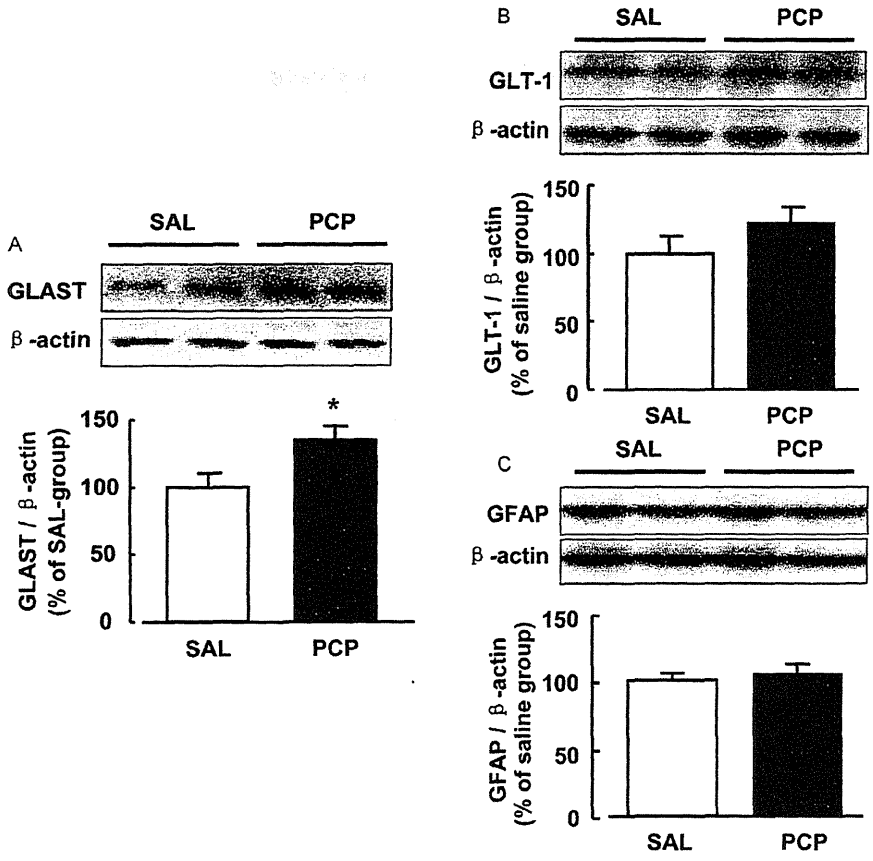

Fig. 4. Changes in the expression of glutamate transporters in the prefrontal cortex of prenatal PCP-treated mice. Representative Western blots band for the expression of GLAST, GLT-1 and GFAP. The amount of protein (30 μg/well) loaded was normalized to that of β-actin. Results are represented as the level of GLAST (A), and GLT-1 (B), as well as GFAP (C) in the prefrontal cortex. *$P < 0.05$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M. for 6–7 mice in each group (Student's *t*-test). SAL, saline; PCP, phencyclidine.

higher dose of DL-TBOA (10 nmol) ($F_{group\,(1,40)} = 24.66$, $P < 0.01$; $F_{treatment\,(2,40)} = 3.96$, $P < 0.05$; $F_{group \times treatment\,(2,40)} = 6.51$, two-way ANOVA; $P < 0.01$, Fig. 5C). However, there were no significant differences in total exploration time in the retention session among each group ($F_{group\,(1,40)} = 0.14$, $P > 0.05$; $F_{treatment\,(2,40)} = 0.02$, $P > 0.05$; $F_{group \times treatment\,(2,40)} = 1.27$ $P > 0.05$; two-way ANOVA; Fig. 5D). These results suggested that DL-TBOA did not affect motivation or curiosity, but ameliorated the impairment of recognition memory in the prenatal PCP-treated mice.

Next, we evaluated the effects of DL-TBOA on the prolonged immobility time in the forced swimming test in the PCP-treated mice. DL-TBOA (10 nmol) significantly reversed the prolonged immobility induced by prenatal PCP exposure in the forced swimming test ($F_{group\,(1,40)} = 18.03$, $P < 0.01$; $F_{treatment\,(2,40)} = 7.42$, $P < 0.01$; $F_{group \times treatment\,(2,40)} = 3.10$, $P = 0.06$; two-way ANOVA; Fig. 5E), but did not change immobility time in the SAL-treated mice. These results indicated DL-TBOA to be effective in correcting emotional abnormalities induced by prenatal exposure to PCP without affecting motility.

Furthermore, we continued to evaluate the effects of DL-TBOA on the reduced extracellular glutamate level and impairment of K^+-induced glutamate release induced by prenatal PCP treatment. After the basal levels of glutamate reached a steady state, DL-TBOA was given through the probe for dialysis and the basal release of glutamate was monitored for 90 min. DL-TBOA tended to normalize the level of extracellular glutamate in the prenatal PCP-treated mice ($F_{(2,18)} = 2.60$, $P = 0.10$; one-way ANOVA; Fig. 6A). Moreover, it clearly improved the high K^+ (100 mM)-induced glutamate release reduced by prenatal exposure to PCP ($F_{group\,(2,18)} = 9.09$, $P < 0.01$; $F_{time\,(4,18)} = 22.45$, $P < 0.01$; $F_{group \times time\,(8,18)} = 2.43$, $P < 0.05$; repeated two-way ANOVA for 10–50 min; Fig. 6B). These results indicated that DL-TBOA reversed the reduction in glutamatergic neurotransmission observed in the prenatal PCP-treated mice.

4. Discussion

The blockade of NMDA receptors by PCP in the developing brain has been found to impair learning and memory. For instance, prenatal exposure to PCP disrupts passive avoidance and pole-climbing avoidance responses [36], and impairs performance in the eight-arm maze and Morris water maze in adult rats [2,51]. In the present study, prenatal PCP treatment produced an impairment of memory in the novel object recognition test, consistently suggested a cognitive deficit in this model. Furthermore, the prenatal PCP-treated mice showed a prolonged immobility in the forced swimming test, which are frequently observed in PCP animal models displaying schizophrenia-like negative symptom [38]. Taken together, these results indicate that the blockade of NMDA receptors by PCP in the prenatal period triggers cognitive and emotional abnormalities in postpubertal mice.

Glutamate neurotransmission plays a critical role in synaptic activity and plasticity throughout the brain, including cognition-, emotion- and reward-related circuits [31]. In schizophrenic patients, evidence of abnormal glutamatergic transmission has been found, such as disturbances of cortical glutamate release

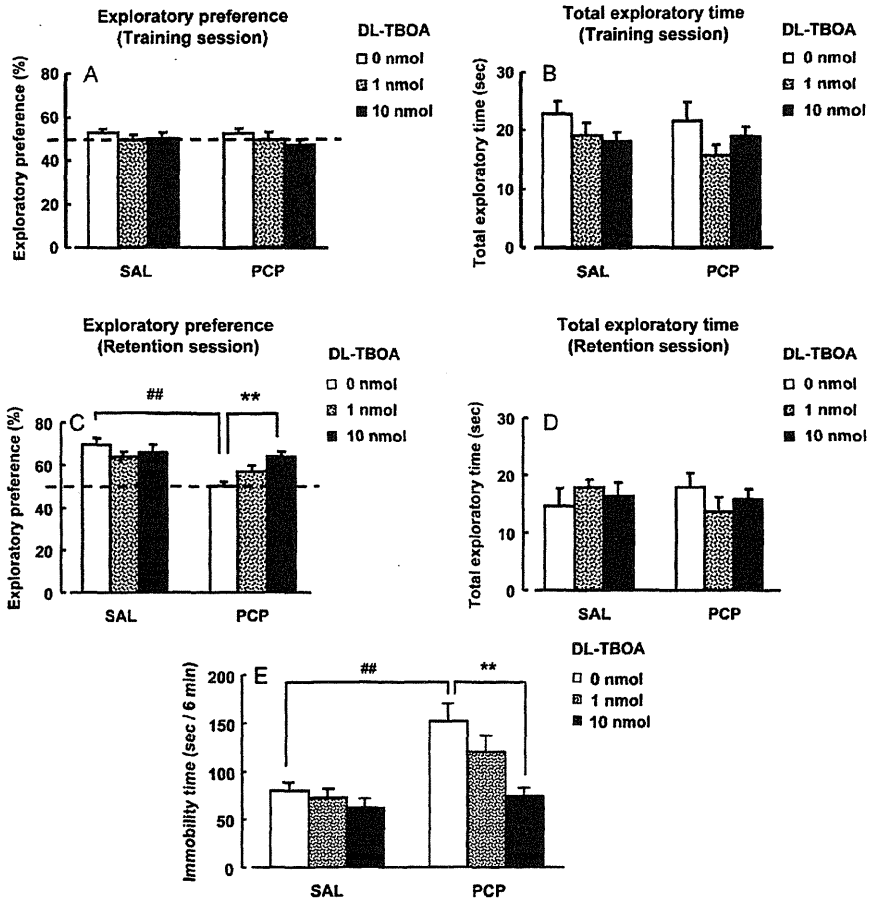

Fig. 5. Effects of DL-TBOA on the behavior in the prenatal PCP-treated mice. DL-TBOA (1 and 10 nmol) was administered by microinjection directly into the prefrontal cortex 30 min before each behavioral test. Exploratory preference (%) in the training session (A) and retention session (C). Total exploration time (s) in the training session (B) and retention session (D) of the novel object recognition test. Immobility time (s) was assessed for 6 min in the forced swimming test (E). Data are expressed as the mean ± S.E.M. for 7–8 mice in each group. $^{\#\#}P<0.01$ compared with the prenatal SAL-treated group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the prenatal PCP-treated group (two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

[7,8,14,24], lower glutamate levels in the PFC [46], and decreased levels of glutamate in cerebrospinal fluid [19], as well as reduced glutamatergic tone in the cortex area [16]. In PCP-treated adult mice, a decrease in spontaneous extracellular glutamate release [34] and in the level of phosphorylated-NR1 [33,34], but an increase in levels of GLAST expression has been observed in the PFC [34]. In the present study, we found a decrease in both the extracellular glutamate concentration and high K^+-induced release of glutamate in the PFC of the PCP-treated mice compared with the SAL-treated mice, suggesting that prenatal exposure to PCP produced a pre-

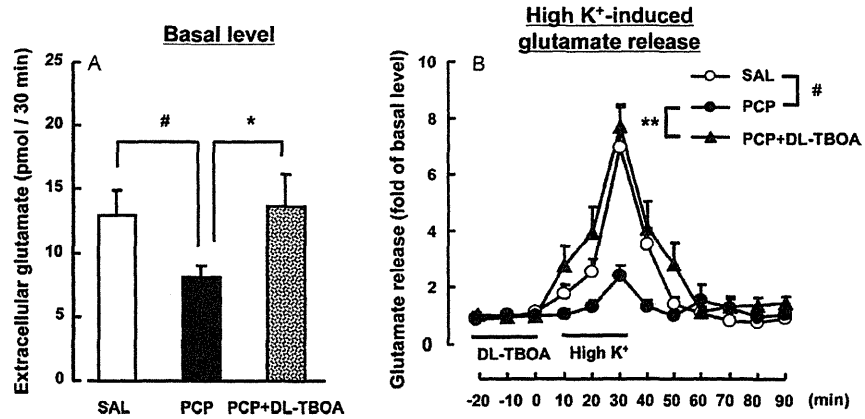

Fig. 6. Effects of DL-TBOA on the reduction of glutamate release in the prenatal PCP-treated mice. DL-TBOA (1 mM) was administered through a micodialysis tube into the prefrontal cortex of mice for 30 min (μl/min). After the administration, basal glutamate release (A) and K^+-evoked (100 mM) glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined. Data are expressed as the mean ± S.E.M. for 7 mice in each group. $^{\#}P<0.05$ compared with the prenatal SAL-treated group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the prenatal PCP-treated group (one-way ANOVA or repeated two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

synaptic hypofunctional glutamatergic neurotransmission. Given that glutamate neurotransmission plays a critical role in synaptic plasticity and long-term potentiation [31], it is possible that the hypofunctional glutamatergic neurotransmission was associated with these behavioral deficits induced by prenatal PCP treatment.

Glutamate transporters, GLAST and GLT-1, are considered to regulate glutamate transmission by rapidly clearing glutamate from extracellular fluid [11]. It is suggested that an increase in GLAST expression contributes to a decrease in extracellular glutamate release, because the regulation of extracellular glutamate levels by membrane transporters is important for terminating synaptic transmission in the brain [43]. Furthermore, increased numbers of binding sites and protein expression of glutamate transporters have been observed in the postmortem brain of schizophrenia patients [9,11,19,37,42]. Additionally, antipsychotics such as clozapine inhibites the increase in glutamate transporters [29,48]. In this study, we observed the overexpression of GLAST protein in the prenatal PCP-treated mice, although no significant change in GLT-1 expression. GLAST is produced during embryogenesis in rodents, while GLT-1 is expressed in the forebrain postnatally [47]. Therefore, prenatal exposure to PCP might lead to different patterns of GLAST and GLT-1 expression. Furthermore, we found that DL-TBOA, a potent glutamate transporter blocker, attenuated the cognitive and emotional deficits by normalizing the extracellular release of glutamate. These results suggested that the elevated expression of GLAST protein was, at least in part, responsible for the dysfunctional glutamate transmission associated with these behavioral changes in the prenatal PCP-treated mice.

GLAST proteins are expressed in glial cells of the adult brain and spinal cord [23,41]. However, in this study, we failed to detect a significant change in the expression of GFAP, a marker of glial cells. Thus, it is unlikely that the increase in GLAST expression is due to the activation of glial cells. It was reported that Ca^{2+} influx inhibits GLAST expression in astrocytes [26]. Since PCP inhibited the influx of Ca^{2+} by blocking the NMDA receptor, one possibility is that the disruption of Ca^{2+} influx into astrocytes is associated with the up-regulation of GLAST expression. Furthermore, any factors which enhance the gene transcription of GLAST or disrupt the protein's degradation might also contribute to the up-regulated expression. The precise mechanism remains to be elucidated.

The NMDA receptors are thought to control the differentiation and migration of immature neurons [4,20]. Many neurons undergo a stage when they are critically dependent on stimulation by glutamate through the NMDA receptors, and sustained deprivation of this input by NMDA receptor antagonists during development activates apoptosis [17]. Therefore, we could not exclude the possibility that a loss of glutamatergic neurons contributes to the glutamatergic hypofunction, although here, we did not observe any significant decrease in the total number of neurons in adult mice. Thus, any neurodevelopmental disturbances caused by prenatal exposure to PCP in development are potentially implicated in these behavioral and biochemical changes.

5. Conclusion

The present findings indicate that prenatal exposure to PCP leads to cognitive impairment and emotional dysfunction, which are accompanied by a disruption to pre-synaptic glutamate neurotransmission through the enhanced expression of glutamate transporters in the PFC. Since the abnormal glutamatergic release and the altered expression of glutamate transporters are involved in the pathophysiology of schizophrenia, this study provides further insights into how psychiatric illnesses develop.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.01.035.

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Current Topics

Translational Research in Neurodevelopmental Disorders: Development of Etiology-Based Animal Models

Genetic Animal Models of Schizophrenia Related with the Hypothesis of Abnormal Neurodevelopment

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Accumulating evidence supports the existence of an overlap in genetic susceptibility with schizophrenia. Translation of human genetic mutations into animals is one of the most important strategies to study the pathogenesis of schizophrenia, identify potential drug targets, and test new medicines for antipsychotic treatment. Recent discoveries of susceptibility genes for schizophrenia make the possibility to develop newer genetic mouse models based on the neurodevelopmental hypotheses of schizophrenia. Although it is not possible to mimic all schizophrenic symptoms by these animal models, the genetic mouse models based on the neurodevelopmental hypothesis are widely developed to reproduce several schizophrenia-like behavioral and biochemical changes in humans. In this mini review, we will discuss the neuropathological and behavioral manifestations of representative genetic mouse models for schizophrenia, associated with the hypothesis of abnormal neurodevelopment.

Key words schizophrenia; genetic mouse model; neurodevelopmental hypothesis

1. INTRODUCTION

Schizophrenia is a heritable mental disorder characterized by chronic psychotic symptoms and cognitive deficits. For more than three decades, the neurodevelopmental hypothesis has prevailed for schizophrenia. This hypothesis posits that schizophrenia is the behavioral outcome of an aberration in neurodevelopmental processes that begins long before the onset of clinical symptoms[1,2] and involves deficits in the genetic program for normal formation of synapses and migration of neurons, as well as their connections in brain development.[3]

With growing evidences on the neurobiology and genetics of schizophrenia, more animal models have been developed to study the molecular mechanisms of pathophysiological changes and to design more effective therapies for schizophrenia. In principle, genetic manipulation offers advantages over pharmacologic models because it is more selective in its molecular targets, it is developmental by nature, and it can be varied in terms of penetrance, allele dose, and temporal characteristics.[4] Genes involved in regulation of cortical cytoarchitecture during development have been suspected to mediate abnormal neurodevelopment in schizophrenia.[5—7] Therefore some of these genes have been employed to make potential animal models of schizophrenia.

In this article, we briefly review the genetic animal models related with the hypothesis of abnormal neurodevelopment of schizophrenia at present.

2. GENETIC EVIDENCE OF NEURODEVELOPMENTAL HYPOTHESIS

Schizophrenia is a clinically heterogeneous psychotic illness whose etiology remains poorly understood. However, clinical, epidemiological, genetic, and neuropathological features of schizophrenia continue to suggest that abnormal neurodevelopment is important for the disorder.[8] Genetic studies have identified several specific genes that are associated with schizophrenia risk in a number of populations. Generally, twin studies have shown that schizophrenia is a predominant genetic disorder, with estimates of heritability risk ranging at 50—60% and there are recent reports of 80%.[9—12] Family studies have found that single effects of a major gene are unlikely; instead, polygenic models effects of multiple-risk genes acting additively or multiplicatively may provide the best explanation for schizophrenia.[13] Several neurodevelopment-related genes are located in chromosomal loci that are associated with potential candidate genes due to their polymorphic status, and to alter their expression during embryonic stages. They might putatively result in the neurodevelopmental abnormalities observed in schizophrenia.[14] Hence identification of genes responsible for this high heritability will be critical to understanding this disorder. Moreover, without parsing based on genotypes, the environmental and epigenetic factors may be more difficult to clarify.

Anatomical abnormalities such as ventricular enlargement, volume reductions of prefrontal cortex and hippocampus, and generalized brain reduction are well established among other features of schizophrenia.[15—17] Obvious alterations in neuron size and morphology as well as synaptic connectivity are also observed in schizophrenic patients.[18] Investigations focusing on cortical and limbic brain regions increasingly demonstrate that structural and molecular integrity of the synaptic complex, glutamate-related receptors, and signal transduction pathways take critical roles in brain develop-

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ment, synaptogenesis, and synaptic plasticity.[8] More importantly, most schizophrenia-related specific genes including disrupted in schizophrenia-1 (DISC1), neuregulin-1 (NRG1), dysbindin-1, and AKT-1 play important roles in neurodevelopment, neurotransmission, and neuroplasticity. Although variations among each gene confer only modest increases in the risk for schizophrenia, the discovery of these genes and identification of candidate proteins and molecular pathways may importantly contribute to the pathophysiology of schizophrenia.[8]

3. GENETIC ANIMAL MODELS RELATED TO THE NEURODEVELOPMENTAL HYPOTHESES OF SCHIZOPHRENIA

Schizophrenia-Susceptibility Genes. Disrupted in Schizophrenia-1 (DISC1) Schizophrenia susceptibility genes have been identified by human genetic studies and enabled us to generate mouse models on the basis of genetic etiology. Because causal mutations have not been identified, there is still debate on the significant roles of each gene. Nonetheless, many of the genetically engineered models for these genes display behavioral abnormalities and morphological alterations that may be relevant to schizophrenia patients. Here, we summarize the behavioral and morphological changes in representative genetic mouse models that are associated with the neurodevelopmental hypotheses of schizophrenia.

Numerous studies have found that DISC1 is highly expressed during brain development and plays critical roles in the growth of the embryonic and postnatal brain.[19,20] It has been confirmed that DISC1 affects the development and maturation of neuronal systems, which is implicated with psychiatric disorders.[21] DISC1 was originally identified as truncated by a translocation that segregated with schizophrenia. Therefore several transgenic animal models expressing this truncated protein have been generated. In several kinds of transgenic mice expressing truncated DISC1, enlarged lateral ventricles,[22,23] reduced cortical thickness, and partial agenesis in the corpus callosum[24] as well as reduced immunoreactivity of parvalbumin (PV) in both the medial prefrontal cortex (mPFC) and the hippocampus[22,24] have been detected as important hallmarks for schizophrenia. Furthermore, transient expression of a dominant-negative DISC1 (C-terminal fragment of DISC1) at postnatal day 7 (PD 7), produces a reduction of hippocampal synaptic transmission[25] as a result of decrease of the hippocampal dendritic complexity. In another type of DISC1 transgenic mice, misorientated and shorter dendrites and decrease in numbers of synaptic spines of hippocampal granule cells[26] have been found, which cause reduced short-term potentiating at CA3/CA1 synapses and indirect working memory deficit.[26]

Recent study has revealed critical roles of DISC1 in regulation of the embryonic and adult neurogenesis.[27] DISC1 is highly expressed in the embryonic ventricular/subventricular zones of the cortex where neural progenitor cells reside, suggesting that this gene regulates their proliferation and/or differentiation. To confirm this idea, DISC1 RNA interference (RNAi) was introduced into neural progenitors in the developing neocortex, using *in utero* electroporation, and a significant reduction of proliferation of progenitor cells and differentiation of premature neurons was observed. Interestingly, overexpression of DISC1 in neural progenitors results in an opposite phenotype. It has been also found that knockdown of DISC1 using a lentivirus to deliver RNAi results in a decrease in the proliferation of adult progenitor cells in the dentate gyrus.[27] Moreover, such transgenic DISC1 mutant mice show hyperlocomotion in a novel environment and increased immobility time in a forced swimming test, which are frequently observed in animal models displaying schizophrenia-like behavior.[27]

In our recent study, by utilizing the methods of in utero electroporation, we have successfully generated a novel mouse model by transferring RNAi of DISC1 at embryonic day 14 (E 14) selectively to disrupt DISC1 gene expression in a lineage for pyramidal neurons mainly in the prefrontal cortex during neurodevelopment.[28] Our results indicate that knockdown of DISC1 leads to maturation-dependent deficits in mesocortical dopaminergic projections and induces a series of schizophrenia-like behavioral abnormalities including hyperlocomotion in a novel environment, enhanced immobility time in forced swimming test, deficits in prepulse inhibition (PPI) of startle response, and memory impairments in novel object recognition test after sexual maturation. Importantly, these behavioral abnormalities are attenuated by treatment with atypical antipsychotic clozapine, indicating their association with schizophrenia.[28] Moreover, we further observed a significant decrease in the extracellular level of dopamine and tyrosine hydroxylase (TH), a marker of mature axonal terminals of the dopaminergic projection, and disturbances of PV interneurons and pyramidal neurons in mPFC of DISC1 knockdown mice at PD 56 but not PD 28 or PD 42.[28] These results suggest that DISC1 may play critical roles in neurodevelopment, and its disruption during development may induce several schizophrenia-like features in adult mice.

Although disruption of DISC1 may be critically involved in many cases of schizophrenia, direct evidence from genetic linkage and association studies suggests that variants of DISC1 may not occur in most cases of the disorder, compared with other psychotic diseases.[21]

Neuregulin-1 (NRG-1) NRG-1 was first identified as a susceptibility gene for schizophrenia in an Icelandic population[29] and further confirmed by subsequent studies.[30,31] NRG-1 plays important roles in brain development, such as neuronal migration and neurite outgrowth, as well as proliferation of glia cells.[32] Furthermore, increased levels of NRG-1 type I mRNA are observed in schizophrenia patients.[33,34] Tissue culture study has revealed that NRG-1 dampens *N*-methyl-D-aspartate (NMDA) receptor function in pyramidal neurons of the prefrontal cortex.[35] Therefore NRG-1 mutant is considered a useful in genetic animal model for schizophrenia.

NRG-1 transgenic mouse models have been generated by manipulation of expression of different NRG1 isoforms by several studies. Several schizophrenia-like behaviors, such as hyperlocomotor activity and impaired PPI, as well as decreased expression of NMDA receptor have been observed in heterozygous NRG-1 knockout mice lacking the transmembrane domain of NRG-1 gene.[29] Moreover, disruption of type III NRG1 in adult mice results in increased volume of lateral ventricles and decreased density of dendritic spines in

hippocampal pyramidal neurons.[36] Knockout of the NRG-1 receptors ErbB2 and ErbB4 (ErbB2/4) at early embryonic stage decreases the density of spine in both the cortex and hippocampus.[37] In behavioral analysis, ErbB2/4 knockout mice display an increase in aggression and a deficit in PPI, as a model of sensorimotor gating that is abnormal in schizophrenia patients.[37] Moreover, ErbB4 knockout mice show a decrease in the power of kainate-induced gamma oscillations[38] and reduction of the density of calbindin-positive GABAergic interneurons in the cortex, as well as PV-positive interneurons in the hippocampus.[39]

Most positive single nucleotide polymorphisms (SNPs) are located upstream of the start site of NRG-1 exons, which suggests a probable effect on its expression. Whereas, the consequences of mutations in the region of NRG-1 remain unknown, and whether the heterozygotes knockout resembles a disease state is arguable. In addition, NRG-1 comprises in at least six major isoforms and many splice variants. The alternations of which one is particularly important for schizophrenia remain unclear, but they are quite critical to design genetically engineered animal models related to this disorder.

Dysbindin Dysbindin is another likely susceptibility gene that has been identified by several studies.[40,41] Irish study of high-density schizophrenia families has suggested that schizophrenic patients with negative symptoms are more likely to inherit the risk of dysbindin mutant, raising the possibility that negative symptoms in psychotic bipolar cases of schizophrenia are likely attributable to heritability of dysbindin mutation. Postmortem study has reported a decrease in the level of gene transcription of dysbindin and its protein expression in brain tissues of schizophrenia patients.[42]

Thus sandy (Sdy) mouse has been designed to mimic a deficiency of dysbindin in human.[43] As reported, Sdy mouse harbors a spontaneously occurring deletion in the DTNBP1 gene and expresses no dysbindin protein, which provides a unique tool to study the role of dysbindin in schizophrenia. Sdy mice also exhibit morphological changes in excitatory asymmetrical synapses on hippocampal CA1 dendritic spines, larger vesicle size, slower vesicle release, and lower release probability, as well as smaller total population of the readily releasable vesicle pool.[44] These mutant mice display deficits of neurosecretion and synaptic morphology in hippocampal neurons, and manifest some schizophrenia-like behavior such as social withdrawal and cognitive deficits. In the hippocampus of Sdy mice, the level of Snapin (a SNAP25-binding protein and a synaptic priming regulator) is reduced, which suggests that destabilization of Snapin in the Sdy mice may lead to abnormal neurotransmission and abnormal behavior.[45] Although more information for the associations between dysbindin gene and schizophrenia is needed, Sdy mice are able to serve as a genetic animal model to identify potential pathways of dysbindin in schizophrenia.

Brain-Derived Neurotrophic Factor (BDNF) BDNF has been found to play important roles in promoting and modifying growth, development, survival of neuronal populations, and activity-dependent neuronal plasticity.[46] BDNF is implicated in the pathogenesis of schizophrenia, since its expression is reduced in some postmortem brains of schizophrenia patients,[47,48] indicating that the downregulated expression of BDNF leads to abnormalities in developing brain. Atypical BDNF knockout mice, in which one allele of BDNF gene is disrupted through the whole developmental stages, exhibit hyperactivity in locomotion and behavioral deficits in spatial learning and memory.[49,50] Dysfunction of non-spatial associative memory is also observed in conditional BDNF knockout mice, in which BDNF gene is disturbed approximately 3 weeks.[51] However, these mutant mice do not show hypersensitivity or deficits in PPI and fear conditioning.[52,53] Moreover, dysfunction of context-dependent fear memory has been found in inducible BDNF knockout mice, in which the disrupted BDNF gene is limited in specific brain regions at certain developmental stages.[54] Further inducible knockout of BDNF from the embryonic stage induces severer context-dependent memory deficits compared with later knockout mice. These data suggest that BDNF plays a critical role in neurodevelopment. However, the genetic linkage between BDNF and schizophrenia is relatively weak, although the biochemical and behavioral changes in BDNF knockout mice resemble several pathological changes of schizophrenia.[55,56]

Reelin Reelin is a glycoprotein that guides neurons and radial glial cells to corrected position in the developing brain. A series of studies suggest that reelin might be a vulnerability gene involved in the development of psychosis including schizophrenia.[57—59] "Reeler mice" is a naturally occurring mutant mice model generated by disrupting the reelin gene. These mice exhibit decreased expression of reelin and glutamic acid decarboxylase 67 (GAD67), as well as lower density of dendritic spine,[57] which resemble some pathological changes of schizophrenic patients.[58] Moreover, these mutant mice show behavioral deficits in PPI, which is specifically involved in schizophrenia, although the reelin mutant mice do not show abnormalities in working memory or social interaction. Nonetheless, the reelin gene appears to play important roles in neuronal development and could mediate outcomes of some causative mutations in other genes and critical environmental insults.

NMDA Receptor Subunit 1 (NR1) Genetic disruption of NR1 by traditional and conditional knockout in mice results in hyperlocomotion, stereotypy, abnormal social behavior, cognitive dysfunction, and abnormal brain development.[60—62] These abnormalities resemble several aspects of schizophrenia. Importantly, some deficits are attenuated by antipsychotic treatment. Roles of NR1 in the pathology of schizophrenia are further supported by decreased expression of NR1 in postmortem tissues from schizophrenic patients and an increase in NR1 expression by chronic antipsychotic treatment.[63—65] Small molecules that enhance the function of NMDA receptor are being tested as novel adjunct therapies for schizophrenia treatment in clinical trials.[66,67]

Recently, Belforte *et al.*[68] characterized a mouse strain in which the essential NR1 subunit of the NMDA receptor is selectively eliminated by 40—50% in cortical and hippocampal interneurons in early postnatal development. Consistent with the NMDA receptor hypofunction hypothesis of schizophrenia, the postnatal NR1-ablated mice exhibit distinct schizophrenia-related symptoms after adolescence, including novelty-induced hyperlocomotion, mating and nest-building deficits, as well as anhedonia-like and anxiety-like behaviors. In addition, impairment of social memory, spatial working memory, and prepulse inhibition are also observed in the mutant mice. Furthermore, reduced expression of GAD67 and

PV is accompanied by disinhibition of cortical excitatory neurons and reduced neuronal synchrony. However, postadolescent deletion of NR1 did not result in such abnormalities, suggesting that early postnatal inhibition of NMDA receptor activity in corticolimbic GABAergic interneurons contributes to the pathophysiology of schizophrenia-related disorders.[68]

Others Studies have focused on a number of interesting candidate genes in neurodevelopmental hypotheses of schizophrenia, such as neural cell adhesion molecule (NCAM), cyclin-dependent kinase-5 (CDK5), V-akt murine thymoma viral oncogene homolog 1 (Akt1), Lis1, and Lhx5 so as to prepare potential genetic models.[69—72] Mice expressing these mutant genes show abnormal development in the brain and some schizophrenia-like behavior, although the patterns of abnormality vary. Clearly, the potential genetically to manipulate genes that affect brain development and to explore phenomenological links to the molecular and behavioral phenotypes related to schizophrenia is virtually limitless.

4. PERSPECTIVES

Schizophrenia has been long recognized as a heritable mental illness that probably involves multiple genes with relatively modest effects across large populations.[73] Most of these identified susceptibility genes such as DISC-1, NRG-1, AKT1, and Reelin are known to have essential functions in neurodevelopment including neuronal differentiation, migration, survival, synaptogenesis, and apoptosis.[27,74—77] Thus some aspects of altered brain development in schizophrenia may be attributable to abnormal expression of genes that are essential for early neurodevelopmental processes. Furthermore, such genetic mechanisms may significantly interact with prenatal and/or perinatal environmental insults to enhance the risk of developing schizophrenia.

Until now, there is still debate whether it is possible to use rodent models to reflect psychiatric disorders in humans. However, genetically engineered mice in which susceptibility genes are modified have potential advantages over human studies. In the case of schizophrenia, initial risks for this disorder occur during neurodevelopment, whereas onset of the disease arises in adulthood, with almost two decades for the full development of pathology to overt incidence. To understand in detail the mechanisms of schizophrenia, it is important to characterize how the disorder etiologies develop over time until development of full-blown disease. Therefore genetically engineered mouse models may be expected to provide further understanding of the disease mechanisms and time course. Another major advantage of genetic mouse models is their usefulness for compound screening in drug development, since rodents are much easier for preclinical drug screening from both economical and ethical viewpoints. For these reasons, genetic mouse models may provide an opportunity to identify novel therapeutic strategies that are directly linked to the mechanisms of psychological disorders.

Many investigators have considered the possibility that brain function and behavior are modulated by a combination of several genetic and environmental factors, and the concept of "pathway" is more likely to mimic the mechanisms than the effect of a single gene product. Therefore it is necessary to co-transfer more than one gene, such as by the methods of in utero electroporation.[28,78,79] This will make it possible to evaluate the synergistic influence or epistatic effect of multiple genetic factors, as well as to test how defects in neuronal network formation in early development lead to the behavioral abnormalities in adulthood.

In summary, we tried to establish a series of abnormalities in genetically engineered mice models based on the neurodevelopmental hypotheses of schizophrenia. It seems clear that the multiple similarities of genetic mouse model with schizophrenic patients indicate the potential for further understanding the pathogenesis of schizophrenia. Generation of these genetic mouse models should shed light on the etiology of schizophrenia and lead to more effective therapies in the future.

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Increases of CRF in the amygdala are responsible for reinstatement of methamphetamine-seeking behavior induced by footshock

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Anxiety

ABSTRACT

Recent evidence suggests the involvement of corticotropin-releasing factor (CRF) in drug abuse. Here, we evaluated whether CRF modulates the reinstatement of methamphetamine (METH)-seeking behavior induced by stress using a drug-self administration paradigm in rats. Rats were trained to lever-press for intravenous METH (0.02 mg/infusion) accompanied by light and tone (drug-associated cues) and then underwent extinction training (saline substituted for METH without cues). Under the extinction condition, the inhibitory effects of a CRF receptor antagonist on the stress-induced reinstatement of METH-seeking behavior were assessed. Anxiety-like behaviors during METH withdrawal in METH self-administered rats were also evaluated. The non-selective CRF receptor antagonist α-helical CRF_{9-41} attenuated METH-seeking behavior induced by footshock stress. CRF levels both in the amygdala and in plasma were significantly increased on day 10 of withdrawal after METH self-administration. However, plasma corticosterone concentrations were unchanged during the withdrawal. In addition, METH-seeking behavior was not affected by an inhibitor of corticosterone synthesis, metyrapone. In the elevated plus maze test, METH self-administered rats showed a decrease in the duration of time spent in the open arms on day 10 of withdrawal. The increased CRF levels in the amygdala may, at least in part, contribute to the footshock-induced reinstatement of METH-seeking behavior and the increase in anxiety-like behavior. The present findings indicate that CRF receptor antagonists would be useful as a therapeutic agent for METH-dependence.

1. Introduction

The biggest challenge to the successful treatment of drug dependence is preventing craving and relapse, which are hallmarks of the disease. Three different kinds of stimuli such as stress, drug associated-cues and drug-priming injections are capable of eliciting drug-seeking behavior in both human addicts (de Wit, 1996; Sinha et al., 2003) and animal reinstatement models (Shaham et al., 1996; Anggadiredja et al., 2004; Hiranita et al., 2008). Methamphetamine (METH) is a highly addictive psychostimulant, with reinforcing properties comparable to those of cocaine (Peltier et al., 1996; Shimosato and Ohkuma, 2000), but only one study has investigated the effect of stress on the reinstatement of METH-seeking behavior (Shepard et al., 2004). Furthermore, responses to stress such as footshock or the administration of corticosterone during drug self-administration differ between METH and cocaine (Moffett and Goeders, 2005). The aim of this study is to clarify mechanisms of stress-induced METH-seeking behavior.

The stress hormone corticotropin-releasing factor (CRF) plays a key role in the neuroendocrine and behavioral responses to stress, primarily via the hypothalamic–pituitary–adrenal (HPA) axis (Chalmers et al., 1996; Carrasco and Van de Kar, 2003). Centrally administered CRF produces several signs of increased anxiety and transgenic mice that over-express CRF exhibit increased anxiogenic behavior (Stenzel-Poore et al., 1994). Conversely, the central administration of either a CRF antisense oligodeoxynucleotide or a CRF receptor antagonist produces anxiolytic effects in the rat (Skutella et al., 1994; Griebel et al., 2002). In addition, clinical study also suggests that CRF_1 receptor antagonists are promising candidates for drug development in stress-related disorders such as depression and anxiety (Ising et al., 2007). Thus, additional functions of CRF as a contributing factor in psychiatric diseases are indicated.

We previously showed a critical role of the amygdala and nucleus accumbens (NAc) in the reinstatement of METH-seeking behavior attenuated by a reversible loss in function of the amygdala or NAc achieved using a local anesthetic, lidocaine (Hiranita et al., 2006). In the amygdala, CRF levels are indicated to rise during the early phase of withdrawal (the first 12 h) after cocaine self-administration (Richter and Weiss, 1999), but whether the levels change during withdrawal from METH in which cravings are elicited has not been elucidated. Therefore, the first aim of this study was to determine whether CRF could be involved in the reinstatement of stress-induced METH-seeking behavior, focusing on the amygdala and NAc.

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Cessation of cocaine intake following chronic drug exposure is typically characterized by a negative emotional state (e.g., anxiety, irritability) (Koob and Le Moal, 1997, 2008). Importantly, anxiety induced during cocaine abstinence is often a provoking factor leading to relapse to cocaine use in human addicts (Gawin and Kleber, 1986; Koob and Bloom, 1988). Also, in a state of withdrawal from METH, addicts commonly experience heightened states of anxiety (Cruickshank and Dyer, 2009). However, it remains obscure whether an increase of anxiety-like behavior would be observed during METH withdrawal in animal models of relapse using METH self-administration. Secondly, we examined the possibility that an anxiety-like behavior changes during withdrawal after METH self-administration.

2. Material and methods

2.1. Animals

The subjects were 69 male Wistar rats (250–350 g, 10 weeks old, Nippon SLC, Hamamatsu), weighing 280–300 g at arrival. Subjects were housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 7:00 a.m.).

Before surgery, the animals had unlimited access to food and water and were housed at 3–4 animals/cage. Each rat was housed individually after surgery and, after 5 days for recovery, food was limited to 15–20 g/day/body. Procedures for animal treatments were conducted in accordance with the Guide for the care and use of Laboratory Animals as adopted and promulgated by the Declaration of Helsinki and Faculty of Pharmaceutical Science, Nagasaki International University Publication, enacted 2009.

2.2. Drugs

Methamphetamine HCl (METH; Dainippon Sumitomo Pharma, Osaka) was dissolved in saline. For self-administration, METH (0.02 mg/0.1 ml/infusion) was delivered intravenously (i.v.). α-Helical CRF_{9-41}, a non-selective CRF receptor antagonist (10 and 30 μg/rat, i.c.v.; Sigma, St Louis, MO), was dissolved in distilled water. NBI27914, a selective CRF_1 receptor antagonist (32 and 100 μg/rat, i.c.v.; Sigma, Louis, MO), was dissolved in a mixture of ethanol, cremophor EL and saline (1:1:9). Both these antagonists were injected intracerebroventricularly (i.c.v.) 10 min before the test of reinstatement. Metyrapone (2-Methyl-1,2-di-3-pyridinyl-1-propanone; Tocris Bioscience, Park Ellisville, MO), an inhibitor of corticosterone synthesis, was dissolved in distilled water containing 2% Tween 80. Metyrapone was injected subcutaneously (s.c.) at a dose of 100 mg/kg 3 h before the test of reinstatement (Shaham et al., 1997).

2.3. Procedure

2.3.1. Surgery

2.3.1.1. Intravenous catheterization. After the completion of food training, rats were anesthetized with isoflurane (Mylan Pharmaceut, Osaka), prior to the surgical implantation of indwelling i.v. catheters. Catheters were constructed from Silastic laboratory grade tubing (0.5 mm i.d., 1.0 mm o.d., Kaneka Medix, Osaka). The catheter was implanted into the right jugular vein and secured in place with silk suture around the silicon nodule. The Silastic tubing ran under the skin to an exit point in the mid-scapular region. Rats were infused i.v. with 0.1 ml of heparinized saline (30 U/ml) daily during the experiment in order to prevent blockage of the catheter.

2.3.1.2. Intraventricular cannulation. During surgery, each rat was implanted with a 21 gage guide cannula from which the injector extended 1 mm to end in the right or left lateral ventricle. Stereotaxic coordinates used were as follows: −0.8 mm from bregma, +1.4 mm lateral from the midline, and −2.5 mm from dura (Paxinos and Watson, 1986) measured from the tip of the injector.

2.3.2. METH self-administration

2.3.2.1. Food training. Rats were restricted to approximately 90% of their normal weight for 2 days prior to the start of food training and trained to press levers for 45-mg food pellets (Bio-Serv, Frenchtown, NJ). The training took place on a fixed ratio 1 (FR1) schedule during which no stimuli were presented. Lever-pressing training ceased when rats could obtain 30 pellets within 250 s for three consecutive sessions.

2.3.2.2. Intravenous METH self-administration. METH self-administration training was conducted in standard operant chambers (30×20×24 cm; Neuroscience, Tokyo) with two fixed levers (5 cm above the chamber floor). White circular stimulus lights were located 4 cm above the levers, and a house light was located on the wall on the same side and top of the chamber. One end of the swivel was connected via polyethylene tubing (Kaneka Medix, Osaka) encased in a protective stainless steel spring tether (Instech Laboratories, Plymouth Meeting, PA) to the animal's catheter while the other end of the swivel was connected via polyethylene tubing to the infusion pump. The self administration apparatus was enclosed in a ventilated, sound-attenuating chamber (Neuroscience, Tokyo). METH was delivered using a computer-controlled infusion pump located inside the sound attenuating chamber. The entire system was computer integrated using MED PC 4 (Actimetrics, Wilmette, IL). Rats were self-administered METH on 10 consecutive days during 2-h sessions. The animals were connected to the drug infusion line and the session was initiated. Each session began with illumination of the house light that remained lit for the entire session. Responses on the active lever resulted in delivery of METH (0.02 mg/0.1 ml) infusion over 6 s; this training was accomplished using an FR1 schedule. Responses on the left lever had no programmed consequences, but were recorded. Each infusion was paired with a 26-s compound stimulus presentation, consisting of the white stimulus light (200 lx) over the right lever and a tone (2.9 kHz, 85 dB) delivered via a programmable audio generator (Neuroscience, Tokyo). Following drug delivery and stimulus presentation, responses on the active lever had no programmed consequences (no drug or stimulus delivery) for 20 s, but lever responses were recorded. Following each self administration session, rats were administered 0.2 ml of heparinized saline (30 U/ml) i.v., and catheter ports were closed to maintain patency.

2.3.2.3. Extinction. After the self-administration sessions, at least five extinction sessions (1-h) were conducted daily during which active lever responding resulted in an infusion of saline instead of METH without presentation of the METH-associated cues. Rats conducted this extinction session until they achieved the extinction criterion of less than 10 responses per session on the previously active lever.

2.3.2.4. Reinstatement. A stress-induced reinstatement test was conducted every 10 days under a FR-1 schedule. The footshock testing session was preceded by 15 min of intermittent footshock. During the test, the levers were extended and rats were not connected to the infusion system to avoid damage resulting from the jumping behavior induced by footshock stress.

2.3.3. Electrical footshock

Footshocks (current intensity 0.8 mA; 1.0-s trains) were delivered through a scrambler (Random Shocker version 7.1; Neuroscience, Tokyo) to the grid floor of the operant chamber. They were administered during a period 15 min in an intermittent manner according to a variable interval schedule (mean interval: 40 s; range: 10–70 s).

2.3.4. Elevated plus maze test

The elevated-plus maze (Neuroscience, Tokyo) was constructed from black plastic and consisted of two open arms (50×10 cm) and two enclosed arms (50×10×50 cm) that extended from a central platform (10×10 cm). The maze was elevated 50 cm above the floor. Experiments began by placing a single rat on the central platform facing a closed arm. During the 10 min of free exploration, time spent in open arms (defined as the animal placing its forepaws onto an arm) and the number of crossings between closed arms were recorded manually. The maze was cleaned thoroughly between animals using water.

2.3.5. Measurement of brain/plasma CRF and plasma corticosterone levels

Blood samples were obtained from the orbital vein with a heparinized capillary (Hirschmann Laborgerate, Germany) under inhalation anesthetic. The blood was collected into tubes containing EDTA-2Na on ice and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was removed and stored at −80 °C until assayed. For the brain CRF assay, the entire brain was removed from the skull immediately after decapitation and placed on ice, then the amygdala, nucleus accumbens and hypothalamus were removed using a stainless-steel brain slicer (RBSC-0.5S, Neuroscience, Tokyo) according to the brain map (16). These tissues were immediately homogenized in an extraction buffer (PBS with 0.2% Nonidet P-40) and centrifuged at 15,000 rpm for 20 min at 4 °C. The sample was stored at −80 °C until assayed. Both blood and brain samples were collected immediately after the self-administration sessions. Brain/plasma CRF levels were determined with a Mouse/Rat CRF-HS ELISA assay kit (Yanaihara Institute, Shizuoka). Plasma corticosterone levels were determined using a Corticosterone EIA kit (Cayman Chemical, Ann Arbor, MI).

2.4. Statistical analysis

Data represent the mean ± SEM number of lever responses or CRF/corticosterone concentrations. Response totals were analyzed by ANOVA (a within-subjects design). A one-way ANOVA was used to compare means, and Bonferroni–Dunn tests were used for post hoc analyses. Differences were considered significant at $p<0.05$. All statistical analyses were performed by using the Stat View software program (v. 5.0; SAS Institute, Cary, NC).

3. Results

3.1. Effects of CRF receptor antagonist on reinstatement of footshock-induced METH-seeking behavior

The number of METH infusions in the last training session (day 10) was 22.0 ± 0.9 and the total number of METH infusions during the

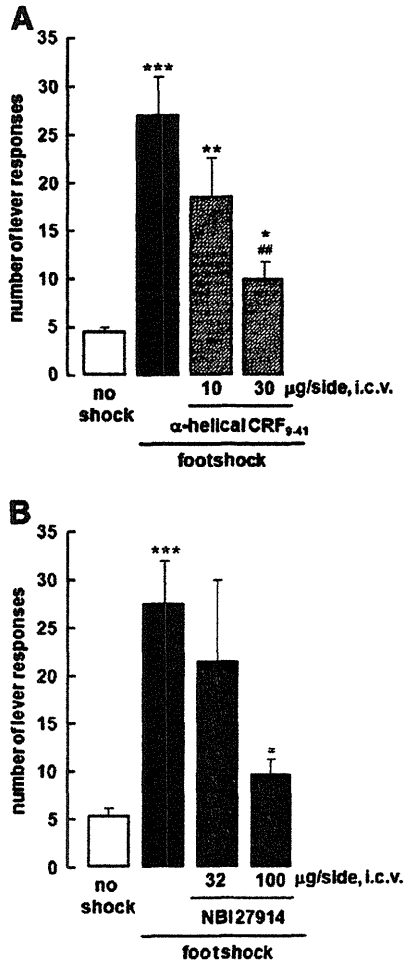

Fig. 1. Effects of CRF receptor antagonists on reinstatement of METH-seeking behavior induced by footshock.Each bar represents the mean (±SEM) number of presses on the previously active lever during the 15-min exposure to footshock stress (or no-shock). A. Rats were pretreated with a non-selective CRF receptor antagonist, α-helical CRF_{9-41} (10, 30 µg, i.c.v.), 10 min before the start of the session (n=8). B. Rats were pretreated with a selective CRF_1 receptor antagonist, NBI27914 (32, 100 µg, i.c.v.), 10 min before the start of the session (n=5). *Different from the no-shock condition (no shock), $p<0.05$; **, $p<0.01$; ***, $p<0.001$. #Different from the footshock condition, $p<0.05$; ##, $p<0.01$.

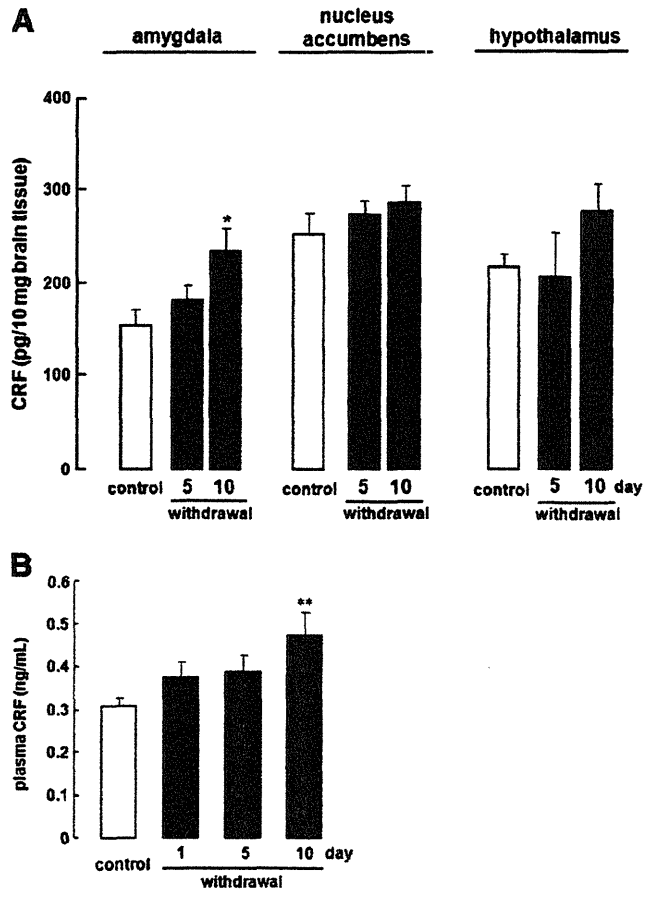

Fig. 2. CRF levels in METH self-administered rats during withdrawal. A. Each bar represents the mean (±SEM) CRF concentration (pg/10 mg brain tissue) in the amygdala, nucleus accumbens and hypothalamus (withdrawal day 10: n=6). *different from the control (naïve animals: n=4), $p<0.05$. B. Bars represent mean (±SEM) CRF (n=13–18) concentrations (ng/ml) in plasma. Blood was taken from the orbital vein under inhalation anesthesia immediately after the end of each session. **Different from the control, $p<0.01$. Control: before the start of METH self-administration acquisition training.

training session was 192.9 ± 8.9. When the drug was replaced with saline, the lever press responses gradually decreased from 17.4 ± 2.7 on the first day, reaching 7.6 ± 2.4 lever presses on day 5 of extinction. Under the extinction condition, footshock stress significantly reinstated lever-pressing behavior, so called METH-seeking behavior (5.4 ± 0.7 to 26.9 ± 4.1; F [1, 25] = 24.7, $p<0.001$), compared with the no-shock condition (no shock). Pretreatment with the CRF receptor antagonist α-helical CRF_{9-41} (10 and 30 μg/rat, i.c.v.) attenuated this METH-seeking behavior in a dose-dependent manner and significantly suppressed the lever presses at 30 μg (26.9 ± 4.1 to 10.0 ± 1.8, F [1, 20] = 8.9, $p<0.01$; Fig. 1A). Furthermore, the METH-seeking behavior was also suppressed by the selective CRF_1 receptor antagonist NBI27914 (100 μg/rat, i.c.v.) (27.6 ± 4.5 to 9.6 ± 1.7; F [1, 19] = 4.7, $p<0.05$; Fig. 1B).

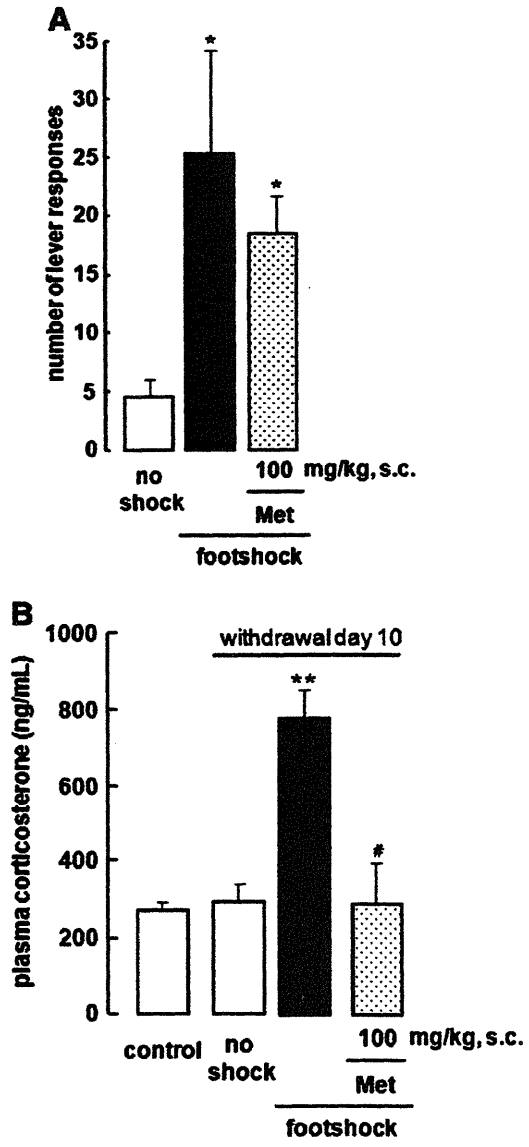

Fig. 3. Effects of an inhibitor of corticosterone synthesis on reinstatement of footshock-induced METH-seeking behavior. A. Each bar represents the mean (±SEM) number of presses on the previously active lever during the 15-min exposure to footshock stress (or no-shock). Rats were pretreated with an inhibitor of corticosterone synthesis, metyrapone (Met, 100 mg, s.c.), 3 h before the start of the session (n = 5). *Different from the no-shock condition (no shock), $p<0.05$. B. Bars represent mean (±SEM) corticosterone (n = 3–5) concentrations (ng/ml) in plasma. Blood was taken from the orbital vein under inhalation anesthesia immediately after the end of each session. **Different from the control, $p<0.01$. #Different from the footshock condition, $p<0.05$. Control: before the start of METH self-administration acquisition training.

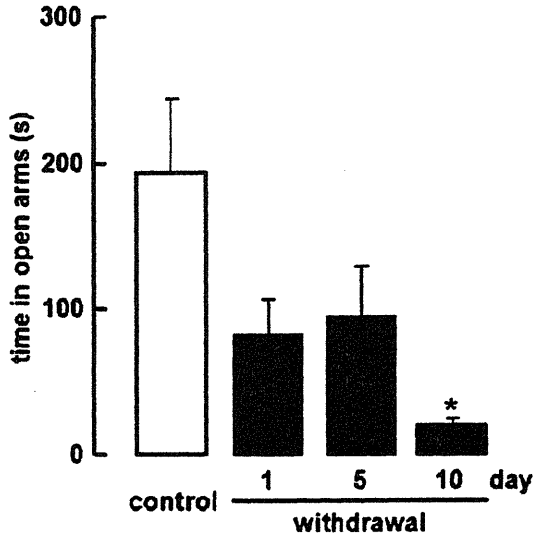

Fig. 4. Anxiety-like behavior in METH self-administered rats during withdrawal.Anxiety-like behavior during withdrawal after METH self-administration in the elevated plus maze test. Each bar represents the mean (±SEM) total time spent in open arms in the 10-min test session (n = 5–7). *Different from the control, $p<0.05$. Control: before the start of METH self-administration acquisition training.

3.2. CRF levels in METH self-administered rats during withdrawal

Fig. 2A shows CRF concentrations (pg/10 mg brain tissue) in the amygdala, NAc, and hypothalamus on days 5 and 10 of withdrawal from METH self-administration. The level of CRF in the amygdala gradually increased after withdrawal and showed a significant increase on day 10 (F [1, 8] = 5.5, $p<0.05$; Fig. 2A). In the nucleus accumbens and hypothalamus, however, the level showed a slight increase, though the change was not significant in either region (Fig. 2A).

Fig. 2B shows the levels of CRF in plasma. The plasma CRF level (ng/ml) increased with no significance on day 1 and day 5 of withdrawal. Then, it significantly increased on day 10 (0.30 ± 0.02 to 0.47 ± 0.06, F [1, 28] = 9.9, $p<0.01$; Fig. 2B).

3.3. Effects of an inhibitor of corticosterone synthesis on reinstatement of footshock-induced METH-seeking behavior

A corticosterone synthesis inhibitor, metyrapone (100 mg/kg, s.c.), did not block the footshock-induced METH-seeking behavior (Fig. 3A). Although the plasma corticosterone concentration (ng/ml) showed no change on day 10 of withdrawal ($p = 0.75$), it increased significantly immediately after the exposure to footshock (275.1 ± 22.1 to 747.8 ± 69.8, F [1, 6] = 29.2, $p<0.01$). Metyrapone completely reversed this increase (747.8 ± 69.81 to 279.1 ± 104.6, F [1, 5] = 15.2, $p<0.05$; Fig. 3B).

3.4. Anxiety-like behavior in METH self-administered rats during withdrawal

In the elevated plus maze test, control rats spent 193.2 ± 52.2 s in the open arms during the 10-min test session, whereas METH self-administered rats on day 10 of withdrawal spent just 20.8 ± 4.9 s in the open arms (F [1, 9] = 8.9, $p<0.05$; Fig. 4). However, the number of crossings between closed arms was not affected in the METH self-administered rats on withdrawal day 10 (Table 1).

Table 1
Number of crossings in the elevated plus maze test.

		Withdrawal after METH self-administration		
	Control	Day 1	Day 5	Day 10
Number of crossings	21.3 ± 3.8	10.7 ± 4.0	12.0 ± 4.4	16.0 ± 4.5

4. Discussion

The METH-seeking behavior induced by footshock was attenuated by both non-selective CRF and selective CRF_1 receptor antagonists. CRF is a primary activator of the HPA axis and an essential mediator of behavioral and autonomic outcomes of stress. It causes the release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn induces the secretion of glucocorticoids such as corticosterone from the adrenal gland. Administration of METH is known to increase plasma corticosterone levels in mice (Ago et al., 2009). Therefore, we investigated the effect of an inhibitor of corticosterone synthesis, metyrapone, as well as CRF receptor antagonists on METH-seeking behavior to determine the involvement of corticosterone in the reinstatement. However, metyrapone did not elicit a suppressing effect on the reinstatement of METH-seeking behavior. Hence, CRF but not corticosterone is likely to have a facilitatory role in footshock-induced METH-seeking behavior. This idea is supported by the present finding that levels of CRF in the amygdala were significantly increased on day 10 of withdrawal after METH self-administration without an accompanying increase in plasma corticosterone levels. The present study is the first to reveal a facilitating role of CRF/CRF_1 receptors in footshock-induced METH-seeking behavior with increases of CRF levels in both the amygdala and plasma during withdrawal from METH in METH self-administered rats. The role of CRF in reinstatement of cocaine-seeking behavior has been demonstrated previously. Treatment with a non-selective CRF receptor antagonist, D-Phe CRF_{12-41}, has been shown to block footshock-induced reinstatement in cocaine-trained rats (Erb et al., 1998). An attenuating effect on METH-seeking behavior by a CRF_1 receptor antagonist has also been shown in a cue-and METH priming-induced reinstatement model (Moffett and Goeders, 2007). Therefore, activation of the CRF receptor is likely to modulate psychostimulant-induced reinstatement and CRF_1 receptors mediate METH-seeking behavior in common with the three main stimuli described above.

Neurons in the amygdala provide an excitatory input, such as glutamate (Glu), to the prefrontal cortex (PFC) and NAc (Kalivas and Volkow, 2005). We previously revealed critical roles of the PFC and NAc in the reinstatement of METH-seeking behavior using lidocaine (Hiranita et al., 2006). Footshock elicits a significant increase in Glu in the PFC and the blockade of this increase attenuated the reinstatement of cocaine-seeking behavior (McFarland et al., 2004). Interestingly, as the administration of CRF significantly increased amygdala-PFC EPSC amplitude in chronic cocaine-treated animals, CRF appears to help facilitate amygdala-PFC glutamatergic transmission in cocaine-treated animals (Orozco-Cabal et al., 2008). There is a possibility that CRF in the amygdala also modulates glutamatergic transmission in the NAc taking into account neural projections from the amygdala into the NAc. Taken together, the increase of CRF levels in the amygdala in METH self-administered rats may be involved in facilitating the transmission of Glu in the PFC or NAc during reinstatement.

The plasma CRF level was significantly increased on day 10 of withdrawal similar to that in the amygdala. This may reflect the level of CRF in the brain because a large amount of CRF injected i.c.v. can diffuse via the specific unidirectional brain-to-blood transport system for CRF (Martins et al., 1996).

In the absence of METH administration, abusers commonly experience heightened states of anxiety (Cruickshank and Dyer, 2009). Similarly, METH self-administered rats in withdrawal showed an increase in anxiety-like behavior in the elevated plus maze test. Spiga et al. (2006) reported that the injection of a CRF receptor agonist, urocortin 1, into the amygdala produced anxiogenic effects in social interaction tests, reducing total interaction time without affecting locomotor activity or exploratory behavior. This report suggests that CRF in the amygdala has a facilitatory role in anxiety-like behaviors. Indeed, an increase in CRF is likely to parallel a heightening of negative emotions such as anxiety-like behavior. Consequently, the activation of CRF in the amygdala during withdrawal from METH may be attributable to the increase of anxiety-like behaviors in METH self-administered rats. Recent clinical study reveals that abstinent cocaine abusers exhibit significantly higher and more persistent stress- and cue-induced drug-craving and negative emotions such as increased anxiety, anger, fear and sadness (Fox et al., 2008). Based on this finding, enhanced sensitivity to stress and negative emotions may result in a decrease in the threshold to reinstate drug craving.

In summary, the present findings indicate that increased CRF levels in the amygdala may, at least in part, play a facilitating role in the reinstatement of METH-seeking behaviors following footshock and the increase in anxiety-like behavior without the participation of corticosterone. Therefore, CRF/CRF_1 receptor antagonists may be useful as an anti-craving agent. Moreover, although further study is required, plasma CRF levels may have a potential as a diagnostic biomarker for measuring the craving risk in METH abusers.

Acknowledgments

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〔CINP2010 発表報告〕

Footshock により誘発される覚せい剤メタンフェタミン探索行動における副腎皮質刺激ホルモン放出因子（CRF）の促進的関与*

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薬物への“渇望”の再燃は，薬物依存症の最大の問題点である．この渇望を誘発する因子としては，類似薬物の少量投与，薬物摂取時の環境刺激ならびにストレスの3種類が挙げられる．一方，副腎皮質刺激ホルモン放出因子（CRF）は，ストレス反応を担う視床下部-下垂体-副腎系の構成分子として知られている．CRF は，このホルモンとしての役割のみならず，腹側被蓋野では，AMPA ならびに NMDA 受容体を介するグルタミン酸（Glu）神経伝達を増強する（Hahn et al, 2009）．また CRF が豊富に発現する分界上床核では，ドパミン（DA）神経の活性化により CRF が遊離され，CRF_1 受容体を介して Glu 神経伝達が促進されることも明らかにされている（Liu et al, 2004; Kash et al, 2008）．このように，CRF は，渇望再燃に関わる Glu 神経や，DA 神経のニューロモデュレーターとして機能する可能性が示唆されている．本研究では，覚せい剤 methamphetaine（MAP）を用い，MAP への渇望再燃（MAP 探索行動の発現）における CRF の関与を追究した．

方　法

1. 実験動物

Wistar-ST 系雄性ラット（（株）日本エスエルシー，300～350 g）を使用した．

2. 使用薬物

Methamphetamine（MAP:（株）大日本製薬）は生理食塩液（（株）大塚製薬）に溶解した．また，α-helical CRF_{9-41}（Sigma-Aldrich, Inc.）ならびに Astressin 2B（Sigma-Aldrich, Inc.）は蒸留水に溶解した．NBI27914（Sigma-Aldrich, Inc.）は，蒸留水，ethanol および cremophor EL の混合溶液（18:1:1）に溶解した．

3. 薬物自己投与実験

薬物自己投与実験には，レバー押しに伴い薬物が注入される active lever と，薬物が得られない inactive lever の2つのレバーが装着されたオペラント箱（29×23×33 cm;（株）ニューロサイエンス）を使用した．ラットが active lever を1回押せば（FR1 条件下），MAP（0.02 mg/100 μl/infusion）が薬物関連刺激（drug-associated cue: 音; 85 dB/2.9 kHz，光; 200 lux）とともに動物の静脈内へ注入される．10 日間の MAP 自己投与実験後，MAP を生理食塩液に置換した自己投与実験（cue 呈示なし）を続けた（消去過程）．レバー押し行動が減弱した時点で，薬物関連刺激の呈示，MAP-priming 投与ならびに footshock 負荷（0.8 mA，ON; 1 秒，variable interval; 10～70 秒）を行い“渇望”の指標としての MAP 探索行動（生理食塩液自己投与下でのレバー押し反応）の発現の有無を調べた．

結　果

MAP 自己投与実験最終日の MAP 注入回数は，22.3±0.9 回であった．消去実験（生理食塩液注入）1日目のレバー押し回数は 16.7±2.5 回で，このレバー押し回数は，経日的に減衰し，最終的には 7.9±1.0 回となり 10 回以下まで低下した．

退薬時における footshock 負荷により，ラットのレバー押し回数は有意に増加し MAP 探索行動が発現した．非選択的 CRF 受容体拮抗薬 α-helical CRF_{9-41}（10, 30 μg/5 μl）は，用量依存的に footshock により誘発される MAP 探索行動を抑制した（$P<0.01$ vs. stress alone）．同様に，MAP 退薬時でのレバー押し回数は，MAP 関連刺激ならびに MAP-priming 投与により増加し MAP 探索行動が誘発された．これらの MAP 探索行動は，α-helical CRF_{9-41}（3.2, 10 μg/side）の投与により有意に抑制された．さらに，選択的 CRF_1 受容体拮抗薬 NBI27914 は，10 および 32 μg/side の用量において MAP 関連刺激により誘発される MAP 探索行動を有意に抑制した．また，MAP-priming 投与により誘発される MAP 探索行動は，NBI27914 の 10 μg/side では抑制されなかったが，32 μg/side の用量により有意に抑制された．一方，選択的 CRF_2 受容体拮抗薬 Astressin 2B は，MAP 関連刺激による MAP 探索行動を 32 および 100 μg/side の用量において有意に抑制したが，MAP-priming 投与により誘発される MAP 探索行動は抑制しなかった．

コントロール群における脳分画 10 mg 当たりの扁桃体内 CRF 濃度は，153.5±17.0 pg であった．MAP 退薬日数の増加に伴いこの CRF 濃度は増加し，退薬5日目では，181.7±16.5 pg，退薬 10 日目では有意な増加が認められ，

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234.0±25.3 pg となった．一方，血漿 CRF 濃度は，MAP 自己投与実験前では 0.31±0.02 ng/ml であった．また MAP 自己投与開始第 1 日目の CRF 濃度は 0.40±0.12 ng/ml で，最終投与日のそれには著明な変化はなく，0.43±0.07 ng/ml であった．MAP 退薬 1 日目では，CRF 濃度の変化は認められなかった（0.38±0.04 ng/ml）．しかし，退薬を重ねるにしたがって CRF 濃度は増加し，10 日目では有意な増加が認められた（0.47±0.06 ng/ml）．

考　察

MAP は，急性投与により血漿コルチコステロン濃度を増加させることが知られている（Moseley et al, 2007）．また，コルチコステロンの末梢投与はマウス扁桃体内 CRF mRNA 量を増加させる（Makino et al, 1994）．このように，血中コルチコステロンの過剰増加に伴う視床下部室傍核内 CRF 産生の抑制的制御（負のフィードバック）とは反し，扁桃体では CRF 産生が亢進することがわかる．これらの知見から，MAP 退薬時における扁桃体内 CRF 濃度の増加は，MAP 自己投与に伴い増加した血中コルチコステロンによる，扁桃体内 CRF システムに対する正のフィードバックに基因する可能性が推測される．

一方，MAP と同様に，footshock により誘発される cocaine 探索行動も α-helicalCRF$_{9\text{-}41}$ により抑制される（Wang et al, 2005）．このことから考えると，footshock による MAP 探索行動の発現における CRF の促進的な関与は，乱用性薬物一般に共通している可能性が示唆される．さらに，footshock による cocaine 探索行動発現時では，腹側被蓋野における Glu 遊離が増加するが，α-helicalCRF$_{9\text{-}41}$ はこれを阻害し，cocaine 探索行動を抑制する（Wang et al, 2005）．また，CRF の腹側被蓋野内微量注入は，cocaine 自己投与履歴を有する動物に限定して Glu 遊離を増加させる（Wang et al, 2005）．これらの知見は，CRF を介した Glu 神経伝達の亢進が，cocaine 探索行動の発現の誘因になっている可能性を指摘するものである．次に，腹側被蓋野で遊離される CRF が脳内のどの場所から供給されているのかに興味がもたれるが，Rodaros らは，この点について明らかにした．すなわち扁桃体で産生される CRF が腹側被蓋野における CRF 発現の主要な供給源であることを見いだした（Rodaros et al, 2007）．以上のような知見から，MAP 退薬時における扁桃体内 CRF 濃度の増加は薬物探索行動発現の準備状況を意味し，footshock 負荷により①扁桃体から腹側被蓋野への CRF 供給の増加，これによる②Glu 神経伝達の亢進によって MAP 探索行動が誘発される可能性が示唆される．

一方，MAP 退薬時では，血漿中においても CRF 濃度の増加が認められた．CRF は，末梢組織と脳組織の両方で産生されている（Boorse and Denver, 2006）．脳に発現する CRF は，血液-脳関門を通して末梢血管へ移行する（Martin et al, 1996）．したがって，血漿 CRF 濃度の変化は，脳組織での CRF 濃度変化を反映している可能性がある．このことから考えると，MAP 退薬時での血漿 CRF 濃度を測定することによる“渇望再燃”のバイオマーカーとしての臨床応用が期待される．

Footshock により誘発される MAP 探索行動の発現における CRF 受容体の促進的な関与が明らかになったことから，その他の誘発因子である MAP 関連刺激ならびに MAP-priming 投与により誘発される MAP 探索行動への関与を調べた．両誘発因子により誘発される MAP 探索行動は，footshock と同様に CRF 受容体の活性化により発現することがわかった．さらに，MAP 関連刺激による MAP 探索行動の発現には CRF_1 ならびに CRF_2 の両受容体が，また MAP-priming 投与による MAP 探索行動の発現には，CRF_1 受容体が促進的に関与することも明らかとなった．CRF_1 受容体は扁桃体や前頭前皮質に多く分布している．一方，CRF_2 受容体は扁桃体に多く分布しているが，前頭前皮質では少ない（Chalmers et al, 1995）．薬物探索行動発現の責任部位は，誘発因子によって異なることが知られている．リドカインを用いた我々の実験から，薬物関連刺激により誘発される MAP 探索行動の脳内責任部位は扁桃体，MAP-priming 投与によるそれは前頭前皮質であることを見いだしている（Hiranita et al, 2006）．したがって，CRF_2 受容体拮抗薬は，MAP-priming 投与による MAP 探索行動の発現を抑制しないが，これは前頭前皮質において CRF_2 受容体の分布が少ないことに基因する可能性がある．

以上をまとめると，①footshock，MAP 関連刺激ならびに MAP-priming 投与により誘発される MAP 探索行動の発現は，共通して CRF 受容体の活性化を介して発現することがわかった．また，②退薬時での扁桃体内 CRF 濃度の増加は，薬物探索行動発現の“準備状況”を作り出す重要な因子の 1 つである可能性が推察される．このことは CRF 受容体拮抗薬の薬物依存症治療薬としての可能性とともに，血漿 CRF 測定による“渇望”のバイオマーカーとしての臨床応用が期待される．

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大麻依存

大麻依存の基礎

アサ（麻）は，一属一種のきわめてまれなアサ科の植物で，雌雄異株の一年生草本である．大麻は，これを乾燥させたものである．アサ（大麻）の学名は*Cannabis sativa*であり，一般的な英語表記ではhemp，俗称としてmarihuana/marijuana（マリファナ）が用いられている．現代の精神医学の分野では，マリファナという用語はカンナビスと呼び換えられてきている（**図1**）．

大麻の起源は古く，最も古い“幻覚生薬／快楽植物”の一つとして登場したのは紀元前3000～5000年といわれ，その幻覚作用を基に儀式やまじない，また麻酔・鎮痛薬として医療にも用いられてきた．大麻には400種を超える化学成分が含まれることが明らかにされているが，そのうちカンナビノイドという70種余りの脂溶性の成分は大麻特有のものである．なかでもΔ^9-テトラヒドロカンナビノール（delta-9-tetrahydrocannabinol: Δ^9-THC）は幻覚作用の主要な活性成分と考えられている．

大麻の中でも幻覚作用成分を含む植物部位は花穂と葉の部分であり，茎，種子，根には幻覚作用成分はほとんど含まれない．これら大麻の未熟花穂や葉の部分を集め乾燥などの加工を施したものが一般にいう大麻である（**表1**）．乾燥した成熟花穂や葉の部分を集めたBhang（バング），乾燥させた葉のみをSawi（サヴィ），実のならない花穂を圧縮したganja（ガンジャ），またその樹脂をhashish（ハッシシ）あるいはcharas（チャラス）と特別な呼称がつけられている（**表1**）．ハッシシは，大麻から直接作られる製品の中で最も効果が強い．さらにその大麻樹脂のエタノール抽出液は，Δ^9-THC含有量が最も高いハッシシオイルとよばれるものである（**表1**）．

一般的には，マリファナタバコ1本約0.5 g

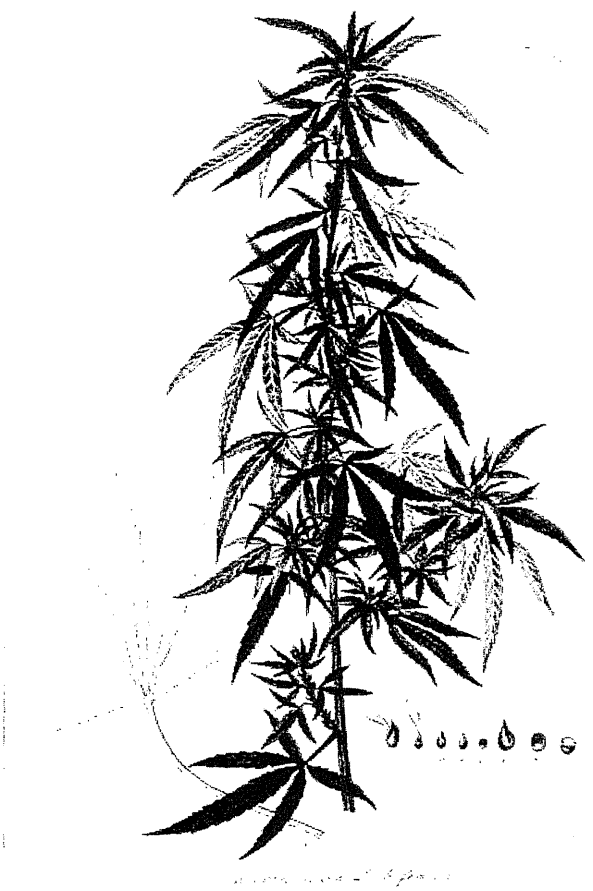

図1　アサ科の1年生草本である大麻草
学名 *Cannabis sativa*. 英名 marijuana; hemp.
（長崎国際大学 正山征洋教授より写真提供.
in Botanik, beschreibung und abbildung,
von Daniel Wagner, 1829）