Developmentally regulated and thalamus-selective induction of leiomodin2 gene by a schizophrenomimetic, phencyclidine, in the rat

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Abstract
The onset of schizophrenia and the schizophrenomimetic effects of an N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine, rarely occur during infancy and childhood, suggesting that schizophrenia-related neuron circuits and molecules in the brain might show an age-related response to an NMDA receptor antagonist. By using a DNA microarray technique, we have identified the developmentally regulated PCP-inducible gene leiomodin2 (Lmod2) that encodes a tropomyosin-binding actin-capping protein enriched in the cardiac and skeletal muscles. PCP caused an increase in the thalamic amounts of Lmod2 transcripts at postnatal days (PD) 32 and 50 without affecting them at PD 8, 13, 20 and 24, while the NMDA antagonist failed to produce a significant change in the gene expression in the adult heart. In-situ hybridization analysis revealed that the basal and PCP-induced expression of the Lmod2 gene is almost confined to the lateral and anterior nuclei of the thalamus among the brain regions at PD 50. The PCP-induced up-regulation of Lmod2 mRNAs in the adult thalamus was mimicked totally (also up-regulated) by another NMDA antagonist, dizocilpine, and partly by the indirect dopamine agonist, methamphetamine. Moreover, pretreatment with a D2-prefering dopamine receptor antagonist, haloperidol, partially antagonizes the increasing effects of PCP on thalamic Lmod2 gene expression. These findings suggest that Lmod2 might be involved in the pathophysiology of the age-dependent onset of drug-induced schizophrenia-like psychosis and schizophrenia and that the limited thalamic nuclei expressing the Lmod2 gene could compose the neuron circuits that are specifically disturbed in these mental disorders.

Key words: Leiomodin2 gene (Lmod2), phencyclidine, postnatal development, schizophrenia, thalamus.

Introduction
Schizophrenia is a serious brain disorder that exhibits high prevalence, frequent onset during youth, and a wide variety of mental dysfunctions including numerous refractory symptoms and often deprives patients of a complete re-entry into society (Buchanan & Carpenter, 2005). To understand the pathophysiology of this complex disorder, the psychotic state induced by phencyclidine [1(1-phenylcyclohexyl)piperidine; PCP] and other antagonists for the N-methyl-D-aspartate (NMDA)-type glutamate receptor has been considered as a most useful pharmacological model for schizophrenia because (1) these drugs produce positive and negative symptoms and cognitive disturbances which are indistinguishable from those of schizophrenia from the cross-sectional view while the longitudinal progressions of NMDA antagonist-induced psychosis and schizophrenia are different (Javitt & Zukin, 1991); (2) a group of schizophrenia patients suffered exacerbation of their psychotic symptoms with a challenge dose of PCP and a chemically PCP-related and non-competitive antagonist for the NMDA receptor, ketamine (Javitt & Zukin, 1991; Lahti et al. 2001; Petersen & Stillman, 1978), and (3) the psychotomimetic effects of the ketamine stereoisomers are closely correlated with their affinities for the NMDA receptor (Gouzoulis-Mayfrank et al. 2005; Vollenweider et al. 1997).
These schizophrenomimetic actions may be explained by the possibility that NMDA antagonists disturb the molecular and cellular equipment in an information-processing system or neuron circuit that specifically malfunctions in schizophrenia (Ito et al. 2007; Sato et al. 1997). Although the exact causative mechanism for schizophrenia remains unclear, the pathophysiological changes in the information-processing system might be associated with the development-dependent nature of schizophrenia and NMDA antagonist-induced psychosis (Ito et al. 2007; Sato et al. 1997). Thus, the onset of schizophrenia typically occurs after adolescence (Buchanan & Carpenter, 2005). It has been consistently reported that PCP-induced clinical symptoms and signs are mostly neurological in infants and young children but mainly psychiatric in adults; however, PCP has been observed to produce some psychiatric symptoms in young people (Schwartz & Einhorn, 1986; Welch & Correa, 1980). Ketamine has been observed to often produce psychotic symptoms in adults, but not in children (Reich & Silvay, 1989; White et al. 1982). In experimental animals, the behavioural effects of NMDA receptor antagonists have also been observed to alter during postnatal development. For instance, PCP (1–4 mg/kg) and dizocilpine (MK-801; 0.1–0.4 mg/kg) produced different dose–effect curves and peak activity levels in inducing hyperactivity between postnatal days (PD) 12 and 19 (Scalzo & Burge, 1994). Repeated treatment with PCP from PD 22–30, but not from PD 1–9, augments the ability of a subsequent challenge of the NMDA antagonist to elicit abnormal behaviour (Scalzo & Holson, 1992), which is thought to be an animal model for the hypersensitivity of schizophrenia patients to PCP and ketamine.

The late-developing features of schizophrenia and its pharmacological models indicate that maturation of the specific neuronal systems could be required for their onset. We can therefore postulate that the schizophrenia-related information-processing system might mature around adolescence in humans or a critical period of the postnatal development in experimental animals and could not play a crucial role in the regulation of mental functions or behaviour before these climactic periods. The hypothetical human system and its animal homolog should contain molecules that are responsive to NMDA antagonists only after adolescence and the critical period for the animal model of schizophrenia.

The plausible maturation process appears to be reflected in our observations that the distribution of brain c-Fos expression, which has long been used as a marker for changes in various brain cellular activities including metabolic and neural signal pathways (Morgan & Curran, 1991) following PCP administration, altered markedly during postnatal development (Nishikawa et al. 1998; Sato et al. 1997). Indeed, we recently found that acute systemic administration of PCP produced marked and insignificant alterations, respectively, of connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed [CCNI = cysteine-rich protein 61 (CYR61)] mRNA expression in the neocortex of rats aged 56 d and 8 d (Ito et al. 2007). To further explore candidates for schizophrenia-linked and developmentally regulated molecules, we compared the effects of PCP on gene expression in the thalamus between the infant and young adult periods in rats using a DNA microarray and the RT–PCR technique.

The thalamus was selected for the screening experiments because (1) not only the neocortex (Sato et al. 1997) but also the thalamus (Nishikawa et al. 1998) exhibits the prominent postnatal changes in PCP-induced abnormal activities as revealed by c-fos gene expression before and after the critical period, (2) schizophrenia patients have been reported to show neurochemical, neuropathological, neurophysiological and in-vivo imaging abnormalities in or closely associated with the thalamus (Andreassen et al. 1977; Clinton & Meador-Woodruff, 2004; Sim et al. 2006), and (3) EEG, neuroimaging and brain activity mapping studies have suggested that the thalamus is one of the essential sites of action of NMDA antagonists in humans (Greifenstein et al. 1958; Vollenweider et al. 1997; Watis et al. 2008) and experimental animals (Dragunow & Faull, 1990; Duncan et al. 1999; Miyasaka & Domino, 1968).

**Materials and methods**

**Animals**

The animal experiments were performed in strict accordance with the guidelines of the Tokyo Medical and Dental University and were approved by the Committee for Animal Experiment Ethics of the University. Male Wistar rats (ST strain, Clea Japan, Japan) at PD 8 (15–25 g), PD 13 (20–30 g), PD 20 (35–45 g), PD 26 (60–80 g), PD 32 (100–120 g), and PD 50 (200–260 g) were used. The animals were housed at 24.0 ± 0.5 °C under a 12-h light/dark cycle (lights on 08:00 hours) and had free access to food and water. In our experiments we used only male rats to study the effects of PCP because onset of the menstrual cycle in females has been suggested to modify behavioural and biochemical responses to a variety of drugs.
Chemicals

Phencyclidine hydrochloride was kindly synthesized and donated by Astellas Pharma Inc. (Japan). Methamphetamine (MAP) hydrochloride was purchased from Dainippon Sumitomo Pharma Co., Ltd (Japan) with official permission of the Tokyo Metropolitan Bureau of Public Health. The other chemicals used were of ultrapure quality and were commercially available. PCP hydrochloride, MAP hydrochloride and dizocilpine hydrogen maleate (MK-801 hydrogen maleate; [5R,10S]-[+]-5-methyl-10,11-dihydro-5Hdibenzo[a,d]
cyclohepten-5,10-imine) were dissolved in saline for subcutaneous (s.c.) injection. Haloperidol (Hal) was dissolved in 0.15% tartaric acid and titrated with 0.05 M NaOH to pH 5.0. Some animals were pretreated with Hal injection intraperitoneally (i.p.) 30 min before PCP (Sakurai et al. 2004). Control animals received the same volume of saline or vehicle. The doses of these drugs always refer to the free bases and were chosen in order to cause robust and typical behavioural and biochemical effects (Sakurai et al. 2004; Shintomi, 1975; Shirayama et al. 2000).

Behavioural ratings

On each day of the experiment, rats were placed individually into plastic observation cages (internal dimensions: 37 cm × 22 cm × 14 cm), allowed at least 90 min to habituate to the new environment, and were monitored for stereotypy, which is considered to be a model for schizophrenia symptomatology according to the PCP behaviour rating scales (Sturgeon et al. 1979), with minor modifications as described previously (Tanii et al. 1994) (see Fig. 2b legend). Behavioural ratings were made every 10 min from 30 to 60 min after acute PCP injection. The cumulative behavioural rating for each animal was determined as the summation of every 10-min score for the last 30 min.

Tissue and total RNA preparation

Rats were killed by cervical dislocation 60 or 90 min after administration of various drugs or saline. The thalamus, other discrete brain regions and the heart were rapidly dissected out in the cold, frozen in liquid nitrogen, and stored at −80 °C until required. The total RNA was prepared from these frozen rat tissues using an RNeasy Midi kit (Qiagen GmbH, Germany). The RNA quality was verified by gel electrophoresis (Agilent Bioanalyzer, USA).

DNA microarray

We performed DNA microarray analysis using the Affymetrix Rat Genome 230 2.0 arrays (Affymetrix, USA) to isolate the developmentally regulated transcripts responsive to PCP in the thalamus. These arrays contained 31 000 probe sets capable of analysing the expression level of over 30 000 transcripts and variants from over 28 000 well-substantiated rat genes. Further details can be obtained at http://www.affymetrix.com.

For this screening step, the following four experimental groups of rats were prepared: five saline-injected control rats at PD 50; five PCP (7.5 mg/kg s.c.)-treated rats at PD 50; five saline-injected control rats at PD 8; five PCP (7.5 mg/kg s.c.)-treated rats at PD 8. Equal amounts of total RNA individually isolated from the five animals of each experimental group were pooled in each experimental group. cDNA synthesis, cRNA labelling, hybridization and scanning were done according to the manufacturer’s instructions (Affymetrix) (see Table 2 note). This first screening step to search for the candidate genes was accomplished by a single microarray comparison on the four pooled cDNAs from the respective four experimental groups. The 3′:5′ ratios of GAPDH, hexokinase, and β-actin of all four samples used were less than 1.2, 2.2, and 3.0, respectively, and thus satisfied the sample quality standard indicated as the signal value ratio <3. The excellent inter- and intra-platform reproducibility of the Affymetrix microarray (more than 88% and 90%, respectively) have been reported elsewhere (MAQC Consortium, 2006), suggesting the reliability of the assay system. To verify the results obtained from the microarray assay, we further achieved expression analyses by RT–PCR and Northern blotting on the candidate transcripts screened from the first single microarray in the individual, but not pooled, samples.

Quantitative RT–PCR

Total RNA was extracted from tissues using the RNeasy Midi kit and was DNase-treated (RNase-Free DNase set; Qiagen, USA). Total RNA (1–2 µg) was reverse-transcribed with an Oligo(dT)20 primer using Superscript III RT (Invitrogen, USA). Real-time quantitative PCR was performed on a LightCycler (Roche Diagnostics, Germany) using a SYBR Premix Ex Taq kit (Takara Bio, Japan) according to the manufacturer’s protocol. The expression levels of individual genes were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative mRNA abundance of each gene was calculated and verified using the standard curve method (Applied Biosystems, USA). In some experiments in order to examine the open reading frame of Lmod2
mRNA and the expression levels in the thalamus and heart, semi-quantitative RT–PCR was performed on GeneAmp PCR system 9700 (Applied Biosystems) using KOD DNA polymerase (Japan). The primer sequences for these experiments are shown in Table 1.

**Northern blot analysis**

Two micrograms of poly(A)$^+$ RNA purified from the rat thalamus using an oligo(dT)-cellulose column (Amersham, UK) was separated by formaldehyde/1.0% agarose gel electrophoresis and transferred to a Hybond-XL membrane (Amersham). A 335-bp cDNA fragment corresponding to the nucleotide position 1549-1883 of rat\textit{Lmod2} cDNA (DDBJ accession no. AB331240) was subcloned into pGEM-T Easy Vector (Promega). Plasmids were linealized, and digoxigenin (DIG)-labelled RNA probes were prepared by\textit{in-vitro} transcription using T7 and SP6 RNA polymerases. Pre-hybridization and hybridization were performed in DIG-Easy Hyb buffer (Roche) at 68°C for 2 h and overnight, respectively. The membranes were washed in 2× SSC/0.1% SDS at room temperature for 1 h and in 0.1× SSC/0.1% SDS at 68°C for 1 h. The hybridized probes were immunodetected with anti-DIG antibody conjugated with alkaline phosphatase (AP) and visualized with a chemiluminescence substrate, CDP-Star (Roche). In some experiments, Rat Multiple Tissue Northern (MTN) Blot (Takara Bio/Clontech) was used for hybridization.

**Real-time quantitative PCR**

In real-time PCR in order to determine the subregional distribution in the brain, the reaction products were separated in 10% polyacrylamide gel electrophoresis in 1× TBE (89 mM Tris–borate and 2 mM EDTA) and visualized by staining with SYBR Green I. PCR was performed under the conditions of annealing temperature at 55°C for 30 cycles (\textit{Lmod2}), for 26 cycles (\textit{Tmod1}), for 25 cycles (\textit{Tmod2}), and for 22 cycles (\textit{GAPDH}), respectively. In the semi-quantitative RT–PCR, the PCR products were electrophoretically separated on 1.5% agarose gel in 1× TAE (40 mM Tris–acetate and 1 mM EDTA) and visualized by staining with ethidium bromide. After 100 times dilution of each cDNA, PCR was performed under the condition of annealing temperature at 55°C for 35 cycles.

**Semi-quantitative RT–PCR**

In the present study we routinely used β-actin expression for the normalization of expression levels of subject genes in the different organs or brain areas in Northern blot analyses in adult rats. Because the basal expression of β-actin, but not GAPDH, has been found to be altered in the developing rat brain whilst PCP does not affect brain expression of the two genes in the adult period, GAPDH was selected as an internal standard housekeeping gene when studying developmental changes in PCP induction of the \textit{Lmod2} gene by RT–PCR techniques.

**In-situ hybridization**

\textit{In-situ} hybridization histochemistry was performed on coronal or sagittal brain sections according to the methods of Kiyama’s research group. For \textit{in-situ} hybridization studies using DIG-labelled RNA probes (nt 1549-1883, Genbank accession no. AB331240) (Nagata et al. 2006; Ohba et al. 2004), slide-mounted frozen brain sections (16-μm-thick) were briefly dried, fixed in 4% paraformaldehyde in PBS for 20 min, rinsed with PBS for 2×15 min, and treated with 5× SSC for 15 min. To detect \textit{Lmod2} mRNA signals following the pre-hybridization and hybridization procedures (see legend of Fig. 4c–e), the sections were shortly rinsed in buffer 3 [100 mM Tris–HCl (pH 9.5), 150 mM NaCl, 50 mM MgCl$_2$], and incubated in buffer 3 containing NBT (Nitro Blue tetrazolium)/BCIP
Table 2. Screening of the developmentally regulated PCP-responsive transcripts in the rat thalamus by DNA microarray

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genbank accession no.</th>
<th>PD 50</th>
<th>PD 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fos: FBJ murine osteosarcoma viral oncogene homolog</td>
<td>BF415939</td>
<td>2.1 (428)</td>
<td>0.00002</td>
</tr>
<tr>
<td>Klf2: predicted: Kruppel-like factor 2 (lung) (predicted)</td>
<td>BF288243</td>
<td>1.6 (303)</td>
<td>0.00002</td>
</tr>
<tr>
<td>Nr4a3: Nuclear receptor subfamily 4, group A, member 3</td>
<td>NM_031628.1</td>
<td>1.4 (264)</td>
<td>0.00002</td>
</tr>
<tr>
<td>Klf4: Kruppel-like factor 4 (gut)</td>
<td>NM_053713.1</td>
<td>1.4 (264)</td>
<td>0.00002</td>
</tr>
<tr>
<td>LOC296935: Similar to leiomodin 2 (cardiac)</td>
<td>AI453854</td>
<td>1.1 (214)</td>
<td>0.000214</td>
</tr>
</tbody>
</table>

The thalamic expression of >30,000 clones was analysed by the Affymetrix Rat Genome 230 2.0 arrays (Affymetrix, USA) in the young adult (PD 50) and infant (PD 8) rats 60 min after acute s.c. administration of PCP (7.5 mg/kg) or saline. The microarrays were scanned with the GeneChip Scanner 3000 and analysed using the GeneChip operating software version 1.2. We used moderately stringent cut-off indices for significance determination (McClung & Nestler, 2003): genes were considered to be detected if the experimental or control signal was >20 (detection \( p \) value \(<0.04\) and were considered to be up-regulated if the \( \log_2 \) ratio experimental value/control value for each gene was >1.0 (\( >200\% \) of control value, change \( p \) value \(<0.002\). We screened the transcripts of the known genes that showed the up-regulation by PCP injection with the \( \log_2 \) ratio experimental (PCP)/control (saline) of >1.0 (change \( p \) value \(<0.002\)) at PD 50 and no significant changes in the ratio at PD 8. ‘Absent’ means that the gene expression was not detected (the experimental or control signal was <20, or detection \( p \) value <0.04).

(5-bromo-4-chloro-3-indolyl phosphate) stock solution (Roche) overnight for up to 3 d at 4 °C. The colour development was terminated by TE [10 mM Tris–HCl (pH 7.4), 1 mM EDTA].

For radioisotope-labelled in-situ hybridization experiments (Tanabe et al., 1998, 1999), \( ^{35}S \)-labelled cRNA probes (nt 1549-1883, GenBank accession no. AB331240) were prepared by in-vitro transcription using T7 and SP6 RNA polymerases and [\( ^{35}S \)]UTP (Amersham, 37 TBq/mmol). The brain sections were dried, fixed in 4% paraformaldehyde in phosphate buffer for 20 min at room temperature, treated with 10 \( \mu \)g/ml proteinase-K in 50 mM Tris–HCl (pH 8.0), and 5 mM EDTA for 5 min, and then fixed again. To detect the hybridized \( ^{35}S \)-labelled cRNA probes following the hybridization procedures (see Fig. 4b legend), the sections were exposed to an X-ray film (BioMax XAR, USA) for 1 wk and subjected to autoradiography.

Statistical analysis

Results are mainly given as means with s.e.m. of the data. Statistical differences among three groups or more with parametric and non-parametric distribution were estimated by the Kruskal–Wallis test followed by the Scheffé post-hoc test. Some developmental and organ differences in the effects of PCP were examined by a two-way ANOVA followed by the Scheffé post-hoc test. We confirmed by Bartlett test that the datasets analysed with the two-way ANOVA had a parametric distribution.

Nucleotide sequences

The DDBJ/GenBank/EMBL accession number for primary nucleotide sequence of rat Lmod2 is AB331240.

Results

Detection of Lmod2 mRNA as a developmentally regulated PCP-responsive transcript by a DNA microarray method

From the DNA microarray data, we screened the thalamic transcripts of the known genes that showed up-regulation by PCP injection (7.5 mg/kg s.c.) with the \( \log_2 \) ratio experimental PCP/control (saline) of >1.0 \([>2\text{-fold} (200\% \text{ of control})]\) with a statistical significant \( p \) value at PD 50 (young adult rat) and no significant changes in the ratio at PD 8 (infant rat). The filtering procedure provided us with five candidate transcripts: c-fos, Klf2, Nr4a3, Klf4 and leiomodin2 (Lmod2)-like (Table 2). Because there has been no study on the expression, functions and neuronal or psychiatric involvement of the Lmod2 (or Lmod2-like) gene in the brain, whereas other genes have already been reported to be regulated by PCP in the rat brain (Kaiser et al., 2004), we characterized the structural, developmental, pharmacological and neuroanatomical properties of the Lmod2/Lmod2-like transcript.
To this end, we first screened Lmod2/Lmod2-like cDNAs from the adult rat thalamus by RT–PCR with the primer set designed based on mouse Lmod2 cDNA (Genbank accession no. NM_053098) and the rat genomic sequence database (Fig. 1a). The isolated rat Lmod2/Lmod2-like cDNA is predicted to encode a protein with a molecular weight of 64.5 kDa that is composed of 549 amino acids showing 97% identity with mouse Lmod2 protein (Genbank accession no. NM_444328, originally designated as C-Lmod protein; Conley et al. 2001). Thus, we concluded that this gene product was a rat ortholog of the mouse Lmod2 protein. Lmod2 is thought to be a member of the tropomodulin (Tmod) family of the actin filament pointed end-capping proteins (Fischer & Fowler, 2003). The structures of the presumed tropomyosin-binding (TM) domain and leucine-rich repeats (LLR), which were characteristic of the Tmod protein family, were well conserved in rat Lmod2 protein (Fig. 1a), suggesting the possible interaction with the other molecules that might regulate actin-cytoskeletal structures (Conley et al. 2001; Fischer & Fowler, 2003). The polyproline (PP) motif near the carboxyl terminal, which was similar to that of Lmod1, is also preserved in rat Lmod2 protein. Additionally, we detected a possible nuclear localization signal (NLS), whose amino-acid sequences are RKKK (480-483; mouse, 481-484), in Lmod2 protein using the PSORTII program (Nakai & Horton, 1999). A similar NLS was reported in Tmod proteins (Kong & Kedes, 2004) but was absent in Lmod1. However, rat Lmod2 protein did not possess a typical nuclear export signal motif, which was identified in Tmod proteins (Kong & Kedes, 2004).
Phencyclidine, Lmod2 and the developing thalamus

Fig. 2. Effects of acute PCP injection on expression of Lmod2, Tmod1 and Tmod2 mRNAs in the thalamus and behaviour in developing rats. Relative expression levels of Lmod2, Tmod1 and Tmod2 mRNAs (Lmod2, Tmod1 or Tmod2 to GAPDH mRNA ratio) were determined by the real-time RT–PCR method 60 min after administration of PCP or saline. Results are the means with S.E.M. of data obtained from five rats per group and are expressed as a percentage of the values of the adult (PD 50) saline-treated animals. (a) Lmod2: adult rats (PD 50), saline 100 ± 8%, PCP 231 ± 7% (p < 0.01 vs. saline-treated controls); infant rats (PD 8), saline 100 ± 8%, PCP 87 ± 10 (p > 0.05 vs. saline-treated controls; Scheffé test) (two-way ANOVA, postnatal days effect: F_{1,8} = 76.878, p < 0.0001; PCP treatment effect: F_{1,8} = 51.066, p < 0.0001; postnatal days × PCP treatment effect: F_{1,8} = 77.456, p < 0.0001). (b) Tmod1: adult rats (PD 50), saline 100 ± 3%, PCP 97 ± 2 (p > 0.05 vs. respective saline-treated controls); infant rats (PD 8), saline 100 ± 4%, PCP 104 ± 3% (p > 0.05 vs. respective saline-treated controls; Scheffé test) (two-way ANOVA, postnatal days effect: F_{1,8} = 0.981, p = 0.34; PCP treatment effect: F_{1,8} = 0.047, p = 0.83; postnatal days × PCP treatment effect: F_{1,8} = 0.944, p = 0.35). (c) Tmod2: adult rats (PD 50), saline 100 ± 2%, PCP 104 ± 2% (p > 0.05 vs. the respective saline-treated controls); infant rats (PD 8), saline 100 ± 5%, PCP 107 ± 3% (p > 0.05 vs. the respective saline-treated controls; Scheffé test) (two-way ANOVA, postnatal days effect: F_{1,8} = 0.241, p = 0.63; PCP treatment effect: F_{1,8} = 2.546, p = 0.13; postnatal days × PCP treatment effect: F_{1,8} = 0.228, p = 0.64). (d) Rats at PD 8, 13, 20, 26, 32 and 50 were treated with 7.5 mg/kg PCP subcutaneously and the relative expression levels of Lmod2 mRNA (Lmod2:GAPDH mRNA ratio) were determined by the real-time RT–PCR method 60 min after administration of PCP or saline. Results are the means with S.E.M. of data obtained from five or six rats per group and are expressed as a percentage of the values of the adult (PD 50) saline-treated animals (p < 0.01 vs. respective saline-treated controls; # p < 0.01 vs. saline-treated rats at PD 8). (e) The intensity of the PCP-induced stereotypy was evaluated by the following stereotyped behavioural rating scale from 30 to 60 min after drug administration: 0, no stereotyped behaviour; 1, increased exploratory activity with occasional sniffing, grooming or rearing; 2, episodic non-directional movement, weaving, reciprocal forepaw treading, higher frequency of sniffing, grooming or rearing; > 1; 3, intermittent turning, backpedalling and weaving with or without bursts of frequent sniffing, grooming or rearing; 4, rapid and continuous turning, backpedalling, weaving, sniffing and gagging; and 5, dyskinetic extension and flexion of limbs, head and neck. Results are the means with S.E.M. of data obtained from five to seven rats per group (p < 0.01 vs. respective saline-treated controls).}

On the Northern blot of the 2μg poly(A)⁺ RNA from the thalamus of adult rats, we detected a single transcript at 2.4 kb with an RNA probe in an anti-sense specific manner (Fig. 1b). This signal was up-regulated in the adult rat thalamus by PCP administration in agreement with the results of the DNA microarray
assay. The intense signal of rat Lmod2 mRNA was predominantly observed in the heart at an extremely high level, followed by the skeletal muscle at a low level but was not detectable in the whole brain tissue at 2 μg poly(A)^+ RNA (Fig. 1a). This distribution pattern was similar to that of human Lmod2 reported previously (Conley et al. 2001).

Using RT–PCR with several different primer sets (Fig. 1a), we confirmed that Lmod2 RNA expressed in the thalamus shares essentially the same primary structure with that in the heart of the rat. This conclusion was obtained by the results in which, in both the thalamus and heart, the identical PCR products of the 1647 bp nucleotide sequence that contains the same open reading frame of Lmod2 were detected as a single band, while the heart expressed much higher levels of Lmod2 transcripts than the thalamus (Fig. 1d). Moreover, the semi-quantitative RT–PCR analysis revealed that Lmod2 mRNA was exclusively expressed in the thalamus and detected at low levels in the hypothalamus and midbrain among the discrete brain areas of the adult rat, while Tmod1 and Tmod2 mRNAs were evenly distributed throughout the brain regions examined (Fig. 1c).

**Effects of acute PCP on expression of Lmod2 transcripts in the thalamus and behaviour in developing rats**

The contrasting responses to PCP between PD 8 and 50 in the thalamus as shown by DNA microarray analysis were semi-quantitatively verified by the real-time RT–PCR method. As indicated in Fig. 2a, PCP treatment (7.5 mg/kg s.c.) caused a significant increase in the ratios of mRNA levels of Lmod2 to those of GAPDH compared to the saline-treated controls in adult rats but no significant changes were seen in infant rats. In contrast, the relative thalamic expressions of other Tmod family genes, Tmod1 (Fig. 2b) and Tmod2 (Fig. 2c), at PD 8 and 50 were not affected by the acute injection of PCP. The similar magnitude of an increase in thalamic Lmod2 expression after PCP injection in the present microarray using the pooled cDNA (% of saline-treated control value: Lmod2, 214%) and RT–PCR (Lmod2, 231%; Fig. 2a) assay using the individual samples adds further reliability to the microarray analysis.

To further clarify the postnatal developmental changes in the basal and PCP-induced expression of thalamic Lmod2, we evaluated the effects of a single injection of saline and PCP (7.5 mg/kg) on the relative mRNA levels of the gene 1 h later at PD 8, 13, 20, 24, 32 and 50. The expression after saline administration (or basal expression) gradually increased in the thalamus of the rat with postnatal days up to PD 20 and then continued at a plateau level thereafter. Acute PCP failed to alter Lmod2 mRNA levels at PD 8, 13, 20 and 24 but significantly augmented the transcript expression at PD 32 and 50 (Fig. 2d).

The same dose of PCP produced a statistically significant increase in the stereotypy scores at PD 20, 26, 32 and 50, but not at PD 8 and 13, and a similar intensity of the abnormal behaviour was seen between PD 32 and 50 (Fig. 2e). These behavioral observations indicated that the apparent adult type stereotypy (behavioural changes) after acute PCP injection seems to occur between PD 26 and PD 32.

In the young adult period, thalamic Lmod2 mRNA expression in adult rats (PD 50) increased rapidly, peaked at 1–3 h and returned to the saline-treated levels within 6 h after acute PCP (7.5 mg/kg s.c.) administration (Fig. 3a). However, there were no changes in Lmod2 mRNA levels in the thalamus of the infant rats (PD 8) up to 6 h post-injection. The time-course of PCP-induced thalamic Lmod2 up-regulation and stereotypy in the adult rats appears to be parallel (Fig. 3a, b).

**Effects of acute PCP injection on expression of Lmod2 transcripts in the heart and brain regions of adult rats as revealed by RT–PCR and in-situ hybridization**

Unlike Lmod2 expression in the thalamus, the gene transcript levels in the heart were unaffected by acute injection of PCP (7.5mg/kg s.c.) in adult rats (Fig. 4a). Further, we tried to clarify the exact brain or thalamic portions where PCP up-regulates Lmod2 gene expression using an in-situ hybridization method. In accordance with the RT–PCR data, Lmod2 mRNA signals were found to be restricted to the thalamus in the saline-treated control rats at PD 50, although the signal intensity was low (Fig. 4b, left panel). Acute PCP treatment (7.5 mg/kg s.c.) augmented thalamic Lmod2 expression without apparent influence on its basal distribution pattern (Fig. 4b, right panel). Therefore, we intensively performed in-situ hybridization studies on the brain samples obtained from PCP-treated adult rats.

With a DIG-labelled anti-sense RNA probe, Lmod2 mRNA signals were observed in the anterior nucleus and the lateral nucleus of the thalamus as shown in the sagittal sections (Fig. 4c, d). In the brain sections counterstained by Methyl Green at higher magnification, the Lmod2 mRNA signal was mainly detected in the cells that possessed a neuron-like morphological
feature with a characteristic large round-shaped nucleus (Fig. 4e). A more sensitive \textit{in-situ} hybridization detection system with a \(^{35}\text{S}\)-labelled anti-sense RNA probe revealed that strong hybridization signals of \textit{Lmod2} mRNA were predominantly observed in the rostral areas of the thalamus including the anterior thalamic complex [i.e. the anteromedial (AM), antero-ventral (AV), and inter-anteromedial (IAM) nucleus], and the ventral anterior-lateral complex (Fig. 4f–l). In addition, much lower hybridization signals were found in limited areas of the caudal thalamus including the ventral medial nucleus, the rhomboid nucleus, the intralaminar thalamic nucleus [consisting of the central medial (CM), paracentral (PCN), and central lateral (CL) nucleus], and the lateral and ventral posterior complex of the thalamus. No hybridization signal was detected in any brain sections studied with the corresponding sense probe (Fig. 4m).

\textbf{Effects of acute injection of NMDA receptor antagonists, dopamine agonist and antagonist on expression of \textit{Lmod2} transcripts in the thalamus of adult rats}

Because \textit{Lmod2} has been detected as a novel candidate for a schizophrenia symptom-related gene in the present study, we evaluated the effects of different psychotomimetics, PCP, dizocilpine and methamphetamine, on thalamic \textit{Lmod2} mRNA expression in adult rats. The acute injection of another non-competitive NMDA receptor antagonist, MK-801 (0.5 mg/kg s.c.), mimicked the up-regulation of the thalamic expression levels of \textit{Lmod2} mRNA seen following PCP administration (7.5 mg/kg s.c.) (Fig. 5a). An indirect dopamine agonist, methamphetamine (MAP, 4.8 mg/kg s.c.) produced a smaller significant increase in \textit{Lmod2} mRNA levels than PCP and MK-801 (Fig. 5a).

We also examined the effects of Hal, which is a \(D_2\)-selective dopamine receptor antagonist, on basal expression and PCP-induced up-regulation of \textit{Lmod2} mRNA. Pretreatment with haloperidol (1.0 mg/kg i.p.) 30 min before PCP injection partially antagonized the ability of PCP to augment \textit{Lmod2} mRNA expression (Fig. 5b). Hal, by itself, showed no significant effect on \textit{Lmod2} mRNA expression.

\textbf{Discussion}

By using a DNA microarray technique, we show that \textit{Lmod2} is a developmentally regulated and PCP-regulated gene in the rat thalamus in that a psychotomimetic respectively causes no and prominent up-regulation of thalamic \textit{Lmod2} gene expression in infant (PD 8) and young adult (PD 50) rats. The significant increase in thalamic \textit{Lmod2} mRNA expression after PCP administration is observed only after PD 32. We further demonstrate that the basal and PCP-induced expression of \textit{Lmod2} is confined to the anterior and lateral nuclei of the thalamus in the brain. The up-regulation of thalamic \textit{Lmod2} transcripts in the
Fig. 4. Effects of acute PCP injection on Lmod2 mRNA expression in the heart and brain regions of adult rats. (a) Relative expression levels of Lmod2 mRNAs (Lmod2 : GAPDH mRNA ratio) were determined by the real-time RT–PCR method in the thalamus and heart of the adult (PD 50) rat 60 min after administration of PCP (7.5 mg/kg s.c.) or saline. Results are the means with S.E.M. of data obtained from five rats per group and are expressed as a percentage of the values of the respective saline-treated controls. Thalamus: saline 100 ± 3%, PCP 171 ± 7% (* p < 0.01 vs. saline-treated controls); heart: saline 100 ± 4%, PCP
adult period is totally and partly mimicked by other
schizophrenimimetics, dizocilpine and MAP, re-
spectively, and moderately attenuated by the D2-
preying dopamine receptor antagonist Hal. These
developmental, neuroanatomical and pharmacologi-
cal profiles of Lmod2 responses suggest that Lmod2 and
its protein products could, at least in part, be asso-
ciated with the late-developing onset and the spe-
cific neuron circuits for the adult type of PCP-induced
abnormal behaviour in the rat.

This is the first study to indicate the profound in-
fluence of psychotomimetics on brain mRNA ex-
pression of the Lmod2 gene. The Tmod gene family
member has so far been considered to represent the
muscular tissue-selective expression without its tran-
scripts in the brain. However, we have demonstrated
that considerable amounts of Lmod2 mRNAs are pre-
dominantly concentrated in the thalamus among the
brain tissues. The expressional changes in the thalamic
Lmod2 by psychotropic drugs may imply the possible
involvement of the molecule in the regulation of
psychological and motor functions. This assumption
appears to be supported by the similar time-course
in up-regulation of Lmod2 expression and stereotyped
behaviour after PCP (Fig. 3a, b). On the other hand, the
present study also revealed the obvious discrepancy
between the postnatal development of the above mol-
ecular and behavioural responses to PCP (Fig. 2d, e).
Thus, the exact regulatory roles of the gene await fur-
ther elucidation.

The possibility that the post-weaning marked in-
crease in Lmod2 transcript levels by PCP could be due
to a non-specific phenomenon appears to be denied by
the observations that PCP failed to influence (1) the
thalamic expression of other Tmod family genes in the
infant and adult periods and (2) Lmod2 mRNA levels

\[ 121 \pm 4 \, \text{(p} < 0.05 \, \text{vs. saline-treated controls, n.s., no significant difference) (two-way ANOVA, organ effect: } F_{1,16} = 25.109, \, p < 0.001; \, \text{PCP treatment effect: } F_{1,16} = 85.035, \, p < 0.0001; \, \text{organ} \times \text{PCP treatment effect: } F_{1,16} = 24.988, \, p < 0.001). \]
Fig. 5. Effects of acute administration of psychotomimetic and antipsychotic drugs on Lmod2 mRNA expression in the thalamus. (a) Effects of PCP, MK-801 and methamphetamine (MAP) on thalamic Lmod2 mRNA. Relative expression levels of Lmod2 mRNA in the thalamus of the adult (PD 50) rat (Lmod2:GAPDH mRNA ratio) were assayed by the real-time RT–PCR method 60 min after acute PCP (7.5 mg/kg s.c.), MK-801 (0.5 mg/kg s.c.), and MAP (4.8 mg/kg s.c.) administration. Results are shown as scatterplots with the means of data (Lmod2:GAPDH mRNA ratio) obtained from six or eight rats per group and are expressed as a percentage of the values of the saline-treated controls. Saline (Sal) 100 ± 5%, PCP 174 ± 8%, MK-801 162 ± 5%, MAP 128 ± 3 (p < 0.01, p < 0.05 vs. saline-treated controls). (b) Effects of pretreatment with haloperidol (Hal) on PCP-induced up-regulation of thalamic Lmod2 mRNA. The adult (PD 50) rats were pretreated with Hal (1.0 mg/kg, i.p.) or vehicle (Veh) 30 min before PCP or saline (Sal) administration, and the relative expression levels of Lmod2 mRNA in the thalamus (Lmod2:GAPDH mRNA ratio) were assayed by the real-time RT–PCR method 60 min after acute PCP (7.5 mg/kg, s.c.) or saline injection. Results are the means with S.E.M. of data (Lmod2:GAPDH mRNA ratio) obtained from six rats per group and are expressed as a percentage of the values of the vehicle-pretreated and saline-injected controls. Vehicle-pretreated saline-injected controls (Veh/Sal) 100 ± 2%, vehicle-pretreated PCP-injected animals (Veh/PCP) 200 ± 6%, Hal-pretreated PCP-injected animals (Hal/PCP) 145 ± 11%, Hal-pretreated saline-injected animals (Hal/Sal) 83 ± 3 (p < 0.01 vs. Veh/Sal controls; p < 0.01 between Veh/PCP and Hal/PCP animals and between Hal/PCP and Hal/Sal animals). n.s., No significant difference. Veh: 0.15% tartaric acid.

in the heart of the adult animal. The ontogenic differences in brain Lmod2 induction by PCP might solely depend on those in the time-course of the pharmacodynamics of PCP or the general responses of the brain. However, this explanation seems to be contrary to the observation that (1) PCP caused no change in thalamic Lmod2 transcript levels even up to 6 h post-injection in the infant rat despite the pronounced increase in those of the adult rat during the same time (Fig. 3a), and (2) a similar time-course of the acute PCP-induced increase in c-fos gene expression was seen in the various brain areas of the rat at PD 8 and 50 (Sato et al. 1997).

The PCP-induced thalamus-selective up-regulation is more likely to be associated with a psychotomimetic action generated by reduced NMDA receptor function and excessive dopaminergic transmission because potent schizophrenimimetic drugs, the selective NMDA antagonist MK-801 and the dopamine signal potentiator MAP, caused an elevation in thalamic Lmod2 expression. However, MAP elicited a smaller magnitude of elevation than PCP and MK-801 (Fig. 5a). The selective D2 dopamine receptor antagonist, Hal, is found to partially attenuate the increasing effects of PCP on Lmod2 expression. Together with the fact that NMDA receptor blocking results in the augmented cerebral dopaminergic activities (Umino et al. 1998), these observations suggest that the mechanisms underlying PCP-induced up-regulation of Lmod2 expression may consist of NMDA receptor-related D2 receptor-sensitive and -insensitive components. These pharmacological features allow us to assume that Lmod2 or its protein products could participate in the molecular cascades that are dysregulated in the dopamine-dependent positive symptoms and NMDA receptor-associated dopamine-uncoupled negative symptoms and cognitive disturbances in PCP psychosis and schizophrenia (Javitt, 2004; Nishikawa et al. 1991; Petersen & Stillman, 1978).

From this pharmacological point of view, it is also plausible that the distinct developmental changes in the responses of Lmod2 to PCP could be attributed to the neuroanatomical and functional development of the NMDA receptor subunits (Watanabe et al. 1992) and/or the cerebral dopaminergic systems (Perez-Navarro et al. 1993) and in turn this transcript might be an excellent marker for the developmental maturation of the response of the thalami to increased dopaminergic transmission. The acquisition by the Lmod2 gene of thalamus-selective responsiveness to PCP after the weaning period (Fig. 2d) further argues that the maturation of a specific information-processing system in the thalamus containing Lmod2 transcripts or proteins as its molecular elements might be required for the
PCP-induced up-regulation of Lmod2. Such a system could be disturbed in the schizophrenomimetic-induced abnormal behaviour in experimental animals, schizophrenia-like psychosis and schizophrenia.

In fact, the restricted rostral thalamic regions including the anterior and lateral nuclei that show PCP-induced Lmod2 expression have been found to display aberrant 2-deoxyglucose uptake, activity-dependent gene expression or cerebral blood flow after ketamine, MK-801, PCP and amphetamine application in experimental animals (Duncan et al. 1999) and/or humans (Långsjo et al. 2003). Furthermore, in-vivo neuroimaging studies describe schizophrenia patients exhibiting reduced activation following cognitive tasks (Andrews et al. 2006), decreased N-acetylaspartate signals in magnetic resonance spectroscopy (Jakary et al. 2005), and increased diffusivity of magnetic resonance diffusion tensor imaging (Rose et al. 2006) in these thalamic regions. Biochemical and histochemical analyses using post-mortem brains from schizophrenia patients have revealed various changes in the glutamate system such as altered expression of the l-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and NMDA-type ionotropic glutamate receptors (Ibrahim et al. 2000) and glutaminase and glutamine synthetase mRNA (Bruneau et al. 2005) in the above Lmod2-associated nuclei. Dopaminergic imbalance has also been noted in the thalamus of schizophrenia patients while the major observations concerning the imbalance concentrate on the mediodorsal and posterior nuclei (Takahashi et al. 2006).

The series of thalamic aberrations in schizophrenia and its pharmacological models, and the glutamate and dopamine signal-related, late-developing and thalamus-specific nature of the PCP-induced modification of Lmod2 reinforce the hypothesis that Lmod2-expressing cells might compose a part of the information-processing system or neuron circuitry that is specifically distorted in schizophrenia. Accordingly, detection of the schizophrenomimetic-induced Lmod2 mRNA signals might be a useful tool for tracing the cells and circuits that play a central role in the pathogenesis or pathophysiology of schizophrenia. Because the distribution pattern of Lmod2 mRNA is similar to that of [3H]muscimol binding to the GABA A receptor in the thalamic area of the adult rat (Palacios et al. 1981), it would be useful to clarify the glutamate–dopamine–GABA interaction in Lmod2-expressing cells that is thought to be dysregulated in schizophrenic brains (Lisman et al. 2008).

The molecular and functional consequences and the pathophysiological significance of the schizophrenomimetic-induced changes in thalamic Lmod2 expression are still unknown. Although the biological roles of Lmod2 protein in brain tissue have not yet been analysed, the characteristic motifs and domain structures of the Lmod2 protein buttress its potential contributions to neuronal and mental functions. Similar to other Tmod family members, the Lomod2 protein has been shown to regulate the organization of the actin-cytoskeletal system through the tropomyosin-binding domain and leucine-rich repeats. A body of evidence has accumulated indicating that actin-based morphological changes in the dendritic spines are involved in synaptic plasticity, which is one of the most essential neural processes for higher brain functions, e.g. learning and memory (Carlisle & Kennedy, 2005).

The polyproline motifs, which the Lmod2 protein possesses at its carboxy-terminal, have been considered to interact with the Src-homology 3 (SH3) domains that are implicated in synaptic organization or reorganization (Segura et al. 2007). Moreover, the nuclear localization signal-like amino-acid sequence was found in Lmod2 protein (see Results section). This could be extrapolated to the idea that the possible intranuclear link between Lmod2 and actin could join the integration of gene expression in the thalamus, because nuclear actin has recently been demonstrated to be required for the chromosomal movement that may be connected to the positioning of genes within the nuclear volume for the appropriate transcriptional activity (Dundr et al. 2007).

As a consequence, we can presuppose that the quantitative or structural alterations of thalamic Lmod2 mRNA or proteins would lead to disintegrated synaptic transmission and/or plasticity that may underlie the characteristic symptoms of schizophrenia and related psychoses. Although no studies identified the expression changes in the mRNA or protein of this gene in schizophrenic brain tissues, and regions of the human genome in which LMOD2 is located (7q31.32) have not been suggested to be associated with an altered risk of schizophrenia, it would be valuable to investigate the possible involvement of LMOD2 in schizophrenia because the chromosome 7q31 region includes the PTPRZ1 (Buxbaum et al. 2008) and FOXP2 (Sanjuán et al. 2006) genes that have been indicated to be related to the susceptibility of schizophrenia.

In conclusion, the present findings indicate that PCP can affect the expression of Lmod2 in an age-dependent, schizophrenomimetic cross-reactive and thalamus-selective manner in mammal brain (the rat). These figures seem to be consistent with the view that a thalamic neuronal system influenced by PCP may be equipped with a signal pathway containing Lmod2 or its protein and be functionally
late-developing. Therefore, our PCP data would suggest that changes in Lmod2 expression should be present in the thalamus of subjects with schizophrenia and a study measuring the expression of that gene in post-mortem CNS is required to confirm this hypothesis.

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Statement of Interest

None.

References


