうる興奮性・抑制性神経・シナプス及びグリア細胞への影響も、ウエスタンブロットで検討したが、変化がみられたのは上記のドーパミン神経系分子のみであった。このようなNRG1のドーパミン神経に対する選択的な作用は、□マウスのドーパミン神経は出生後発達が特に盛んであり、NRG1 シグナル変化に高感受性であったため、もしくは□内在性NRG1等、既存する神経栄養因子作用との競合関係がドーパミン神経でのみ少ないことが原因であると推察された。

これまでの報告によるとNRG1遺伝子ノックアウトマウスは今回のNRG1投与マウスと類似した統合失調症様の認知機能異常を示すことが知られている。一過的なNRG1 投与によって同様の認知行動異常が再現されたことは、NRG1 過剰シグナルも神経発達障害を誘発できることを示唆するものである。これらの知見はNRG1による統合失調症病態の発症の背景に神経発達障害が深く関わっていることを示しているとともに、ドーパミン神経の機能異常が、PPI等、一連の統合失調症に関連する認知機能異常に関連していることを示している。

E. 結論

統合失調症の関連遺伝子ニューレグリン 1はドーパミン神経発達に密接に関連しているので、ドーパミン関連分子が治療薬標的として探索・再評価されるべきである。

F. 健康危険情報

なし

G. 研究発表

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H．知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑 誌（主なもの）

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Relationships between psychological distress, coping styles, and HPA axis reactivity in healthy adults

Hiroaki Hori [a,c,d], Yuji Ozeki [a], Toshiya Teraishi [a,c], Junko Matsuo [a], Yumiko Kawamoto [a], Yukiko Kinoshita [a], Shiho Suto [a], Sumio Terada [c], Teruhiko Higuchi [b], Hiroshi Kunugi [a,d,*]

[a] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo, 187-8502, Japan
[b] National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo, 187-0031, Japan
[c] Section of Neuroanatomy and Cellular Neurobiology, Department of Systems Neuroscience and COE Program for Brain Integration and Its Disorders, Tokyo Medical and Dental University Graduate School, 1-5-45, Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan
[d] CREST, JST (Japan Science and Technology Agency), Saitama, 332-0012, Japan

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ABSTRACT

Psychological distress and coping styles have been suggested to relate to altered function in the hypothalamic-pituitary-adrenal (HPA) axis, although there remains much to be understood about their relationships. High and low cortisol levels (or reactivity) both represent HPA axis dysfunction, with accumulated evidence suggesting that they are linked to different types of psychopathology. The dexamethasone (DEX)/corticotropin-releasing hormone (CRH) test has been extensively used to identify HPA axis abnormalities in various psychiatric conditions including mood disorders; however, the possible associations of psychological distress and coping styles with HPA axis function have not been well documented using this test. Here, we examined the relationships of HPA axis reactivity as measured by the DEX/CRH test with subjectively perceived psychological distress and coping styles, both of which were assessed with self-report questionnaires, in 121 healthy volunteers. Subjects were divided into three groups by the cortisol suppression pattern, namely the incomplete-suppressors (DST-Cortisol $\geq$ 5 μg/dL or DEX/CRH-Cortisol $\geq$ 5 μg/dL), moderate-suppressors (DST-Cortisol $<$ 5 μg/dL and 1 μg/dL $\leq$ DEX/CRH -Cortisol $<$ 5 μg/dL), and enhanced-suppressors (DST-Cortisol $<$ 5 μg/dL and DEX/CRH-Cortisol $<$ 1 μg/dL). The enhanced-suppressors showed significantly higher scores in obsessive-compulsive, interpersonal sensitivity and anxiety symptoms and significantly more frequent use of avoidant coping strategy, compared to the other two groups. These results point to the important role of enhanced suppression of cortisol, or blunted cortisol reactivity, in non-clinical psychopathology such as avoidant coping strategy and greater psychological distress.

1. Introduction

A wide variety of stress is associated with alteration in the hypothalamic-pituitary-adrenal (HPA) axis function. Studies looking at cortisol as the main output substance of the HPA axis have thus been critical to advancing our understanding of psychobiological underpinnings of various stress-related conditions (de Kloet et al., 2005; Heim et al., 2000). For instance, perceived stress in everyday life (Pruessner et al., 1999), stressful situations such as academic examinations and seafaring (Droogleever Fortuyn et al., 2004; Liberzon et al., 2008), self-reported symptoms (Van den Bergh et al., 2008), psychological coping styles (Nicolson, 1992; O'Donnell et al., 2008), rejection sensitivity (Tops et al., 2008), sleep status (Backhaus et al., 2004; Lasikiewicz et al., 2008; Wright et al., 2007) and personality profile (Tyrka et al., 2007) have been reported to be associated with alteration in cortisol levels. These studies in healthy subjects have investigated HPA axis function using several different cortisol measures including diurnal cortisol profiles, cortisol awakening response, and cortisol reactivity to psychosocial challenge tests such as Trier Social Stress Test (Kirschbaum et al., 1993).

On the other hand, HPA axis function in clinical populations, particularly in patients with major depression, has been investigated with pharmacological challenge tests including dexamethasone (DEX) suppression test (DST, Carroll et al., 1976) and DEX/corticotropin-releasing hormone (CRH) test (Heuser et al., 1994a;

* Corresponding author. Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo, 187-8502, Japan. Tel./fax: +81 42 346 1714.
E-mail address: hkunugi@ncnp.go.jp (H. Kunugi).

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Holsboer et al., 1987). The DEX/CRH test is an integrated challenge test for HPA axis function that combines DEX-pretreatment with CRH administration on the following day; thus, it is essentially a DST followed by CRH challenge. The merit of this combined test is that at the moment of CRH infusion the HPA axis is downregulated due to negative feedback induced by the DEX. In the DEX/CRH test a relatively high dose (i.e., 1.5 mg) of DEX is usually used, whereas DST studies, in particular those which examine the HPA function of post-traumatic stress disorder (PTSD), have used a lower dose (i.e., 0.5 mg or 1 mg) of DEX (e.g., Grossman et al., 2003; Yehuda et al., 2004). Sensitivity of the DEX/CRH test in depressed patients has been shown to be high in prior studies including ours (Heuser et al., 1994a; Kunugi et al., 2004, 2006; Watson et al., 2006b). Moreover, this test has revealed altered HPA axis function in those individuals with specific characteristics: dampened cortisol reactivity in healthy adults reporting childhood emotional abuse (Carpenter et al., 2009), increased cortisol responses in healthy adults reporting childhood parental loss with the exception of attenuated cortisol responses in those with parental desertion and low levels of care (Tyrka et al., 2008b), increased cortisol responses in healthy adults with certain personality traits (Tyrka et al., 2006, 2008a), and attenuated cortisol responses in depressed women on job-stress-related longterm sickleave (Rydmark et al., 2006; Wahlberg et al., 2009). On the other hand, the possible associations of more commonly presented psychopathology such as perceived distress in everyday life and coping styles with HPA axis function have not been well documented using the DEX/CRH test. However, these psychological measures are suggested to relate to altered cortisol level (Heim et al., 2000, 2002; Nicolson, 1992; O'Donnell et al., 2008; Pruessner et al., 1999; Van den Bergh et al., 2008). For instance, severity of daily hassles in the past month was negatively related to cortisol concentrations (Heim et al., 2002). Perceived stress was positively, and burnout was negatively, associated with cortisol levels after DEX administration (Pruessner et al., 1999). Passive coping is suggested to relate to hypocortisolism (Heim et al., 2000). Healthy adults scoring high in either problem engagement or seeking social support showed lower cortisol levels (O'Donnell et al., 2008). Given these findings, it would be of interest to examine HPA axis function in relation to psychopathology at a non-clinical level such as psychological distress and coping styles by using the DEX/CRH test.

Various kinds of psychiatric disorders have been shown to be associated with HPA axis hyperactivity as reflected by the high cortisol levels and impaired negative feedback inhibition due to an impaired corticosteroid receptor function (Holsboer, 2000). On the other hand, a number of psychoneuroendocrinological studies have demonstrated that a variety of conditions are associated with hypocortisolism, including low basal cortisol levels, enhanced sensitivity to the negative feedback, and blunted reactivity of provoked cortisol. Examples of psychiatric conditions characterized by hypocortisolism include PTSD, chronic fatigue syndrome, fibromyalgia and atypical depression (Fries et al., 2005; Heim et al., 2000). Together, while both of these two extremes of cortisol activity can represent HPA axis dysfunction, they are likely to be linked to different types of psychopathology. Concerning hypocortisolism, there remains much to be clarified as to its natural course and meaning. Although hypocortisolism is considered to represent the result of prolonged stress exposure (Fries et al., 2005; Heim et al., 2000; Ising et al., 2005), a condition so-called "allostasis" (McEwen, 2003), there also exists some evidence suggesting that this state could be a preexisting vulnerability to stress-related disorders (Delahanty et al., 2000; Wahlberg et al., 2009; Yehuda et al., 2000).

Arginine vasopressin (AVP), in addition to CRH, is an HPA axis secretagogue. AVP produced in parvocellular neurons of hypothalamic paraventricular nucleus (PVN) and secreted into pituitary portal vein system plays an important role in stress response (Herman, 1995; Romero and Sapolsky, 1996). It is reported that, in chronic stress paradigms, the expression of AVP in parvocellular neurons increases and pituitary V1b receptor, through which AVP stimulates the ACTH secretion, up-regulates (Aguilera et al., 1994; Aguilera and Rabadan-Diehl, 2000). There also exist clinical studies that support this notion. For example, de Kloet et al. (2008) have recently reported elevated plasma AVP levels in veterans with PTSD. Watson et al. (2006a) measured plasma AVP levels after pre-treatment of DEX in patients with chronic depression and those with bipolar disorder, and found significantly higher post-DEX AVP levels in the patient groups than in healthy controls, suggesting that post-DEX AVP levels could be more sensitive than baseline AVP levels in detecting HPA axis abnormalities. These findings raise the possibility that the post-DEX AVP measure may help understand whether the hypocortisolism, if present, is a result of chronic HPA axis overactivity or a preexisting vulnerability factor for psychopathology.

In this context, the present study sought to examine the relationships between subjectively perceived psychological distress, psychological coping styles and the cortisol suppression pattern to the DEX/CRH test in non-clinical volunteers. We also examined the relationships of these psychological measures with the post-DEX AVP level to see whether the possible low cortisol levels would reflect allostatic shift or preexisting vulnerability. The study hypothesis was that the higher cortisol levels (or less suppression of cortisol) and/or lower cortisol levels (or more suppression of cortisol) would be related to greater distress and a unique pattern of coping strategies. If the low cortisol, together with elevation of AVP, is related to these psychological measures, it would indicate allostatic shift; while if the low cortisol, together with no elevation of AVP, is related to such psychological measures, it may indicate preexisting vulnerability.

2. Materials and methods

2.1. Participants

From February 2006 to December 2008, 121 healthy volunteers (age range: 20–70; male, 28, female, 93) were recruited from the community, through advertisements in free local information magazines which contained a wide variety of information including healthcare-related information and by our website announcement. Participants were interviewed using the Japanese version of the Mini-International Neuropsychiatric Interview (MINI, Otsubo et al., 2005; Sheehan et al., 1998) by research psychiatrists (H.H., Y.O., T.T. and H.K.), and only those who demonstrated no current Axis I psychiatric disorders, including PTSD, were enrolled in this study. In addition, those who demonstrated one or more of the following conditions during a non-structured interview by an experienced psychiatrist were excluded from this study: past or current contact to psychiatric services, taking psychotropic drugs or had a history of regular use of psychotropics, and the other obvious self-reported signs of past primary psychotic and mood disorders as well as PTSD. Additional exclusion criteria were as follows: having a prior medical history of central nervous system disease or severe head injury, having major systemic medical illnesses, having a history of substance dependence or abuse, or taking corticosteroids or antihypertensive medication. No subjects reported that they were on oral contraceptives or estrogen replacement therapies. The present experiments on our subjects were conducted in accordance with the Declaration of Helsinki. After the nature of the study procedures had been fully explained, written informed consent was obtained

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from all subjects. The study was approved by the ethics committee of the National Center of Neurology and Psychiatry, Japan.

2.2. DEX/CRH test procedure and presentation for neuroendocrine data

The DEX/CRH test was administered to all subjects by a single examiner (H.H.) according to a protocol proposed in a previous report (Kunugi et al., 2006). First, they took 1.5 mg of DEX (Banyu Pharmaceutical Corporation, Tokyo, Japan) orally at 2300 h. On the next day, they attended our laboratory and sat on a comfortable couch in a calm room. A vein was cannulated at 1430 h to collect blood at 1500 and 1600 h via an intravenous catheter. Human CRH (100 μg) (hCRH 'Mitsubishi', Mitsubishi Pharma Corporation, Tokyo, Japan) was administered intravenously at 1500 h, immediately after the first blood collection. Subjects fasted and rested semi-supine throughout the testing. Blood samples were immediately centrifuged and stored at −20 °C. Plasma concentrations of cortisol and AVP were measured by radioimmunoassay at SRL Corporation (Tokyo, Japan). The detection limits for cortisol and AVP were 1.0 μg/dL and 0.2 pg/mL, respectively (SRL Corporation, Tokyo, Japan). Cortisol and AVP values under the detection limits were treated as 0 μg/dL and 0 pg/mL, respectively. For cortisol, the intra-assay coefficients of variation at 2.37 μg/dL, 13.02 μg/dL, and 36.73 μg/dL were 6.90%, 4.94%, and 5.78%, respectively. The inter-assay coefficients of variation at 2.55 μg/dL, 13.04 μg/dL, and 34.17 μg/dL were 8.91%, 6.03%, and 6.44%, respectively. For AVP, the intra-assay coefficients of variation at 0.97 pg/mL, 1.64 pg/mL, and 2.88 pg/mL were 1.7%, 7.2%, and 3.5%, respectively. The inter-assay coefficients of variation at 0.94 pg/mL, 1.59 pg/mL, and 2.88 pg/mL were 3.9%, 10.3%, and 6.9%, respectively (SRL Corporation, Tokyo, Japan). Outcome measures of this neuroendocrine test were the DST-Cortisol (i.e., the concentration of cortisol [μg/dl] at 1500 h), DEX/CRH-Cortisol (i.e., the concentration of cortisol at 1600 h) and DST-AVP (i.e., the concentration of AVP [pg/ml] at 1500 h). To further dissect the extent to which the subject's HPA axis responded to the CRH challenge, the magnitude of change from DST-Cortisol to DEX/CRH-Cortisol, namely ΔCortisol, was calculated for each subject. For DST-AVP, data were available for only 106 of the total 121 subjects. This reduction of subjects was because we started to collect the AVP data on May 2006, which was about 3 months after the study initiation.

A cut-off criterion for suppression status was considered as follows; 'Incomplete-suppressors' were defined *a priori* to be individuals where either or both of DST- and DEX/CRH-Cortisols were equal to or more than 5 μg/dL. This cut-off value was based on our previous studies (Kunugi et al., 2004, 2006), where the cortisol value of 5 μg/dL was shown to sensitively distinguish depressed patients from healthy controls. Based on these reports of ours, recent studies (Ising et al., 2007; Schüle et al., 2009) also used the same cut-off value of cortisol. Given these findings, in the present study we assumed that the cortisol value of 5 μg/dL would be also useful in detecting those participants whose negative feedback of cortisol was "incomplete". On the other hand, 'Enhanced-suppressors' were defined as those individuals whose DST-Cortisol was less than 5 μg/dL and DEX/CRH-Cortisol was less than 1 μg/dL, because this DEX/CRH-Cortisol value corresponded to its detection limit and can therefore be regarded as an extremely low cortisol level. The remaining individuals were considered to be 'Moderate-suppressors'. We would like to note that the 'Incomplete-suppressors' were the sum total of the 'Intermediate suppressors' and 'Non-suppressors' as defined in our previous studies (Kunugi et al., 2004, 2006). This slight modification on the grouping criterion was because it was expected that very few subjects would fall into the 'Non-suppressors' group since the present study included only healthy subjects.

2.3. Psychological assessment

To assess subjectively perceived psychological distress and psychological coping styles, the following two self-report questionnaires were distributed to the participants.

2.3.1. The hopkins symptom checklist (HSCL)

Subjectively perceived psychological distress during one week preceding the neuroendocrine test was assessed via the Hopkins Symptom Checklist (HSCL, Derogatis et al., 1974), a self-report questionnaire consisting of 58 (or 54) items which are scored on 5 underlying symptom dimensions, i.e., somatization, obsessive-compulsive, interpersonal sensitivity, anxiety, and depression symptoms. In the present study, a validated Japanese version of the HSCL (Nakano, 2005) comprising 54 items was used. In this questionnaire, subjects were instructed to rate each item based on the distress perceived during the previous week, using a four-point scale of frequency, with "not-at-all" being scored 1, "occasionally", 2, "sometimes", 3, and "frequently", 4. All of the 121 participants answered this questionnaire.

2.3.2. The ways of coping checklist (WCCL)

Psychological coping can be defined as the thoughts and behaviors used to manage the internal and external demands of situations that are appraised as stressful (Folkman and Moskowitz, 2004). Coping styles of the participants were assessed using the Japanese version of the Ways of Coping Checklist (WCCL) (Folkman and Lazarus, 1985; Nakano, 1991), a self-report questionnaire consisting of 47 items which measure each participant's preferred coping styles using a four-point scale of frequency, with "not used" being scored 0, "not frequently used", 1, "sometimes used", 2, and "regularly used", 3. The 47 items were grouped into 6 coping strategies, namely planful problem-solving, positive reappraisal, seeking social support, self-blame, wishful thinking, and escape-avoidance (Nakano, 1991), thus measuring both problem-focused and emotion-focused coping strategies. The WCCL data were obtained from 102 of the total 121 participants. This reduction of participants was because we started to collect the WCCL data on June 2006.

2.4. Statistical analysis

Averages are reported as means ± SD (standard deviation). Categorical variables were compared using the χ^2 test. Mann–Whitney *U*-test was used to compare hormonal measures between two groups. The analysis of variance (ANOVA) or Kruskal–Wallis test was used to examine differences between three groups. Pearson's r was used to examine correlations among psychological measures, while Spearman's ρ was used to examine correlations among hormonal data or between hormonal data and psychological measures. To examine the difference between DST- and DEX/CRH-Cortisols, Wilcoxon signed rank test was performed. The analysis of covariance (ANCOVA), controlling for confounding variables, was performed to compare the scores of questionnaires between the three participant groups. Since age and sex have been shown to significantly influence the cortisol levels (e.g., Heuser et al., 1994b; Kunugi et al., 2006; Kunzel et al., 2003), these variables were considered as confounders regardless of the present data. Statistical significance was set at two-tailed $p < 0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 11.0 (SPSS Japan, Tokyo).

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Table 1
Correlations between coping styles and psychological distress (Pearson's r).

	Somatization	Obsessive-compulsive	Interpersonal sensitivity	Anxiety	Depression
Problem-solving	−0.10	−0.22*	−0.24*	−0.17	−0.25*
Positive reappraisal	−0.08	−0.16	−0.26**	−0.20*	−0.32**
Social support	0.11	0.14	0.14	0.16	−0.03
Self-blame	0.29**	0.47***	0.52***	0.49***	0.48***
Wishful thinking	0.22*	0.42***	0.37***	0.40***	0.31**
Escape-avoidance	0.08	0.30**	0.33***	0.30**	0.20*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results

3.1. Demographic characteristics of the subjects

The numbers of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 55, 54, and 12, respectively, indicating that the enhanced-suppressors corresponded to approximately the bottom 10% of total subjects for cortisol levels. The mean ages of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 46.8 ± 14.3, 42.7 ± 15.1, and 44.4 ± 14.7, respectively. These three suppression groups did not significantly differ in age [$F(2,118) = 1.49$, $p = 0.23$]. Male/female ratios of incomplete-suppressors, moderate-suppressors, and enhanced-suppressors were 6/49, 15/39, and 7/5, respectively. There was a significant difference in sex distribution [$\chi^2(2) = 13.6$, $p = 0.001$]; males demonstrated significantly greater suppression than females.

3.2. Correlations between coping styles and psychological distress

Table 1 shows the correlations between coping styles assessed with the WCCL and psychological distress assessed with the HSCL. Significant negative correlations were seen between problem-focused coping strategies (i.e., problem-solving and positive reappraisal) and greater psychological symptoms including interpersonal sensitivity and depression. In contrast, significant positive correlations were observed between emotion-focused coping strategies (i.e., self-blame, wishful thinking and escape-avoidance) and most of the symptom dimensions. Social support was not significantly related to any of the symptom dimensions.

3.3. Relationships between hormonal measures

The cortisol values for the three suppressor groups on the three cortisol indices are provided in Table 2. DST-Cortisol of 64 subjects and DEX/CRH-Cortisol of 12 subjects fell under the detection limit, while DST-AVP did not fall under the detection limit in any subjects. DEX/CRH-Cortisol was significantly higher than DST-Cortisol in the whole sample, as expected ($p < 0.001$; Wilcoxon signed rank test). There was no significant correlation of DST-AVP with DST-Cortisol ($\rho = 0.07$, $p = 0.50$), DEX/CRH-Cortisol ($\rho = 0.03$, $p = 0.75$), or ΔCortisol ($\rho = 0.02$, $p = 0.83$). The three suppression groups did not significantly differ in DST-AVP [Kruskal–Wallis $\chi^2(2) = 0.14$, $p = 0.93$].

3.4. Correlations between hormonal and psychological measures

No significant correlations were seen between DST-Cortisol and any measures of the two questionnaires (all $p > 0.2$). No significant correlations were seen between DEX/CRH-Cortisol and any measures of the two questionnaires (all $p > 0.2$) except for interpersonal sensitivity ($\rho = -0.20$, $p = 0.03$) in the HSCL. Similarly, no significant correlations were observed between ΔCortisol and any measures of the two questionnaires (all $p > 0.2$) except for interpersonal sensitivity ($\rho = -0.21$, $p = 0.02$) in the HSCL. No significant correlation was found between DST-AVP and any of the outcomes of the two questionnaires (all $p > 0.1$).

3.5. Relationships between psychological measures and DEX/CRH outcomes

3.5.1. Psychological distress and DEX/CRH outcomes

Fig. 1 shows the relationships between 5 symptom dimensions of the HSCL and DEX/CRH suppression status. The ANCOVA on 5 symptoms controlling for age and sex showed significant main effects of the suppression status on obsessive-compulsive [$F(2,114) = 5.19$, $p = 0.007$], interpersonal sensitivity [$F(2,114) = 5.43$, $p = 0.006$], and anxiety [$F(2,114) = 5.86$, $p = 0.004$], symptoms. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to moderate-suppressors alone, had significantly greater scores on these three symptom dimensions, while no significant differences were seen between incomplete- and moderate-suppressors (Fig. 1).

However, a considerable portion of the subjects fell into the incomplete-suppressors and thus we considered that this group would not necessarily represent those individuals whose cortisol levels were abnormally high. Therefore, to confirm the results obtained by the main analysis, the same ANCOVA was repeated with another grouping criterion as follows: 'incomplete-suppressors' to be individuals where either or both of DST- and DEX/CRH-

Table 2
Plasma cortisol concentrations (mean ± SD (range)) for the three subject groups, based on the suppression pattern.

	Incomplete-suppressors ($n = 55$)[d]	Moderate-suppressors ($n = 54$)[e]	Enhanced-suppressors ($n = 12$)[f]
DST-Cortisol[a]	1.4 ± 1.5 (0 ~ 5.8)	0.4 ± 0.7 (0 ~ 1.9)	0.1 ± 0.3 (0 ~ 1.1)
DEX/CRH-Cortisol[b]	10.0 ± 4.6 (5.0 ~ 25.1)	2.5 ± 1.1 (1.1 ~ 4.9)	0 ± 0 (0 ~ 0)
ΔCortisol[c]	8.6 ± 4.4 (2.3 ~ 20.2)	2.1 ± 1.3 (−0.3 ~ 4.8)	−0.1 ± 0.3(−1.1 ~ 0)

[a] The concentration of cortisol [μg/dl] at 1500 h (i.e., immediately before the CRH challenge).
[b] The concentration of cortisol [μg/dl] at 1600 h (i.e., 1 h after the CRH challenge).
[c] Defined as "DEX/CRH-Cortisol minus DST-Cortisol".
[d] Defined as "DST-Cortisol ≥ 5 or DEX/CRH-Cortisol ≥ 5".
[e] Defined as "DST-Cortisol < 5 and 1 ≤ DEX/CRH -Cortisol < 5".
[f] Defined as "DST-Cortisol < 5 and DEX/CRH-Cortisol < 1".

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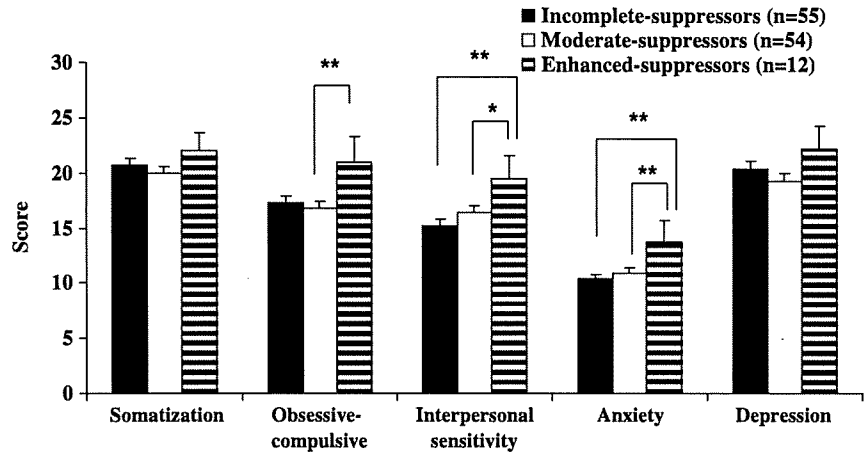

Fig. 1. Comparisons of scores on the 5 dimensions of the Hopkins Symptom Checklist (HSCL) between the three suppression groups. Black, white, and borderline bars are incomplete-suppressors (defined as "DST-Cortisol ≥5 or DEX/CRH-Cortisol ≥5"; $n = 55$), moderate-suppressors (defined as "DST-Cortisol <5 and 1 ≤ DEX/CRH -Cortisol <5"; $n = 54$), and enhanced-suppressors (defined as "DST-Cortisol <5 and DEX/CRH-Cortisol <1"; $n = 12$), respectively. $^{*}p < 0.05$; $^{**}p < 0.01$. Error bars represent standard errors of the mean.

Cortisols were equal to or more than 13 μg/dL, 'enhanced-suppressors' to be those whose DST-Cortisol was less than 13 μg/dL and DEX/CRH-Cortisol was less than 1 μg/dL, and 'moderate-suppressors' to be the remaining individuals. The reason why we here used the cortisol level of 13 μg/dL, instead of the original 5 μg/dL, as the cut-off value for the 'incomplete-suppressors' was that this value corresponded to approximately the top 10% of total subjects for cortisol levels. This 10% derived from the fact that the cortisol value of "enhanced-suppressors" corresponded to approximately the bottom 10% of total subjects. Using this new grouping, additional ANCOVA on the 5 symptoms controlling for age and sex was performed, again showing significant main effects of the suppression status on obsessive-compulsive [$F(2,114) = 4.63$, $p = 0.012$], interpersonal sensitivity [$F(2,114) = 5.50$, $p = 0.005$] and anxiety [$F(2,114) = 5.81$, $p = 0.004$] symptoms. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to moderate-suppressors alone, scored significantly higher on these three symptom dimensions, while no significant differences were seen between incomplete- and moderate-suppressors (data not shown).

3.5.2. Coping styles and DEX/CRH outcomes

The relations between the 6 different coping styles of WCCL and suppression status are provided in Fig. 2. The ANCOVA on the 6 coping subscales controlling for age and sex showed a significant main effect of the suppression status on escape-avoidance [$F(2,95) = 5.26$, $p = 0.007$]. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups, had significantly greater scores on this coping strategy, while no significant differences were found between incomplete- and moderate-suppressors (Fig. 2).

The additional ANCOVA with the other grouping criterion of suppression status on the 6 coping subscales showed significant main effects of the suppression status on wishful thinking [$F(2,95) = 3.31$, $p = 0.041$] and escape-avoidance [$F(2,95) = 5.56$, $p = 0.005$]. Post-hoc analyses with Bonferroni correction revealed that the enhanced-suppressors, compared to the other two groups or to incomplete-suppressors alone, scored significantly higher on these two coping strategies, while no significant differences were seen between incomplete- and moderate-suppressors (data not shown).

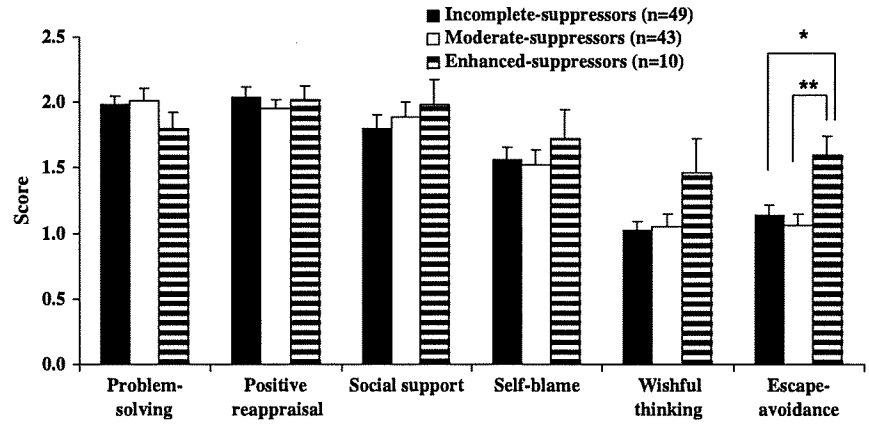

Fig. 2. Comparisons of scores on the 6 subscales of the Ways of Coping Checklist (WCCL) between the three suppression groups. Black, white, and borderline bars are incomplete-suppressors (defined as "DST-Cortisol ≥5 or DEX/CRH-Cortisol ≥5"; $n = 49$), moderate-suppressors (defined as "DST-Cortisol <5 and 1 ≤ DEX/CRH-Cortisol <5"; $n = 43$), and enhanced-suppressors (defined as "DST-Cortisol <5 and DEX/CRH-Cortisol <1"; $n = 10$), respectively. $^{*}p < 0.05$; $^{**}p < 0.01$. Error bars represent standard errors of the mean.

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4. Discussion

We examined the relationships of cortisol reactivity to the DEX/CRH test with subjectively perceived psychological distress and psychological coping styles as assessed with the self-report questionnaires in a non-clinical population. The most salient finding was that the enhanced cortisol suppression to the DEX/CRH test, or blunted cortisol response to CRH challenge, was significantly related to various psychological distress and avoidant coping style.

Besides the well-established relation between acute stress and elevated cortisol levels, numerous studies have linked low cortisol levels to various kinds of stress, in particular to chronic stress (reviewed in Heim et al., 2000). In line with this, a large number of DST studies using a low dose of DEX have observed enhanced suppression of cortisol in a variety of psychiatric conditions including PTSD (Grossman et al., 2003; Yehuda et al., 1993). To our knowledge, however, the present study is the first DEX/CRH study where the overtly defined enhanced suppression, in addition to the incomplete suppression, was examined in the context of non-clinical psychological distress and coping styles. Based on the previous literature, it was hypothesized that both incomplete and enhanced suppression of cortisol due to the negative feedback by DEX administration would be related to greater distress and/or a unique pattern of coping strategies. The significant associations of enhanced suppression with greater distress and more frequent use of avoidant coping strategy supported the hypothesis, while contrary to our prediction incomplete suppression was not significantly related to any of the psychological measures.

We observed significant negative correlations between interpersonal sensitivity in the HSCL and cortisol values, namely DEX/CRH-Cortisol and ΔCortisol. A significant relation between interpersonal sensitivity and enhanced cortisol suppression was also found. These results were in line with a recent study showing the association between higher rejection sensitivity and lower cortisol awakening responses in community women (Tops et al., 2008). In addition, the significant relationships of enhanced cortisol suppression with obsessive-compulsive and anxiety symptoms, but not depressive symptom, point to the possibility that enhanced suppression is more related to anxiety symptoms than depressive symptoms in healthy populations. Several studies investigated cortisol levels as measured by DST in patients with obsessive-compulsive disorder (OCD), and the majority of these studies found that OCD patients did not show non-suppression or their baseline cortisol levels did not differ from healthy controls (e.g., Kuloğlu et al., 2007; Lieberman et al., 1985). These previous findings, combined with the present result, might suggest that obsessive-compulsive symptoms are associated with normal cortisol suppression to DEX and subsequent blunted response to CRH challenge.

Several lines of research have documented the relationship of coping styles with psychobiological measures including cortisol levels (Frecska et al., 1988; Nicolson, 1992; O'Donnell et al., 2008). While cortisol activity has been considered to reflect the effectiveness of coping strategies (Nicolson, 1992), how differential coping styles in everyday settings relate to cortisol reactivity has not been well documented using pharmacological challenge tests. The present study found that blunted, but not exaggerated, cortisol reactivity was significantly associated with the avoidant coping style. This finding is consistent with the previous study reporting the association between passive coping and low cortisol levels (Heim et al., 2000). However, the findings to date on the association between coping styles and cortisol activity have not been unequivocal. A 1-mg DST study (Frecska et al., 1988), for example, observed an association between high post-DEX cortisol levels and denial and passivity. Furthermore, O'Donnell et al. (2008) found no significant association between the avoidant coping style and cortisol levels in healthy older adults. Instead, they found that individuals who scored higher in either problem engagement or seeking social support had lower cortisol output over the day. These inconsistent findings might be due to different instruments for the measurement of coping styles and/or to different measures for the assessment of HPA axis function (e.g., DST vs. DEX/CRH and high- vs. low-dose of DEX) between studies. Still, the present finding of the association between blunted cortisol reactivity and more frequent use of avoidant coping style might be intriguing, taking into account that atypical depression, a disorder known to relate to down-regulation of HPA axis (Gold and Chrousos, 2002), has been reported to be associated with avoidant personality (Alpert et al., 1997; Parker et al., 2005). Similarly, PTSD has been found to be associated with avoidant coping styles (Bryant and Harvey, 1995; Krause et al., 2008) as well as with low cortisol levels, including somewhat low baseline cortisol levels (Meewisse et al., 2007) and enhanced suppression of cortisol to the low dose of DEX (Grossman et al., 2003; Yehuda et al., 1993).

The associations between coping styles and psychological distress, more specifically the significant positive correlation between more frequent use of avoidant coping strategy and greater psychological distress, were in line with previous studies (Goossens et al., 2008; Spira et al., 2004). Taken together, we observed significant relationships between greater distress, avoidant coping style, and blunted cortisol response. A feasible scenario for this relation would be that psychological distress and avoidant coping style result from the failure to mobilize cortisol to adequately cope with stressors. Alternatively, persistent psychological distress and/or avoidant coping style may end up in blunted cortisol reactivity.

The potential mechanism by which the enhanced suppression was related to the psychological distress and avoidant coping style could be discussed as follows. Since the negative feedback by DEX occurs mainly at the level of the pituitary (Cole et al., 2000), the excessively suppressed cortisol response to the DEX/CRH challenge is likely to stem from high sensitivity of pituitary glucocorticoid receptor. In line with this, enhanced negative feedback inhibition at the level of the pituitary caused by the low dose of DEX (0.5 mg) is considered to underlie the enhanced suppression of cortisol and ACTH in PTSD (Yehuda et al., 2004). The present study, using a higher dose of DEX (1.5 mg), was not primarily aimed to test the association of enhanced feedback inhibition by DEX itself with psychological measures, and actually cortisol levels of a considerable portion of our subjects fell under detection limit. A DST with the higher dose of DEX pre-treatment is optimized for the detection of decreased HPA axis feedback sensitivity whereas a DST with the lower dose of DEX is more sensitive for the detection of increased HPA axis feedback sensitivity. Indeed, using a 0.5-mg DST, a number of studies have found individuals with PTSD to display enhanced suppression of cortisol relative to those without PTSD (e.g., Grossman et al., 2003; Yehuda et al., 2004). Nevertheless, the fact that no significant correlational relationships were seen between DST-Cortisol and the psychological measures might indicate that DST is not very sensitive in detecting HPA axis alteration in relation to the psychopathology at a non-clinical level. Instead, the significant associations between the enhanced suppression to the combined DEX/CRH challenge and the unfavorable psychological outcomes may suggest that HPA axis alteration in relation to the psychopathology in healthy populations would be accounted for, at least in part, by the down-regulation of CRH receptors on the level of the pituitary rather than by the enhanced feedback inhibition detectable by the DST. However, to draw any conclusions as to where in the HPA axis the alteration exists, more adequate dose of DEX for pre-treatment should be further explored.

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Based on the preclinical and clinical evidence that elevation of AVP occurs as a consequence of chronic stress (De Goeij et al., 1992; Watson et al., 2006a), we measured the post-DEX AVP level to investigate whether the blunted cortisol response would reflect allostatic shift toward elevation of AVP or preexisting vulnerability, as stated earlier. Actually, no significant association was seen between the post-DEX AVP level and psychological measures, suggesting that the relation of blunted cortisol response with several psychological measures would not be attributable to the allostatic shift. Rather, the blunted cortisol response observed here could be considered to relate to preexisting non-clinical psychopathology, including the psychological distress and avoidant coping style. This corresponds well to the evidence that low cortisol levels may be a risk factor for psychopathological conditions, in particular PTSD (Yehuda et al., 2000). Simeon et al. (2007) also demonstrated the positive association between resilience and higher urinary cortisol levels in healthy adults. However, caution should be exercised in accepting this argument because evidence from clinical studies that the peripheral AVP level is elevated after chronic stress has not been sufficient to date.

Findings reported here should be interpreted in the context of a number of limitations. First, since the DEX/CRH test used here was based on a simple test protocol (i.e., measuring hormones only twice and omitting the ACTH measures), it may have provided less information on HPA axis hormones than the standard DEX/CRH test measuring both cortisol and ACTH levels at 5 time points between 1500 h and 1615 h. Moreover, we did not measure baseline levels of cotisol or AVP, i.e., those before the DEX challenge, which precluded us from knowing the extent to which each participant suppressed his/her cortisol and AVP secretions in response to the 1.5 mg of DEX. Second, the criteria for the suppression pattern employed here do not have sufficient empirical basis of the literature; however, the consistency between the *a priori* defined grouping (where cut-off values of cortisol were 1 μg/dL and 5 μg/dL) and the other grouping (where cut-off values of cortisol were 1 μg/dL and 13 μg/dL) in terms of their associations with the psychological measures might justify the grouping criteria. Third, this cross-sectional study cannot provide information as to whether the psychological outcomes assessed with the questionnaires were temporary or prolonged ones, nor can it address the natural history of the alteration in HPA axis function. Fourth, we cannot determine from the peripheral AVP measures alone whether they originated from the parvocellular or magnocellular system of PVN. Fifth, we did not collect data on menstrual cycle in the female participants, which may have affected HPA axis function. Sixth, one might think that there would be some biases in our sampling because none of the 93 female subjects reported that they were on oral contraceptives or hormone replacement therapies at the time of the neuroendocrine test. However, this issue should be considered in the context of considerable ethnic differences in the prevalence of these medications; some data show that approximately 1% and 4% of Japanese women were on low-dose oral contraceptives and hormone replacement therapies, respectively (Katanoda et al., 2003; Matsumoto et al., 2003), while that approximately 35–60% and 20–30% of women in Western countries were on low-dose oral contraceptives and hormone replacement therapies, respectively (Mishra et al., 2006; Tanis et al., 2003; Terry et al., 2002). Therefore, the absence of the use of such medications in the present sample could be attributed to the very low prevalence of these medications in Japan, unlike in most Western countries. Finally, as we did not collect data on the history of childhood trauma or maltreatment, which has been repeatedly reported to lead to HPA axis dysfunction in adulthood (Carpenter et al., 2007, 2009; Heim et al., 2008), some findings of the present study (e.g., the association of avoidant coping strategy with enhanced suppression of cortisol) might be confounded by such a history of early-life adversity. However, even if this is the case, our purpose of investigating the cross-sectional relations between HPA axis function and its psychological correlates in non-clinical adults will not be hampered.

In conclusion, the present study found that enhanced suppression, or blunted response, of cortisol in the DEX/CRH test was associated with greater psychological distress and avoidant coping style in a healthy population. This finding further suggests that impaired ability to mount an adequate cortisol response to pharmacological challenge may serve as a biomarker to define certain psychopathology in a non-clinical population. Such a biomarker might be useful to better understand the etiology of mental disorders and risk for symptom development.

Conflict of interest

All authors declare no conflict of interest.

Contributors

HH and HK conceptualized and designed the study, including the literature searches and analyses. HH, YO, TT, JM, YumK, YukK, SS and HK collected the data. HH performed the neuroendocrine testing, undertook the statistical analyses, and wrote the first draft of the manuscript, under the supervision of HK. ST and TH gave critical comment on the manuscript. All authors contributed to and have approved the final manuscript.

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