was a good correlation in the pathway specificity between the regulation of the EphA4/EphA5-ephrinA5 system and phosphorylation of Erk1/2 in the SNr neurons. This correlation suggests that the Erk1/2 signaling downstream of ephrin actions in the SNr is important for the cocaine-induced behavioral responses.

Discussion

The principal striatal neurons receive inputs from the cerebral cortex and thalamus and send their inputs to the SNr through two parallel pathways (1, 7). In the basal ganglia circuit, cocaine inhibits the dopamine transporter and massively increases dopamine levels in the striatum and the NAc (8). This rapid increase in dopamine activates both the low-affinity D1 receptor in the direct pathway and the high-affinity D2 receptor in the indirect pathway (22). The chronic cocaine exposure then persistently activates the D1 and D2 receptors and differentially induces long-term potentiation at striatonigral neurons of the direct pathway and long-term depression at striatopallidal neurons of the indirect pathway (23). The long-term potentiation of the direct pathway is thought to be critical for inducing the adaptive response to chronic cocaine exposure (9, 23). However, the pathway-specific regulatory mechanisms of cocaine actions at the convergent SNr remained to be clarified. This investigation has revealed an important mechanism, in which the ephrinA5 ligand-EphA4/EphA5 receptors are regulated in the SNr via a direct pathway-specific mechanism in both the acute and chronic phases of cocaine responses. These ephrin-Eph molecules were up-regulated specifically by blocking inputs of the direct pathway after cocaine administration. Conversely, the EphA4 and EphA5 receptors in the SNr suppressed adaptive response to repeated cocaine administration. Furthermore, cocaine exposure activated the Erk1/2 signaling cascade in ephrinA5-expressing SNr cells in a direct pathway-dependent manner. These results indicate that the ephrinA5-EphA4/EphA5 signaling molecules are specifically regulated by inputs of the direct pathway and play an important role in the acute and adaptive responses to cocaine exposure.

The ephrin-Eph system consists of the large family of both ephrins and Eph receptors and controls a large variety of cellular responses, including contact-mediated attraction or repulsion, synapse formation, spine morphogenesis, and neural plasticity (24, 25). One of the characteristic features of the ephrin-Eph system is its bidirectional signaling cascade, in which the interaction of ephrin with the Eph receptor induces a forward signaling in the Eph-bearing cells and simultaneously elicits a reverse signaling in the ephrin-bearing cells (20, 26). Although ephrinA5, EphA4, and EphA5 were all up-regulated by blocking the direct pathway at least at the acute phase of cocaine administration, activation by EphA4 and EphA5 was more effective than activation by ephrinA5 in suppressing the cocaine sensitization. This reverse signaling could thus play a predominant role in the transmission regulation of the direct pathway in the SNr. This regulation may occur by interaction of the presynaptic EphA4/EphA5 of striatal cells (27, 28) and the postsynaptic ephrinA5 of the SNr neurons. Astrocytes also highly express EphA4 and EphA5, which may stimulate ephrinA5 in neurons (26). Recently, the ephrin-Eph *cis* interaction within the same cellular membrane has been shown to transduce a key signaling in the ephrin-Eph system (26, 29, 30). Because both ephrinA5 and EphA4/EphA5 are commonly distributed in most of the SNr neurons, the *cis* interaction of this system could be involved in the direct pathway-specific regulation of cocaine responses. Whatever the mechanisms of the ephrinA5-EphA4/EphA5 system in the SNr, our finding that the Erk1/2 signaling is pathway-specifically regulated in ephrinA5-expressing cells strongly suggests that the ephrinA5-EphA4/EphA5–expressing SNr neurons play an important role in cocaine-induced input transmission of the direct pathway.

No alteration of the ephrinA5-EphA4/EphA5 system was observed in saline-treated D-RNB mice, indicating that the observed changes in this ephrin-Eph system were linked to the action of cocaine and not a consequence of impaired transmission per se of the direct pathway. Our previous study using the RNB technique revealed that blockade of either the direct or the indirect pathway abolished the acute cocaine response (9). The dual stimulation of the two pathways is thus necessary for the rapid response to cocaine administration (9). In the chronic response to repeated cocaine administration, blockade of the direct pathway—but not that of the indirect pathway—severely impaired cocaine-induced adaptive responses, indicating that the direct pathway plays a predominant role in input transmission for the adaptive response to cocaine (9). However, despite the defectiveness of the acute response by blockade of either of the two pathways (9), up-regulation of the ephrinA5-EphA4/EphA5 system as well as activation of Erk1/2 was observed in a direct pathway-selective manner at both acute and chronic phases of cocaine administration. The ephrinA5-EphA4/EphA5 system is thus most likely to contribute to triggering the acute response and then inducing the adaptive response to cocaine actions. The cellular response to ephrin-Eph engagement is often repulsive between the two cells, although a repulsive or attractive response depends on the cellular context (26). This ephrin-Eph system is also important for cell-cell communication by controlling spine morphogenesis and neural plasticity (18, 24, 26, 31). Present findings of the pathway-selective ephrin-Eph engagement in cocaine-induced responses thus shed light on the action of cocaine and would provide valuable therapeutic targets for the treatment of drug addiction.

Materials and Methods

Animals and Behavioral Analysis. All animal handling procedures were performed according to the guidelines of the Osaka Bioscience Institute. The RNB mice, in which transmission of either the direct or the indirect pathway was selectively blocked, was generated as described previously (9). Briefly, the expression of TN was driven in the TN mice by the TRE and induced by interaction with the tTA (32). The expression of tTA was restricted to the direct or indirect pathway by injecting one of two types of the recombinant AAVs into the NAc, in which tTA was exclusively expressed in either the direct or the indirect pathway under the control of the substance P promoter or the enkephalin promoter, respectively (9). The recombinant AAV was bilaterally injected into four sites of the NAc by stereotaxic techniques (9). The RNB mice and their WT littermates were used for all experiments.

Locomotor activity was measured with an infrared activity monitor (MED Associates). For measurement of cocaine-induced hyperlocomotion, animals received intraperitoneal saline once a day and were habituated to a novel chamber for 3 d. Cocaine (10 mg/kg) or saline was then intraperitoneally injected once a day from day 1 to day 4, and immediately thereafter the locomotor activity was counted for a 10-min period.

Microdissection of the SNr. One hour after cocaine or saline administration, mice were killed, and frozen coronal sections (40 μm) were obtained from the brain embedded in OCT compound. Microdissection was performed by using a Micro Dissector PPMD (Eppendorf), consisting of a 1-mm diameter stainless-steel needle (Eppendorf) set at a 45° angle to the surface of the microscope table. A micropipette was mounted on a 3-axis–controlled, motorized micromanipulator (Eppendorf) attached to the microscope. After cryosections were covered with a pool of 15 μL of xylene for visualization, the SNr was dissected as an ultrasonically oscillating needle was moved along a selected tissue area.

Microarray Analysis. Total RNA of dissected SNrs was extracted with the reagents of an RNeasy Mini Kit (Qiagen) after evaporation of xylene in a vacuum concentrator. Approximately 5 ng of total RNA was labeled by using GeneChip Two-Cycle Target Labeling and Control Reagents (Affymetrix). Hybridization signals were calculated by analyzing raw data with Microarray Suite 5.0 (Affymetrix) and further analyzed with GeneSpring GX 11.0 software (Agilent Technologies) and Ingenuity Pathway Analysis 6.0 software (Ingenuity Systems). The data were normalized to the 75th percentile for per-chip normalization.

Quantitative RT-PCR. Reverse transcription was carried out by using the SuperScript First-Strand Synthesis System (Affymetrix) with the T7-oligo(dT) primer. cDNAs thus synthesized were amplified by a cycle of T7 amplification by using the MEGAscript High Yield Transcription Kit (Applied Biosystems). Specific primers were designed to generate 60- to 150-bp PCR products corresponding to the 3′ region of each mRNA. All reactions were performed in duplicate, and β-actin mRNA was used as an internal control for mRNA quantification.

Immunohistological Analysis. Immunohistochemistry of frozen coronal sections (20 μm) of the adult mouse brain was performed as described by Schneider Gasser et al. (33) by using the primary antibodies against ephrinA5 (Abcam), EphA4, EphA5 (for both, Abcam or Santa Cruz), pErk1/2 (Santa Cruz), GAD67, TH (Millipore), gephyrin (Synaptic Systems), and GFAP (Sigma Aldrich). The secondary antibodies used were Alexa488- or Alexa594-conjugated goat IgG (Molecular Probes), and specific immunoreactivity was confirmed by performing immunohistochemical analysis without addition of the primary antibody.

Immunoadhesin Analysis. Three different immunoadhesins were used; that is, fusion proteins consisting of the binding domain of either ephrinA5, EphA4, or EphA5 attached to the Fc domain of human IgG (R&D Systems). To prevent diffusion of immunoadhesins into other brain regions, we attached fluorescent microspheres (Lumafluor) to each immunoadhesin, as described by Riddle et al. (19). Immunoadhesin was injected stereotaxically at four sites in the SNr of WT mice (3.4-mm and 3.6-mm posterior to the bregma, ± 1.5-mm lateral from the midline, 4.0-mm depth from the dura). Four days after immunoadhesin injection, locomotor activity was measured immediately after daily administration of cocaine (10 mg/kg). After the behavioral analysis, injection sites of immunoadhesins were confirmed by visualization of immunoadhesin-attached fluorescent microspheres in the SNr of brain-slice preparations.

Statistical Analysis. Statistical analysis was conducted by using Graph Pad PRISM 5.0 (GraphPad Software). Data were analyzed by one-way ANOVA or repeated-measure ANOVA and were presented as the mean ± SEM.

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精神疾患の分子遺伝学：最近の知見

疋田貴俊 [1, 2, 3]

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精神疾患の発症には遺伝要因が関与しているが，その遺伝形式は複雑で，環境要因や複数の遺伝要因により発症に至るメカニズムは明らかにされていない．精神疾患の生物学的研究を進めるうえで，分子遺伝学的研究からの知見を欠かすことができない．近年，全ゲノム関連解析や候補遺伝子に対する精力的な研究が行われてきた．これらの研究の最近の知見を紹介する．

Key Words: 精神疾患，統合失調症，CNV，22q11.2 欠失症候群，DISC1

Ⅰ．はじめに

精神疾患の発症には遺伝要因が大きく関与しているが，多因子性で複数の遺伝要因や環境要因により最終的に発症に至る．今日まで，多数の家系からの連鎖解析や精神薬理学，死後脳解析からの機能的候補遺伝子などから関連遺伝子の解析が進められている[6]．また，高密度DNAマイクロアレイによる全ゲノム関連解析（genome-wide association study: GWAS）が行われ，特にコピー数多型（copy number variance: CNV）に注目が集まっている．さらに，単一家系より同定された候補遺伝子のDISC1に関する研究から，精神疾患の生物学的研究が進められている．

本稿では，これらの精神疾患の分子遺伝学的研究から最近の知見を考察する．

Ⅱ．CNV

特定のゲノム領域にCNVが起きやすいが，CNV成立の機構はわかっていない．統合失調症の患者のゲノム全域CNV解析研究から，新生CNVが健常人の1.3%に対して孤発例の統合失調症患者に10%と多く認められている[18]．統合失調症と関連があるCNVを表に示す[12]．これらの結果は2つの大規模統合失調症解析で確認されている[8, 17]．どのCNVも今までの関連遺伝子より高いオッズ比となり，さらに興味深いことに，これらのCNV変異は統合失調症に特異性は低く，自閉症などでも高頻度に認められる[13, 17]．CNV変異による遺伝子機能への影響として複数の遺伝子の欠失あるいは部分欠失，転写調節領域に変異による遺伝子転写量変化，新規融合転写産物，イ

表　統合失調症で見られるCNV変異（文献12より改変のうえ引用）

染色体部位	変異型	サイズ（Mb）	統合失調症患者群（%）	対照群（%）	オッズ比	含有遺伝子数（n）	統合失調症以外の関連疾患
1q21.1	欠失	1.5	0.2	0.02	9	10	小頭症，精神遅滞
2q16.3	欠失	変動有	0.2	0.04	4	1	自閉症
15q11.2	欠失	0.6	0.6	0.22	2-3	4	精神遅滞
15q13.3	欠失	1.3	0.2	0.02	11	6	精神遅滞，てんかん
16p13.1	重複	1.4	0.3-0.5	0.1-0.25	2-3	12	自閉症
16p11.2	重複	0.6	0.3	0.03	8	26	自閉症
22q11.2	欠失	2.4	0.2		30	43	ディジョージ症候群

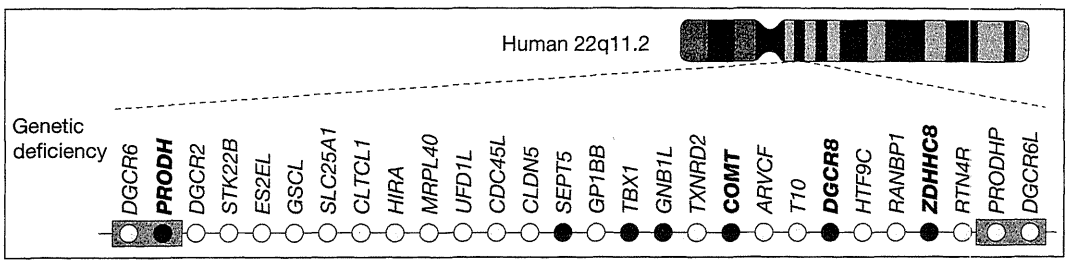

図1　22q11.2領域に含まれる遺伝子群（文献11より一部改変のうえ引用）
22q11.2欠失疾患群でよく見られる1.5Mbの染色体欠失領域を示す．両側の四角で示すlow copy sequence（LCR）で囲まれた領域で欠失が起こりやすい．●は機能がよく調べられている遺伝子を示す．***PRODH*** と ***COMT*** はドーパミン代謝に，***DGCR8*** はマイクロRNAプロセシングに，***ZDHHC8*** は蛋白のパルミトイル化にそれぞれ関与する．●は変異マウスで行動異常を呈する遺伝子を示す．

ンプリンティングの影響などが挙げられている[2]．次項では22q11.2のCNVに絞って解説する．

Ⅲ．22q11.2欠失症候群

全統合失調症患者の1%で22q11.2微小欠失が見られる．22q11.2欠失症候群は，臨床上の特徴として，眼裂狭小，眼間解離，鼻根部扁平，小顎症，耳介下方付着，口蓋裂といった特異的な顔貌，胸腺の低形成による免疫不全，副甲状腺の低形成による低カルシウム血症，心室流出路から大血管の異常を伴った先天性心疾患（ファロー四徴症，肺動脈閉鎖兼心室中隔欠損，総動脈幹遺残，大動脈弓離断など）を合併することが多い．ディジョージ症候群とも言われる．

統合失調症のリスクが一般の30倍程度で（**表**参照），22q11.2欠損症候群の1/4～1/3に統合失調症が発症する[8]．22q11.2の1.5Mb染色体領域には**図1**で示す複数の遺伝子があり，これらの遺伝子欠失の相互作用が統合失調症発症に関与することが考えられている[11]．ヒト染色体22q11.2のマウス相同領域を欠失したマウスが作製されており，作業記憶の低下と課題遂行時の海馬と大脳皮

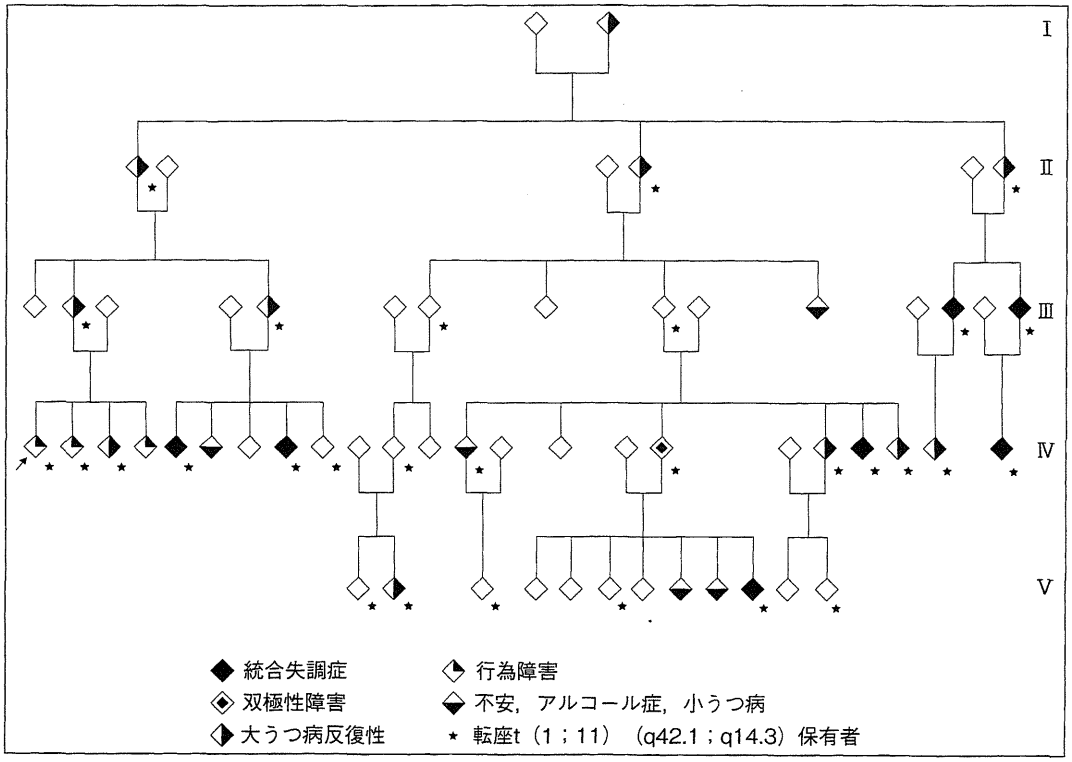

図2　DISC1遺伝子が同定されたスコットランドの家系図（文献1より一部改変のうえ引用）

質の同調性の異常が見いだされている[16]．このように，CNV変異が精神疾患で見られる行動異常と神経回路異常を引き起こすことがモデル動物を用いて示されている．

Ⅳ．DISC1

パーキンソン病やアルツハイマー病といった神経疾患が，特異例である家族性疾患を解析することによって分子病理研究が発展したように，まれな集団や家系から同定される候補遺伝子から疾患の生物学的理解が進むことが多い．精神疾患においては，DISC1研究がその嚆矢となっている．

DISC1（Disrupted-In-Schizophrenia）はスコットランドにおける精神疾患多発単一家系により発見された（**図2**）[1]．この家系において，37人のt（1；11）（q42.1；q14.3）の均衡型染色体転座保有者を確認しており，このうち精神医学的診断が可能であった29人のうち統合失調症7例，双極性障害1例，大うつ病10例などの精神疾患を患っていた．これに対し，転座を持たない38人には統合失調症や双極性障害といった精神疾患はなかった[1]．

興味深いことに，転座保有者は精神疾患を発症していない対象者も含めて脳波の事象関連電位P300の潜時延長と振幅低下が報告されている[1]．第1染色体の転座部位にはDISC1遺伝子が同定

され，転座によって全854アミノ酸のうちC末端の257アミノ酸が切断される．このC末端欠損型変異DISC1蛋白は野生型DISC1蛋白に対して，*in vitro* および *in vivo* においてドミナントネガティブに作用する[10, 15]．変異DISC1蛋白を神経細胞に過剰発現させたトランスジェニックマウスでは，過活動，prepulse inhibitionの異常，強制水泳試験での無動時間延長といった行動異常や，統合失調症患者で見られるMRI撮像における側脳室拡大や大脳皮質でのパルブアルブミン発現の減少といった形態異常が観察された[4]．これらの行動異常や形態異常は環境要因の付加で増大する．

周産期のウイルス感染は統合失調症のリスク要因として知られている．合成dsRNAであるpolyI:C（polyriboinosinic-polyribocytidylic acid）を妊娠マウスに投与し母胎の免疫系を賦活することで周産期感染を模倣すると，変異DISC1トランスジェニックマウスの行動異常が悪化し，大脳皮質のパルブアルブミン発現がより減少する[7]．統合失調症発症における遺伝要因と環境要因の組み合わせの重要性が再現される結果となっている．

DISC1は脳発達期および成熟期において，神経細胞の核，シナプス，細胞骨格のそれぞれでさまざまな機能に関与していることが示されている[3, 9]．しかし変異DISC1が，脳神経回路のどこに，さらに脳発達の時系列のいつに，関与することで病態発現に至るかは明らかにされていない．子宮内電気穿孔法を用いた発達段階における前頭前野のDISC1の発現をRNAiによりノックダウンしたマウスでは，錐体細胞樹状突起の形態異常に加え，思春期後にドーパミン系の障害や行動異常を呈した[14]．これらの行動異常は思春期前には認めず，DISC1の発達期における機能がその後の神経回路と行動に影響を与えることが示唆された．精神疾患の生物学を研究するうえで，病態の時間軸に沿ったアプローチが必要になってきている[5]．

V．おわりに

精神疾患の分子遺伝学の最近の知見を，統合失調症を中心に紹介した．しかし，これらの遺伝要因の多くは統合失調症のみならず双極性障害や自閉症といった他の精神疾患への脆弱性にも関与しており，発症メカニズムを研究するうえで注意が必要である．それにもかかわらず，これらの分子遺伝学的知見を基に作製された遺伝子改変動物は，精神疾患遺伝モデル動物として，精神疾患の生物学的研究には必須であり，研究の発展が期待される．

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Unusual presentation of more common disease/injury

Memory deficits due to brain injury: unique PET findings and dream alterations

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Summary

The authors herein report the case of a young male with memory deficits due to a traumatic head injury, who presented with sleep-related symptoms such as hypersomnia and dream alterations. Although MRI and polysomnography showed no abnormalities, ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) and ^{11}C flumazenil (FMZ)-PET revealed findings consistent with cerebral damage to the affected temporal region. The memory deficit of the patient gradually improved in parallel with the relief of the sleep-related symptoms. FDG-PET showed considerable improvement in glucose metabolism when he had recovered, however, evidence of neural loss remained in the FMZ-PET findings.

BACKGROUND

Traumatic brain injury may cause various types of memory disturbance, such as amnesia and difficulty of recall. In addition to mnemonic differences, patients also may manifest psychiatric and neurological symptoms. Although neuroimaging techniques have progressed, the neural mechanism of brain injury and the process of recovery still remain to be fully elucidated.

CASE PRESENTATION

In July 2009, an 18-year-old male with memory loss was referred to our hospital. The patient had experienced trauma to the right of his head while playing handball in April 2008. Although his response and reflexes were normal after the accident, the patient repeatedly talked about the same things. The patient was unable to recall incidents that had happened more than three or four days earlier.

Since the beginning of 2009, the patient manifested hypersomnia that lasted half of the day and claimed that the pattern of his dreams had changed. After the accident, the patient only dreamed about what he had actually experienced during the daytime, such as playing baseball or having dinner with his family. The patient's family would confirm that the dreams were just replays of actual past experiences. The patient was diagnosed with idiopathic hypersomnia or dissociative disorder at another hospital.

Upon admission, cognitive tests were performed. The mini mental state examination and Wechsler memory scale-revised demonstrated that the patient had no short-term memory deficits. No physical abnormalities were detected through blood tests, brain MRI or electroencephalography. However, the patient had difficulties visually recalling incidents that had happened more than 3 days earlier.

Over the course of hospitalisation, the patient's hypersomnia remitted without medication. The patient was told to keep a diary everyday to record his dreams. The recorded contents of the dream were always what the patient had actually seen or experienced previously. Although polysomnography revealed that there was a slight increase in his sleep latency, most of the sleep architecture remained intact, including rapid eye movement (REM) sleep.

INVESTIGATIONS

^{18}F-Fluorodeoxyglucose (FDG) positron emission tomography (PET) showed a significant decrease in glucose metabolism within the right supramargical and inferior temporal gyrus. ^{11}C-flumazenil (FMZ) PET was used to test the neuronal viability of the brain. The study showed the FMZ uptake to decrease in the right middle temporal gyrus; the reduction in binding likely represented neuronal damage, thus indicating a loss of neuronal integrity (figure 1A,B).

DIFFERENTIAL DIAGNOSIS

- Dissociative disorder
- Idiopathic hypersomnia.

TREATMENT

The patient was started on memory rehabilitation, focusing on memory restoration. For example, writing down the events to memorise in a notebook. A couple of months later, his memory performance gradually improved, accompanied by changes in the contents of the dreams. No medication was prescribed for his treatment.

OUTCOME AND FOLLOW-UP

The patient started to dream again about foreign incidents, such as Hollywood movie stars visiting his home. His memory recall also gradually recovered during the hospitalisation, and the duration of memory recall was prolonged. When the patient was discharged in February 2010, the patient showed a remarkable memory

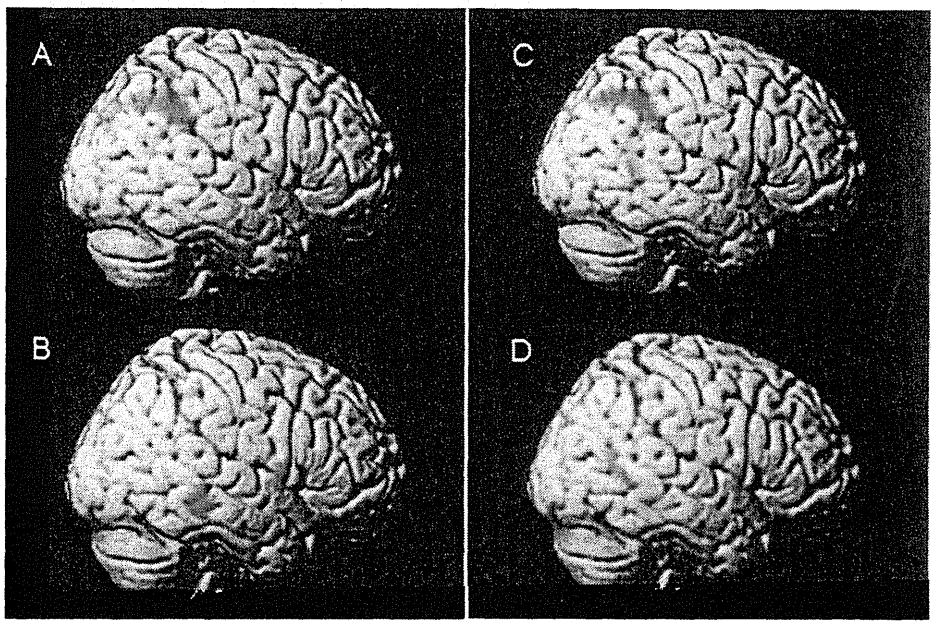

Figure 1 Initial positron emission tomography (PET). Z-score brain surface maps from ^{18}F-fluorodeoxyglucose (FDG)-PET showed decreased values in the supramargical and inferior temporal gyrus (A). ^{11}C-flumazenil (FMZ)-PET showed decreased values in the right middle temporal gyrus (B). The spatial preprocessing and the statistical analysis of the images were done using the SPM2 software program (available at www.fil.ion.ucl.ac.uk/spm). Follow-up PET. An FDG-PET examination 6 months later (C) showed improvement in the inferior temporal gyrus, while FMZ-PET 14 months later (D) showed no improvement in the supramargical gyrus.

improvement, no disturbances in memory recall and had no difficulties in managing daily life. FDG-PET showed a substantial improvement in glucose metabolism within the inferior temporal gyrus, while FMZ-PET demonstrated no significant regional improvement in the FMZ uptake (figure 1C,D).

DISCUSSION

It is well established that sleep facilitates the offline reactivation of formed memories, and that dream experiences reflect this memory processing.[1] Torda reported that subjects with hippocampal lesions had dreams with a stereotyped repetition of past experiences.[2] Although our case did not have hippocampal lesions, the affected cerebral regions including the inferior temporal lesion that the initial PET findings demonstrated may alter the dream contents, as well as memory loss.

We were unable to find any abnormality of REM sleep that is thought to generate dreams from neural signals in the brainstem,[3] that is, different brain regions from that of our case. Previous sleep studies of head trauma patients showed a similar result.[4] It was previously reported that the cessation of dreaming generally follows damage to the bilateral temporo-parieto-occipital junctions.[5] Along with the PET measurements, the unilateral lesion in this case coincided with the supramargical gyrus, which may thus have been responsible for the temporary alterations in his dream contents. As the function of the inferior temporal gyrus improved to compensate for the neural damage to the supramargical gyrus, the patient's mnemonic function was also improved, accompanied by normalisation of his dream contents. Taken together, the neuronal mechanism underlying the improvement in memory retrieval was likely associated with the alteration of brain plasticity, and with the physiology of sleep, incorporating sleep-related memory organisation.

Learning points

- Memory deficit due to traumatic brain injury is a diagnosis of exclusion.
- If you see a patient with amnesia or sleep-related symptoms, ask about the patient's history of brain trauma.
- PET is often necessary for a definitive diagnosis.
- FMZ-PET has the potential to detect neuronal damage.
- Sleep, including dreams, has an influence on memory process, and alters the underlying brain plasticity.

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Competing interests None.

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Effects of novelty stress on hippocampal gene expression, corticosterone and motor activity in mice

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ABSTRACT

Exposure to novelty, a mild psychological stressor, induces neuronal activations in the hippocampus of rodents, which may play an important role in the adaptation to stress. We examined the changes in three parameters, i.e., gene expression in the hippocampus using a RT-PCR method, corticosterone and motor activity, in mice exposed to a new environment for 120 min. A sharp and short-lasting increase in the gene expression of a set of stress-related genes previously reported, e.g., Fos and Nr4a1, was observed during the stress, with a similar pattern of changes in corticosterone. The motor activity gradually decreased during the novelty stress, indicating a process of adaptation to the new environment. In addition, in order to minimize the effects of elevated adrenal hormones by the stress, we carried out experiments on adrenalectomized (ADX) mice. However, the adrenalectomy produced minimal changes in the pattern and the magnitude of the gene response after the stress, while the motor activity showed a relatively slower pattern of adaptation in the ADX mice. Hence, the present study suggests that there was a coordinated adaptation process to the new environment in mice, and that the transcriptional response was mediated by neuronal networks rather than by adrenal hormones.

1. Introduction

A subjective state of sensing potentially adverse changes in internal homeostasis or in the external environment can be called stress (Joëls and Baram, 2009; Pacák and Palkovits, 2001). A highly coordinated system in the central nervous system (CNS) responds to adapt to the stress (McEwen, 2007). Various molecules mediating the stress-response, e.g., monoamines, peptides and steroid hormones, are released and bind to receptor targets distributed in a characteristic pattern, resulting in anatomically and temporally coordinated responses of the neurobiology including transcriptional regulations. The activated transmission of the monoamines and peptides leads to a change in the functions of transcriptional factors such as activator protein-1 (AP-1) and cAMP response element-binding protein (CREB) in a second-to-minutes time frame, whereas a complex of steroid hormones and the receptors is moved to the nucleus and acts as regulators of gene transcription in a time frame of hours (Joëls and Baram, 2009). These genomic effects of stress may inhibit the lingering effects of the acute response to stress and may modulate the ability to respond to a future stress (Oitzl et al., 2010). In addition, there is growing evidence that rapid glucocorticoid action within minutes is mediated by membrane-associated receptors, e.g., G-protein-coupled receptors, as well as other membrane-associated proteins, e.g., ion channels, modulating intracellular signaling cascades downstream from the effectors (Tasker et al., 2006; Riedeman et al., 2010).

Many studies indicate that the hippocampus is one of the most important brain regions subject to the coordinate stress-response system (Joëls and Baram, 2009; De Kloet et al., 2005; McEwen, 2007; Oitzl et al., 2010). The sensations of alterations in stress are conveyed to the CNS by neurochemical pathways and are integrated at the hypothalamic level, resulting in the increase in a release of corticotropin-releasing factor (CRF) neurosecretory neurons of the paraventricular nucleus. The neuropeptide drives an activation of the hypothalamic-pituitary aderenocortical (HPA) axis with the consequence of an increased secretion of glucocorticoids from the adrenal glands. CRF as well as glucocorticoid hormones produces a various effects on the hippocampus, because the brain region expresses a high level of the receptors for these molecules. CRF exerts its action through G-protein-coupled receptors, and glucocorticoids bind to mineralcorticoid receptors (MRs) and glucocorticoid receptors (GRs). The stimulations of the steroid receptors in the hippocampus after stress activate a negative feed-back system of the HPA axis and can modulate the behavioral adaptation to stress and cognitive functions such as memory (Tasker et al., 2006).

Exposure to a novel environment can be one of the psychological stressors presenting no physical burden to animals, activating the HPA axis resulting in an elevated circulating level of glucocorticoid

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hormones (Chandramohan et al., 2007). The hippocampus may play a key role in recognition and habituation to the novelty (Knight, 1996; Hampson et al., 1999; Lee, 2005; Jeewajee et al., 2008). The novelty stress induces a prominent increase in the release of acetylcholine (Aloisi et al., 1997; Giovannini et al., 2001), GABA (Giovannini et al., 2001; De Groote and Linthorst, 2007) and glutamate (Takeda et al., 2006) with a close relation to exploratory motor activity. Moreover, the response to novelty in the hippocampus involves an induction of chromatin remodeling events known to be associated with transcriptional regulation (Chandramohan et al., 2007; Hunter et al., 2009), which may induce a modulation of the responsive ability to a further stressor.

We have previously reported that expressions of a set of genes were activated in the neocortex of mice after treatment with a pharmacological stressor (FG7142) and immobilization stress (Kurumaji et al., 2008). In the present study, we examined whether or not a time-scheduled study of novelty stress produces any changes in the expression of the set of genes in the hippocampus, in addition to the levels of corticosterone and amounts of motor activity. The present study demonstrated that there were time-dependent changes in three parameters during the novelty stress. In addition, in order to minimize the actions of stress-elevated adrenal hormones on the hippocampus through the specific receptors, aderenalectomized mice were used in the stress experiment. The stress-induced rise in the hormones can produce genomic and non-genomic effects on the hippocampus, although the time frame of the effect is different in each case (Joëls and Baram, 2009).

2. Materials and methods

2.1. Animals and chemicals

The present animal experiments were performed in strict accordance with the guidelines of the Tokyo Medical and Dental University and were approved by the Animal Investigation Committee. Male C57BL mice were purchased from Japan Clea Laboratories (Tokyo, Japan). The mice were kept at 24.0 ± 0.5 °C in a humidity-controlled room under a 12-h light–dark cycle (lights on at 8:00 am) with free access to food and water. The mice were kept in groups of three per cage, and three infant animals were housed with their mother in a cage until use at postnatal week 8. All mice were habituated to the housing conditions for at least 7 days before the experiments commenced.

2.2. Surgery

Animals at postnatal week 6 were anesthetized with pentobarbital (40 mg/kg, i.p.) and placed on a heat-pad (37 °C) during surgery. The skin on the back was shaved and disinfected and an incision was made above and parallel to the spinal cord. Though a small opening in the muscle layer left and right of the spinal cord the adrenals were removed from the surrounding fat tissues. The skin was closed using a simple running suture. Sham-operated animals were treated similarly with the exception of actual removal of the adrenals. After surgery, all animals were given free access to 0.9% NaCl in addition to normal drinking water. The mice with adrenalectomy or the sham-operation at postnatal week 8 were used for novelty stress.

2.3. Time-scheduled experiment of novelty stress

All experiments were conducted during the light cycle in a soundproof and temperature-controlled room (24.0 ± 0.5 °C). To induce novelty stress, mice were individually placed in a new cage (a clean with no sawdust, no food or no water) (24.5 × 17.5 × 12.5 cm) the same size as that of their home cage for various times (15, 30, 60, 90 and 120 min) in a room adjacent to the room where the home cage was placed, while the control animals (time point "0") were left undisturbed in the home cage until they were sacrificed.

2.4. Behavioral analyses

To evaluate the behavioral change in the novel environment, the spontaneous vertical and horizontal movements including locomotion, rearing, and head movements were quantified by automatically counting the number of heat changes in the multiple zones of the test cage by means of the heat sensor with a Supermex instrument (Muromachikiki Co. Ltd., Tokyo, Japan) (Kaneko et al., 2007). The mouse was singly placed in the new cage within a soundproof and illuminated wood box at an ambient temperature of 24.0 ± 0.5 °C. The Supermex consists of a monitor that was mounted above the test cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses. The body heat radiated by an animal was detected by the sensor head of the monitor, which contained paired infrared light ray pyroelectric detectors. The behavioral data were obtained every 15 min.

2.5. Radioimmunoassay of corticosterone

Trunk blood was obtained from mice used in the stress experiments at the time of sacrifice. Serum obtained after centrifugation at 3500 rpm for 30 min was stored at −30 °C until the assay. Serum corticosterone was measured by radioimmunoassay (Corticosterone Double Antibody, [^{125}I] Ria Kit, MP Biomedicals, Inc., Irvine, CA, USA) with a highly specific corticosterone antiserum having a detection threshold of 7.7 ng/ml.

2.6. Extract of total RNA

The mice were killed by cervical dislocation after the time-scheduled study of the novelty stress. Both sides of the hippocampus were rapidly removed in the cold, frozen in liquid nitrogen, and stored at −80 °C until use. Each of the frozen hippocampal tissues was homogenized using a Polytron Homogenizer (Kinematica AG, Littau/Luzern, Switzerland) at 20,000 rpm for 10 sec, and its total RNA was extracted using the Quiagen Rneasy Midi System (Quiagen, Valencia, CA, USA).

2.7. Quantitative RT-PCR

The RNA sample was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) to remove any contaminating genomic DNA. The single-stranded cDNA was then synthesized from 1 μg of the DNase I-treated neocortical RNA using a SuperScript Preamplification system (Invitrogen, Carlsbad, CA, USA). The remaining RNAs were digested using Ribonuclease H (Invitrogen, Carlsbad, CA, USA), and the resulting cDNA suspended in 10 volumes of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) was used for the quantitative RT-PCR analysis described below.

Five μL of a diluted sample of each first strand product was amplified using 2 μL of LightCycler FastStart DNA Master SYBER Green I (Roche Diagnostics, Mannheim, Germany) (Castello et al., 2002) and a pair of primers (Table 1) at the final concentration of 0.5 μM each and $MgCl_2$ at the final concentration of 3 mM. PCR was performed in a total volume of 20 μL for 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 5 s at 65 °C and 10 s at 72 °C. The melting curve analysis was done by continuous acquisition from 65 °C to 95 °C with a temperature transition rate of 0.1 °C/s. In each assay, standard curves were generated from four increasing amounts of the pooled cDNA templates of equal volumes of the individual samples. The results were automatically calculated using the respective

Table 1
Primer sequences for the semi-quantitative RT-PCR.

Official symbol of gene Gene name	GeneBank ID	Bases spanned	Forward primer (5 to 3′)	Reverse primer (5′ to 3′)
Gapdh Glyceraldehyde-3-phosphate dehydrogenase	NM_001001303	211–439	CGGCAAATTCAACGGCACAGTCAA	TGGGGGCATCGGCAGAAGG
Cyr6 Cystein rich protein 61	NM_010516	630–858	CCCCCGGCTGGTGAAAGTC	ATGGGCGTGCAGAGGGTTGAAAAG
Fos FBJ osteosarcoma oncogene	NM_010234	323–502	CCCACGGTGACAGCCATCTCCA	CTGCGCTCTGCCTCCTGACACG
Btg2 B-cell translocation gene 2	NM_007570	479–597	CCCCGGTGGCTGCCTCCTATG	GGGTCGGGTGGCTCCTATCTA
Adamts 1 A disintegrin-like and metalloprotease with thrombospondin type 1	NM_009621	550–896	GGCGCCCCACGGAGGAAG	AGGCGCTGGCTGAATGAAGAAC
Rgs 2 Regulator of G-protein signaling 2	NM_009061	325–652	AGGGCGTTTTTAAAGTCCGAGTT	CCGTGGTGATCTGTGGCTTTTTAC
Gem GTP binding protein (gene overexpressed in skeletal muscle)	NM_010276	241–524	CTCCGAAACCGCCACTCTACTGCT	GTTCTCCCCCTTATTTTCCCACAT
Nr4a1 Nuclear receptor subfamily 4, group A, member 1	NM_010444	668–968	CTCCGCCACCTCCAACCTTCTTCT	GTGCGGACCCCATAGTGCTGACA

standard curves by the LightCycler analysis software version 3.5. Amplification of the single product in the RT-PCR was confirmed by monitoring the melting curve and by agarose gel electrophoresis. Expression of the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was determined as the internal standard for each sample, because the Gapdh gene has been reported to be a housekeeping gene that is constantly expressed in neural tissues (Thellin et al., 1999). The ratios of the results of the mRNAs of each gene to those of the Gapdh mRNAs were used for the statistical comparison as the normalized values of the mRNA of each gene.

2.8. Statistical analysis

Differences between two groups were tested with the two-tailed Student's *t*-test. Data of RT-PCR and corticosterone in the time-scheduled experiment of stress were analyzed by one (exposure time)- or two (operation and exposure time)-way ANOVA followed by the post hoc Student's *t*-test with Bonferroni's correction or Newman–Keuls test. Data of behavioral activity were analyzed by one (exposure time)- or two (operation and exposure time)-way ANOVA with repeated measure followed by Student's *t*-test with Bonferroni's correction. p values of 5% or less were considered statistically significant.

3. Results

3.1. Time-scheduled novelty stress on mice

The time-scheduled study of novelty stress produced a sharp and short-lasting increase in the mRNAs of five genes, i.e., Fos [$F(5, 53) = 13.4$, $p < 0.0001$], Btg2 [$F(5, 53) = 42.123$, $p < 0.0001$], Cyr61 [$F(5, 53) = 8.765$, $p < 0.0001$], Nr4a1 [$F(5, 53) = 12.036$, $p < 0.0001$] and Gem [$F(5, 53) = 15.046$, $p < 0.0001$], but not in the mRNAs of Adamsts1 or Rgs2 in the hippocampus of the mice. The significant increases in the gene expressions were observed after 15- to 60-min exposure to the novelty stress, and the levels of the mRNAs after the 90-min stress returned to the levels of the control (time point '0') (Fig. 1).

The time-scheduled stress induced a bell-shaped increase in the serum concentration of corticosterone in the mice [$F(5, 53) = 16.447$, $p < 0.0001$]. A significant increase compared to the control was observed in the content of corticosterone after the exposure to the stress for 15–90 min, and then the concentration after the 120-min stress returned to that of the control (Fig. 2).

The amount of motor activity gradually decreased during the exposure to the novelty environment for 120 min [$F(7, 49) = 39.825$, $p < 0.0001$]. A significant decrease in the count of motor activity compared to the first 15-min interval was observed in from the fourth (45–60 min) to the last (105–120 min) interval (Fig. 3).

3.2. Time-scheduled novelty stress on adrenalectomized mice

The novelty stress induced a time-dependent increase in the hippocampal gene expression of mice with adrenalectomy as well as with the SHAM operation. Two-way ANOVA revealed a significant effect of exposure time on the five genes (Fos: $F(4, 70) = 47.135$, $p < 0.0001$; Btg2: $F(4, 70) = 23.452$, $p < 0.0001$; Cyr61: $F(4, 70) = 11.804$, $p < 0.0001$; Nr4a1: $F(4, 70) = 19.876$, $p < 0.0001$; gem: $F(4, 70) = 9.244$, $p < 0.0001$). The analysis revealed a significant effect of the operation on the mRNAs of Cyr61 ($F(1, 70) = 5.869$, $p < 0.018$) and Nr4a1 ($F(1, 70) = 6.117$, $p < 0.0159$), but not on either of the other genes, and a significant effect of the interaction of operation × exposure time only on the mRNA of Btg2 ($F(4, 70) = 2.719$, $p = 0.0364$). The post hoc multiple comparison tests indicated that the mRNA of Btg2 after 15-min stress and that of Nr4a1 after 60-min stress in the ADX mice were statistically significantly decreased compared to that of each counterpart of the SHAM in the time-scheduled study (Fig. 4).

Two-way ANOVA revealed a significant effect of exposure time [$F(4, 75) = 22.323$, $p < 0.0001$], operation [$F(1, 75) = 254.337$, $p < 0.0001$] and the interaction of operation × exposure time [$F(4, 75) = 18.631$, $p < 0.0001$] on the serum concentrations of adrenalectomized and SHAM mice. A significantly increased level of conticosterone compared to the level for the non-stressed was observed after the stress for 15, 30 and 60 min in the SHAM mice, while there were no stress-induced changes in the level of the ADX mice during the time-scheduled study. The level of the non-stressed adrenalectomized mice was significantly low in comparison with that of the SHAM (Fig. 5).

In the amounts of motor activity during the exposure to the new environment for 120 min, two-way ANOVA with repeated measure revealed a significant effect of the operation [$F(1, 98) = 5.78$, $p < 0.05$], exposure time [$F(7, 98) = 69.284$, $p < 0.0001$] and the interaction of operation × exposure time [$F(7, 98) = 4.156$, $p < 0.001$]. A gradual decrease in the amount of activity during the stress procedure was observed in both the ADX and SHAM, and there was a significantly greater activity of the ADX than that in the SHAM in

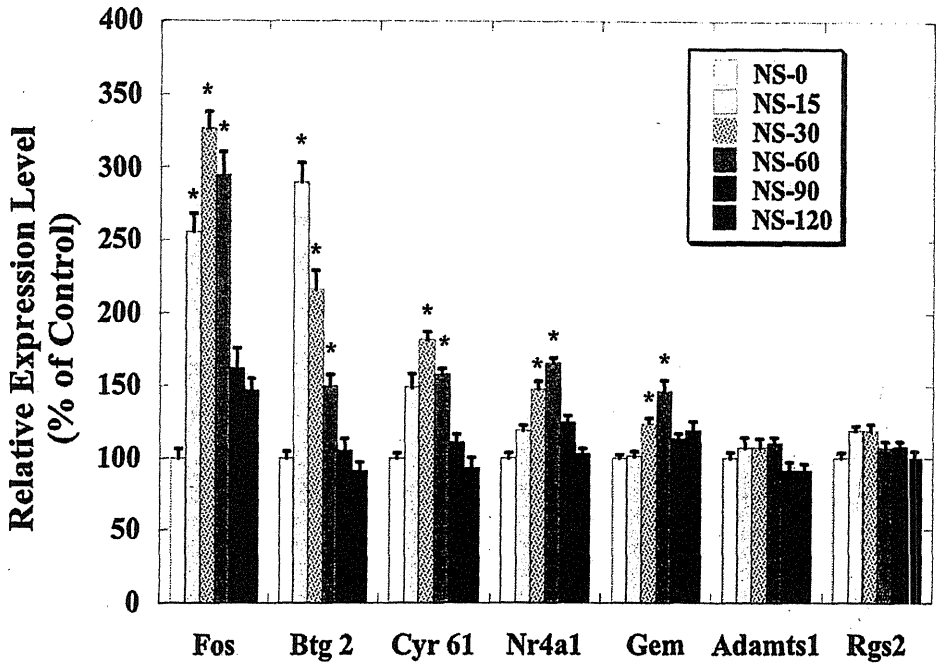

Fig. 1. Results of RT-PCR in the hippocampus of mice in the time-scheduled study of novelty stress. Mice (postnatal week 8) were individually placed in a new cage for various times, i.e., 15 min (NS-15; $n = 7$), 30 min (NS-30; $n = 12$), 60 min (NS-60; $n = 12$) and 120 min (NS-120; $n = 8$) to induce novelty stress, while the control animals (NS-0; $n = 15$) were left undisturbed in their home cage until they were killed. Results are mean with S.E.M. of data (normalized to Gapdh) and are expressed as a percentage of the mean value of the respective control mice. The data were analyzed using one-way ANOVA followed by the post hoc Student's t-test with Bonferroni's correction. $^*p < 0.05$ vs control (NS-0).

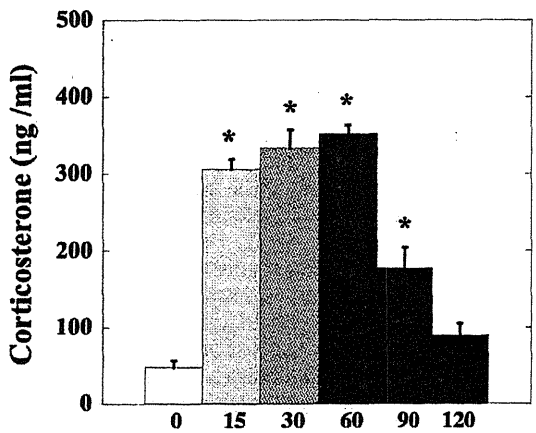

Fig. 2. Results of serum concentration of corticosterone in the mice in the time-scheduled study of novelty stress. Mice (postnatal week 8) were individually placed in a new cage for various times, i.e., 15 min (NS-15; $n = 7$), 30 min (NS-30; $n = 12$), 60 min (NS-60; $n = 12$) and 120 min (NS-120; $n = 7$) to induce novelty stress, while the control animals (time point '0'; $n = 15$) were left undisturbed in their home cage until they were killed. Results were mean with S.E.M. and were analyzed by one-way ANOVA followed by the post hoc Student's t-test with Bonferroni's correction. $^*p < 0.05$ vs control (time point 0).

the later part of the testing interval from the sixth (75–90 min) to the last (Fig. 6).

4. Discussion

The present study demonstrated that the time-scheduled study of the novelty stress for 2 h induced a bell-shaped activation in gene expression of five of the seven genes examined. The pattern of change in the serum corticosterone during the study of novelty stress was similar to that of the activated gene expression, and a gradually decreased count in the motor activity was observed during the stress for 120 min. It appears that the time course of the change in each parameter was subject to a coordinated adaptation process of the animals to the new environment, involving the process of activation and then negative feedback in the HPA axis as well as the neurobiological consequences of released molecules, e.g., monoamines and peptides, in the hippocampus (De Kloet et al., 2005; McEwen, 2007; Joëls and Baram, 2009).

Another finding in the present study was that the pattern of stress-induced activation of the gene expression in the ADX mice was quite similar to that of the sham-operated mice, while a subtle attenuated enhancement induced by the stress was observed in the gene expression of Btg2 and Nr4a1. The low basal level of cor-

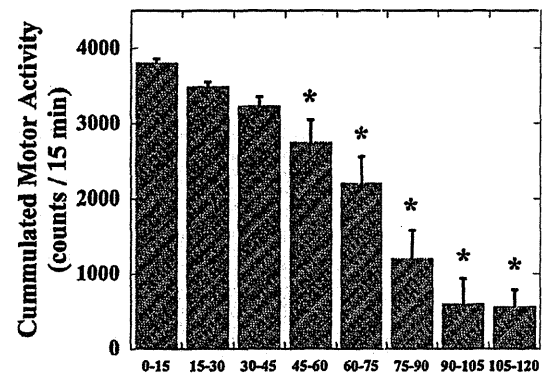

Fig. 3. Results of the behavioral activity of mice during the exposure to novelty stress for 120 min. The behavioral activities of mice (postnatal week 8) placed in new cages was assessed by the Supermex instrument. The results were mean with S.E.M. of the cumulated motor activities at an interval of 15 min obtained from eight animals. The data were analyzed using one-way ANOVA for repeated measure followed by the post hoc Student's t-test with Bonferroni's correction. $^*p < 0.05$ vs the first interval of 15 min (0–15).

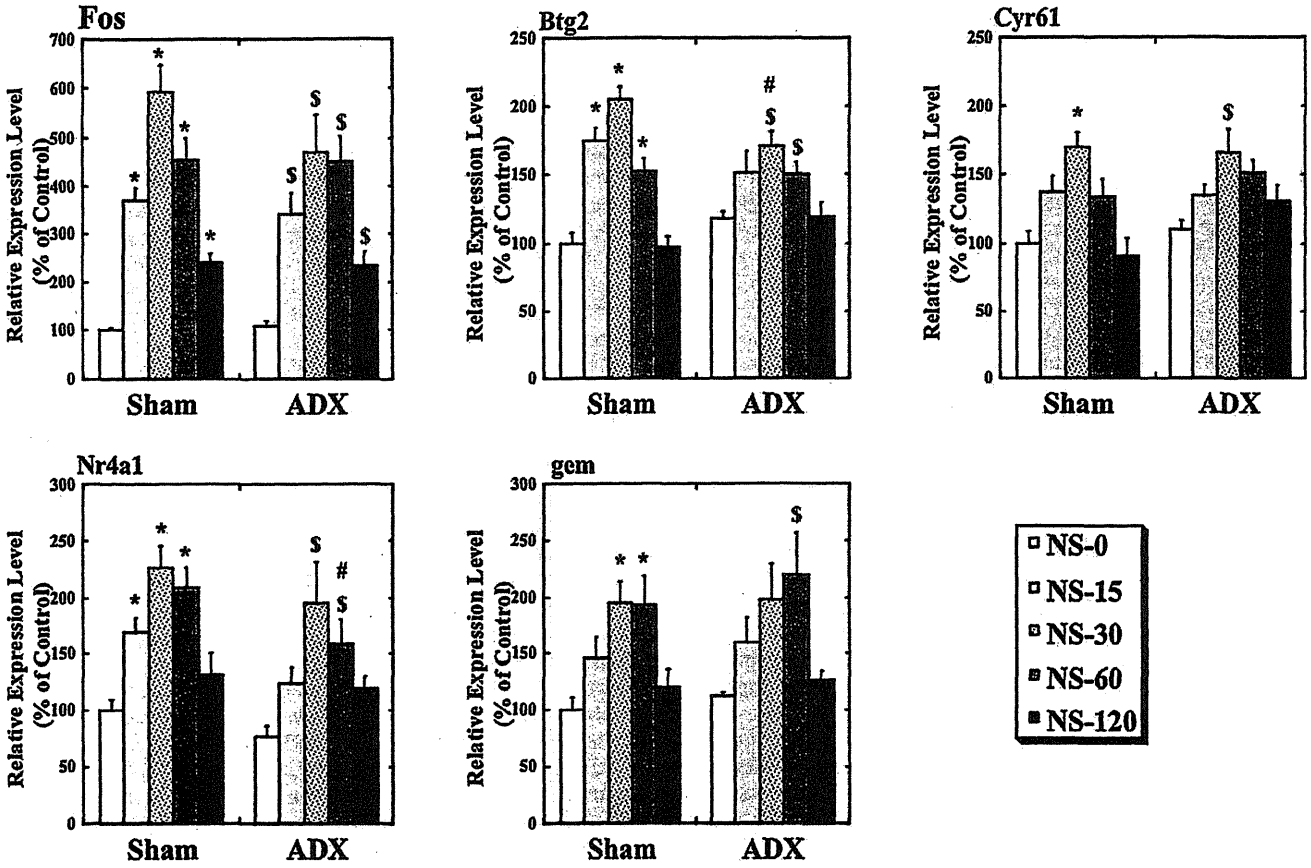

Fig. 4. Results of RT-PCR in the hippocampus of adrenalectomized mice in the time-scheduled study of novelty stress.
Mice (postnatal week 8) with adrenalectomy (ADX) or the sham operation (SHAM) were individually placed in a new cage for various times, i.e., 15 min (NS-15; ADX (n = 7), SHAM (n = 9)), 30 min (NS-30; ADX (n = 7), SHAM (n = 12)), 60 min (NS-60; ADX (n = 8), SHAM (n = 10)) and 120 min (NS-120; ADX (n = 8), SHAM (n = 8)) to induce novelty stress, while the control animals (NS-0; ADX (n = 7), SHAM (n = 13)) were left undisturbed in their home cage until they were killed. Results are mean with S.E.M. of data (normalized to Gapdh) and are expressed as a percentage of the mean value of the SHAM control (NS-0). The data were analyzed using two-way ANOVA followed by the post hoc Newman–Keuls test. $^{*}p < 0.05$ vs control (NS-0) of ADX, $^{\$}p < 0.05$ vs control (NS-0) of SHAM, $^{\#}p < 0.05$ vs the counterpart group of the time-scheduled study of stress in the SHAM.

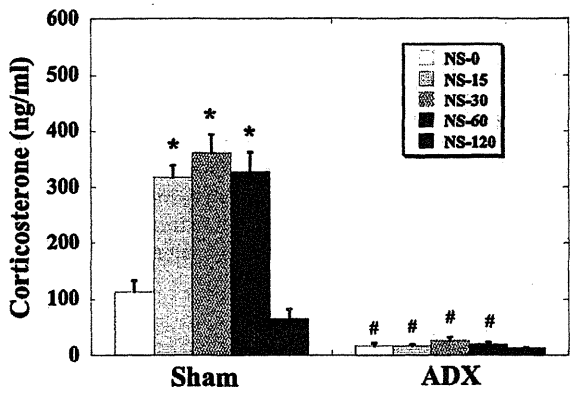

Fig. 5. Results of serum concentration of corticosterone in the adrenalectomized mice in the time-scheduled study of novelty stress. Mice (postnatal week 8) with adrenalectomy (ADX) or the sham operation (SHAM) were individually placed in a new cage for various times, i.e., 15 min (NS-15; ADX (n = 7), SHAM (n = 9)), 30 min (NS-30; ADX (n = 7), SHAM (n = 12)), 60 min (NS-60; ADX (n = 8), SHAM (n = 10)) and 120 min (NS-120; ADX (n = 8), SHAM (n = 8)) to induce novelty stress, while the control animals (NS-0; ADX (n = 7), SHAM (n = 13)) were left undisturbed in their home cage until they were killed. Results are mean with S.E.M. and were analyzed using two-way ANOVA followed by the post hoc Newman–Keuls test. $^{*}p < 0.05$ vs control (NS-0) of SHAM, $^{\#}p < 0.05$ vs the counterpart group of the time-scheduled study of stress in the SHAM.

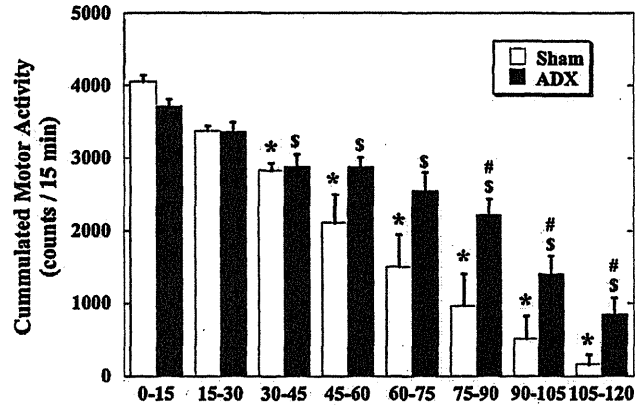

Fig. 6. Results of behavioral activity of the adrenalectomized mice during the exposure to novelty stress. The behavioral activities of mice placed in new cages were assessed by the Supermex instrument. The two-way ANOVA of repeated measure revealed significant main effects of the operation [$F(1, 98) = 5.78$, $p < 0.05$], exposure time [$F(7, 98) = 69.284$, $p < 0.0001$] and the interaction of operation × exposure time [$F(7, 98) = 4.156$, $p = 0.0005$]. * and $^{\$}p < 0.05$ vs the first 15-min interval for each group of mice (Student's t-test with Bonferroni's correction), $^{\#}p < 0.05$ vs the counterpart group of the time-scheduled study of stress in the SHAM (Student's t-test).

ticosterone in the ADX mice did not respond to the stress, which might be produced by accessory nodules (Hummel, 1958). Corticosteroids bind to two specific cytosolic receptors: the GRs or the MRs. MRs have a high affinity for corticosterone and are almost saturated under basal conditions. In contrast, GRs have a 10-fold lower affinity for corticosterone than MRs and become occupied only during stress and at the circadian peak of the hormones (De Kloet et al., 2005). The complex of steroid hormones and the receptors is moved to the nucleus and acts as regulators of gene transcription with a time frame of hours (Joëls and Baram, 2009). For example, the receptor-mediated regulations of gene expression in the hippocampus of rodents were detected 3 or 4 h (Hansson et al., 2000; Datson et al., 2001) after injections of the hormones. Thus, the results of the present study indicate that the bell-shaped activations in the hippocampal gene expression during the stress for 2 h were almost independent of the genomic effect of the elevated hormones.

The motor activity of the ADX mice during the stress differed from that of the sham-operated mice, showing a higher activity at the later part of stress exposure. Behavioral studies revealed interaction between MRs- and GRs-mediated effects on the coordinated control of various aspects of behavior (Korte, 2001; McEwen, 2007). A rapid, within 30 min, and non-genomic effect of glucocorticoids was observed in behavioral experiments with rodents (Tasker et al., 2006; Riedeman et al., 2010), such as locomotion (Sandi et al., 1996), risk assessment (Mikics et al., 2005), and aggressive behavior (Mikics et al., 2004). In the present study, the slower decreasing process of motor activities during the stress for 120 min was observed in the ADX mice. Thus, it is likely that the elevated corticosterone due to the stress played a role in the process of behavioral adaptation through the neurobiological cascades, while it cannot be excluded that other hormones secreted by the adrenal glands modulated the motor activity during the stress.

However, it has been reported that adrenalectomy of adult rats resulted in a selective loss of granule cells, but not pyramidal cells, in the hippocampus 3–4 months after surgery (Sloviter et al., 1989). Although the ADX mice in the present study were used in the experiment 2 weeks after the surgery, it cannot be negligible that the hippocampus of the ADX mice involved somewhat of a structural alteration associated with the reduced circulating corticosterone, which resulted in modifications of the stress response of the other parameters such as gene expression and behavior.

The present study examined the set of stress-related genes previously reported (Kurumaji et al., 2008). After the screening for stress-related genes in the neocortex of mice having received systemic injections of a pharmacological stressor, FG7142 (a partial inverse agonist for benzodiazepine binding sites in the $GABA_A$ receptors), by means of a DNA microarray method, the candidate genes were further characterized after treatment with the drug as well as after immobilization stress by quantitative RT-PCR methods. The systemic administration of FG7142 also produced an enhanced gene expression of the set of genes except for Rgs2 in the hippocampus of mice, mediating at the benzodiazepine binding sites (data not shown). The expressions of five genes in the hippocampus were activated by the exposure to the novelty stress. Fos is an immediate early gene and an established marker of neuronal activation used in many studies of stress including novelty stress (Cullinan et al., 1995; Pace et al., 2005; Senba and Ueyama, 1997; VanElzakker et al., 2008). Nr4a1, also known as nerve growth factor-inducible gene B (NGFI-B), nur77 or tetradecanoyl phorbol acetate-inducible sequence (TIS) 1 belongs to a family of immediate early transcription factors different from that of Fos and is well-characterized in the experiment of stress (Malkani and Rosen, 2000; Lévesque and Rouillard, 2006; Schiltz et al., 2007). Cyr61 is a member of the cystein-rich 61, connective tissue growth factor, and nephroblastoma overexpressed (CCN) family of secreted matricellular proteins that bridge the functional and physical gap between the extracellular matrix-associated proteins and cell surface molecules (Perbal, 2004; Chaqour and Goppelt-Struebe, 2006). Btg2 is a member of the pheocromocytoma cell-3 (PC3)/B-cell translocation gene (BTG)/transducer of ErbB-2 (TOB) family (Tirone, 2001). The potential roles of Btg2 have been suggested to be a transcriptional co-regulator, and differentiation and an anti-apoptotic factor in neurogenesis (Iacopetti et al., 1999; Lim, 2006). Gem belongs to a superfamily of small GTP-binding proteins which behave as molecular signal transducers by interacting with calmodulin (Maguire et al., 1994; Béguin et al., 2001). The activated genes in the present study may be sensitive markers for stressors in the hippocampus.

Many mediators other than the steroid hormone may play a role in the coordinated and complex system response to the novelty stress, mediating the regulation of gene expression and behavior. A release of monoamines is generally induced within minutes after the onset of the stressors, acting at the specific receptors localized in the hippocampus (Pacák and Palkovits, 2001; Joëls and Baram, 2009). The novelty stress induces a prominent increase in the release of acetylcholine (Aloisi et al., 1997; Giovannini et al., 2001), GABA (Giovannini et al., 2001; De Groote and Linthorst, 2007) and glutamate (Takeda et al., 2006) with a close relation to exploratory motor activity. Centrally administered CRF produced a rapid (30–60 min) induction of Fos mRNA expression in the hippocampus (Imaki et al., 1993) as well as a number of physiological and behavioral changes akin to those elicited by exposure to acute stress (Arzt and Holsboer, 2006). CRF in hippocampal neurons can be released by stress, resulting in a transcriptional activation of pyramidal cells through acting at the specific receptors (Chen et al., 2004). A rapid action of glucocorticoids within minutes can be mediated by receptors and proteins associated with membrane through the intracellular signaling cascades of the effectors (Tasker et al., 2006; Riedeman et al., 2010). Consequently, the present study could not explain the neurobiological mechanism whereby the novelty stress induces the activation of the gene expression in the hippocampus. A further examination of other brain areas containing the receptors in a different anatomical distribution may provide an insight into the mechanism. It is also required to clarify neural networks of the response to stress including a cognitive process of a new circumstance.

5. Conclusions

The response to the novelty stress, one of the psychological stressors, involved the transcriptional regulation of several genes in the hippocampus, independent of genomic effects of stress-activated adrenal hormones. The time course of the change in each parameter, i.e., gene expression, corticosterone and behavior, suggested a coordinated process of adaptation to the new environment. However, further studies are required to elucidate the neurobiological process of the coordination.

Acknowledgements

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Review

Analysis of free D-serine in mammals and its biological relevance☆

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Synapse–glia interaction

ABSTRACT

D-Serine is a unique endogenous substance enriched in the brain at the exceptionally high concentrations as a free D-amino acid in mammals throughout their life. Peripheral tissues and blood contain low or trace levels of the D-amino acid. In the nervous systems, D-serine appears to act as an intrinsic coagonist for the *N*-methyl-D-aspartate type glutamate receptor (NMDA receptor) based upon the following characteristics: (i) D-serine stereoselectively binds to and stimulates the glycine-regulatory site of the NMDA receptor consisting of GRIN1/GRIN2 subunits more potently than glycine with an affinity and ED50 at high nanomolar ranges, (ii) the selective elimination of D-serine in brain tissues attenuates the NMDA receptor functions, indicating an indispensable role in physiological activation of the glutamate receptor, and (iii) the distribution of D-serine is uneven and closely correlated with that of the binding densities of the various NMDA receptor sites, and especially of the GRIN2B subunit in the brain. Moreover, D-serine exerts substantial influence on the GRIN1/GRIN3-NMDA and δ2 glutamate receptor. In the brain and retina, metabolic processes of D-serine, such as biosynthesis, extracellular release, uptake, and degradation, are observed and some candidate molecules that mediate these processes have been isolated. The fact that the mode of extracellular release of D-serine in the brain differs from that of classical neurotransmitters is likely to be related to the detection of D-serine in both glial cells and neurons, suggesting that D-serine signals could be required for the glia–synapse interaction. Moreover, the findings from the basic experiments and clinical observations support the views that the signaling system of endogenous free D-serine plays important roles, at least, through the action on the NMDA receptors in the brain wiring development and the regulation of higher brain functions, including cognitive, emotional and sensorimotor function. Based upon these data, aberrant D-serine–NMDA receptor interactions have been considered to be involved in the pathophysiology of a variety of neuropsychiatric disorders including schizophrenia and ischemic neuronal cell death. The molecular and cellular mechanisms for regulating the D-serine signals in the nervous system are, therefore, suitable targets for studies aiming to elucidate the causes of neuropsychiatric disorders and for the development of new treatments for intractable neuropsychiatric symptoms.

Contents

Abbreviations: Aβ, amyloid β peptide; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Asc-1, Na^+-independent alanine–serine–cysteine transporter 1; ASCT2, Na^+-dependent broad-spectrum neutral amino acid transporter 2; BBB, blood–brain barrier; CBT, cognitive behavioral therapy; CNS, central nervous system; DAO, D-amino acid oxidase; DAOA, D-amino acid oxidase activator; DA, dopamine; DPAG, dorsal periaqueductal gray matter; D-ser, D-serine; Dsm-1/PAPST-1, D-serine modulator-1/3′-phosphoadenocine 5′-phosphosulfate transporter-1; GABA, γ-aminobutyric acid; GCS, glycine cleavage system; Gly, glycine; GLYT, glycine transporter; GRIP, glutamate receptor interacting protein; L-glu, L-glutamate; NMDA, *N*-methyl-D-aspartate; 3-PGDH, 3-phosphoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; PSPH, phosphoserine phosphatase; PTSD, post-traumatic stress disorder; SHMT, serine hydroxymethyltransferase; SNARE, soluble *N*-methylmaleimide susceptibility factor attachment protein receptor; SNP, single nucleotide polymorphisms; SOD1, superoxide dismutase 1; SRR, serine racemase; THF, tetrahydrofolate.

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1. Introduction – detection of endogenous D-serine in mammals

Prior to the 1990s, D-serine, like the other D-amino acids, had long been believed to be absent in mammalian tissues even though the L-type homo-chirality hypothesis for the amino acids in the organisms had been challenged by the following facts; (a) the occurrence of D-amino acid oxidase in the organisms, including mammals, was identified in 1935 [1], and since then this observation has suggested the existence of the endogenous D-amino acids that serve as the substrates for the enzyme [1], (b) the D-aspartate residues had already been proved in the long-living proteins such as crystalline in mammalian eyes [2], (c) the presence of incorporated or free D-serine had also been reported in the cell wall of bacteria [3], and in silkworm [4–6] and earthworm tissues [7–10] and (d) the transient presence of substantial amounts of free D-aspartate and high ratios with respect to the L-enantiomer had been demonstrated in the mammalian brain and peripheral tissues during the early stage of postnatal development [11]. In the early 1990s, the present author with coworkers at the National Institute of Neuroscience (Japan) discovered by gas chromatography (GC) and GC with mass spectrometry (GC–MS) that free D-serine is present and enriched in the brain at a high concentration comparable with those of classical neurotransmitters and at a large ratio to the L-serine content over the lifetime in the rat [12], under way of the present author's research project exploring new therapies for intractable schizophrenia [13], innovating in the biological significance of D-amino acid. In parallel, Nagata and collaborators [14] revealed using two-dimensional thin-layer chromatography in combination with high-performance liquid chromatography (HPLC) that free D-serine naturally occurs at low levels in the mammalian periphery including the blood and kidney, while studying D-amino acids and D-amino acid oxidase. These pioneering investigations of D-serine carefully exclude the possibility that D-serine is chiefly produced by the enteric bacteria or by the non-enzymatic racemization of L-serine during the assay procedures [12,14]. This review will begin with a brief introduction of the background of the present author's research leading to the detection of brain D-serine as this step in the history of D-serine research was important for gaining an understanding of the physiological functions of D-serine and their association with neuropsychiatric disorders.

One serious and persistent problem in clinical psychiatry is that sufficient social rehabilitation is precluded by symptoms resistant to pharmacotherapy, as is commonly seen in patients with schizophrenia [13,15]. To obtain a clue for a strategy to solve the problem, the present author has paid particular attention to an anesthetic phencyclidine (PCP), which causes schizophrenia-like symptoms that are both responsive and resistant to antipsychotics, a currently available class of drugs for the treatment of schizophrenia [13,15]. In 1983, PCP was found to be a potent non-competitive antagonist for the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor (NMDA receptor) [16]. In the mid-1980s, this author started investigating a comprehensive treatment strategy for treatment-resistant schizophrenia, which facilitates NMDA receptor functions [13,15,17,18], albeit not via the glutamate site agonists that often bring about cell death or convulsions, but rather via the glycine-site stimulators which are unlikely to have such harmful effects [13,15,19].

To this end, the two D-amino acids, D-serine, and D-alanine, were chosen for further investigation for the following reasons: D-serine, and D-alanine, but not glycine itself, show a site- and stereo-selective actions on the NMDA receptor glycine site in that they have a much higher affinity for the NMDA glycine site than the corresponding L-amino acids, and they have very low affinity for the inhibitory glycine receptor [13,15]. According to this author's idea to overcome the poor ability of these polar D-amino acids to cross the blood–brain barrier (BBB) that their lipid-modified compounds could more easily permeate the BBB and thus would serve as a more useful prototypes for the future clinical systemic application, Hidehiko Hibino, Ph.D. at Nippon Oil and Fats, Co. Ltd., invented and supplied the materials, *N*-myristoyl-D-serine and *N*-myristoyl-D-alanine, for our experiments [20]. Our rat studies demonstrated that not only the intracerebroventiricular injection of D-serine and D-alanine [21] but also the systemic administration of their myristoylated compounds antagonized the PCP-induced abnormal behavior, an animal model of antipsychotic-resistant schizophrenia