1. Introduction

It is well documented that the single or repeated use of N-methyl-D-aspartate (NMDA) receptor antagonists including phencyclidine (1(1-phenylcyclohexyl)-piperidine) (PCP) and ketamine cause positive and negative symptomatologies indistinguishable from those of schizophrenia (Javitt and Zukin, 1991; Lahti et al., 2001). Furthermore, a group of schizophrenic patients suffered exacerbation of their psychotic symptoms by a challenge dose of PCP and ketamine (Javitt and Zukin, 1991; Lahti et al., 2001). These clinical observations indicated that NMDA antagonists may cause a dysfunction in a specific neuronal system that is involved in the pathophysiology of schizophrenia. The facts that children seldom have an onset attack of schizophrenia (American Psychiatric Association, 2000), ketamine psychosis (Reich and Silvay, 1989; White et al., 1982) and PCP-induced clinical psychiatric symptoms and signs (Schwartz and Einhorn, 1986; Welch and Correa, 1980) allow us to postulate that the typical or adult type psychotic symptoms of these psychoses could appear only after the functional maturation of the specific neuronal system around the adolescence period. Because marked postnatal changes have also been observed in PCP- or dizocilpine-induced abnormal behavior as a model of schizophrenia (Scalzo and Burge, 1994; Scalzo and Holson, 1992; Sato et al., 1997) across a critical period, non-human mammalian brains would be provided with a similar neuronal system. Such a mammalian system should, therefore, be equipped with a molecular cascade that differentially responds to NMDA antagonists before and after the critical period.

In support of this hypothesis, our recent study of the development of brain responsiveness by monitoring the c-Fos expression pattern, which is a well-known marker of stimulus-coupled information processing (Morgan and Curran, 1991; Umino et al., 1995), has revealed that the infant distribution pattern of PCP-induced c-Fos shifts to the adult pattern after the weaning period around postnatal week 3 (Sato et al., 1997). The most prominent developmental changes were seen in the neocortex including the mesocortices, cingulate and retrosplenial cortex, while the pyriform cortex, mid-lateral striatum, olfactory tubercle and septum exhibited the minimal postnatal alterations (Sato et al., 1997). Furthermore, Nakki et al. (1996) demonstrated that there is a postnatal development of an expression pattern of another stimulus-inducible molecule, the heat shock protein 70, in the rat cerebellum. The results from the c-Fos and heat shock protein 70 experiments suggest that the plausible functional maturation of the PCP-responsive and schizophrenia symptoms-related neuron circuits could occur, at least, in the neocortex.

To obtain insight into the molecular pathophysiology of the NMDA antagonist-induced psychosis and schizophrenia, we have explored the hypothetical molecules in the infant and young adult rats that are developmentally regulated by a schizophrenic NMDA antagonist PCP using a differential cloning technique, the RNA arbitrarily-primed PCR (RAP-PCR). For this screening procedure, we chose the RAP-PCR method, but not the differential display or the DNA array techniques, because this method enables us to detect even the transcripts that has the entirely unknown nucleotide sequences and/or no poly-A signals (Welsh et al., 1992) although, unlike the DNA arrays, the RAP-PCR does not provide us an overview of the gene expression profiles.

2. Experimental procedures

2.1. Animals

The present animal experiments were performed in strict accordance with the guidance of the National Institute of Neuroscience, National Center of Neurology and Psychiatry and of the Tokyo Medical and Dental University, and were approved by the Animal Investigation Committee of each institute. Male Wistar rats (ST strain, Clea Japan, Tokyo, Japan) at postnatal days 8 and 50 were used.

2.2. Chemicals

The PCP hydrochloride was kindly synthesized and donated by Yamanouchi Pharmaceutical, Co., Ltd. (Tsukuba, Japan). The methamphetamine (MAP) hydrochloride was purchased from Dainippon Pharmaceutical Co., Ltd., with official permission of the Tokyo Metropolitan Bureau of Public Health. All other chemicals were of ultrapure quality and commercially available. The PCP hydrochloride, MAP hydrochloride, cocaine hydrochloride, dizocilpine hydrogen maleate ((+)-MK-801: (+)-5-methyl-10,11-dihydroxy-5H-dibenzo-(a,d) cyclohepten-5,10-imine hydrogen maleate) and SCH23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2, 3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride were dissolved in physiological saline for subcutaneous (s.c.) or intraperitoneal (i.p.) injection. An ampoule solution of pentobarbital and of haloperidol was diluted with physiological saline and injected i.p. The control animals received only the vehicle. The doses always refer to the free bases.

2.3. Screening procedures of the candidates for the developmentally-regulated and PCP-responsive molecules

The gene expression analysis using an RNA fingerprinting was achieved in the rats as the first step to screen the developmentally-regulated and PCP-responsive genes under the conditions that our series of animal experiments concerning schizophrenomimetics and development have been done in the rats (Sato et al., 1997; Kajii et al., 2003), because the systemic administration of PCP caused different behavioral changes between the infant (PD 8) and young adult (PD 50 and 56) rats (Ito et al., 2007: Sato et al., 1997; Scalzo and Burge, 1994; Scalzo and Holson, 1992; Takebayashi et al., 2009). We have confirmed by the behavioral rating experiments that phencyclidine does not elicit an adult type stereotypy as a model of schizophrenia on the PD 8 but elicits the abnormal behavior on the PD 50 (Takebayashi et al., 2009). Based upon the results of the RNA fingerprinting, we picked up the genes markedly altered by PCP at PD 50 in the neocortex, and further selected the transcript that represented the most prominent response to the acute PCP injection at PD 50 with the minimal change at PD 8. Using a semi-quantitative RT-PCR technique, we subsequently studied the effects of PCP on the expression of the selected gene transcript in the neocortex of the rat at PD 50 and/or 8. The doses of 5 and 10 mg/kg were chosen for the screening procedures of the developmentally-regulated PCP-responsive genes, because PCP has generally been injected at the doses ranging from 5 to 10 mg/kg in the studies for its acute psychotomimetic actions in the rodents (Toth and Lajtha, 1986: Tanii et al., 1994).

2.4. RNA fingerprinting

We performed the RNA fingerprinting using a 12-mer arbitrary primer (RNA arbitrarily-primed PCR (RAP-PCR))(Kajii et al., 2003; Tsuchida et al., 2001; Welsh et al., 1992) to isolate the

developmentally-regulated neocortical transcripts responsive to PCP (5 mg/kg, s.c.). Rats at PD 8 or 50 were s.c. injected with a moderate dose (5 mg/kg) of PCP or saline and killed 1 h later by cervical dislocation. The dose and timing of the administration of the PCP were chosen because 1) the aim of this study is to obtain an insight into the initial or early step of the putative molecular cascade associated with the PCP-induced abnormal behavior as a model for schizophrenia, 2) single or repeated treatment with doses ranging from 3 to 10 mg/kg of PCP has been shown to exhibit no apparent neural necrosis (Linden et al., 2001; Olney et al., 1989), and 3) 5 mg/kg of PCP causes an antipsychotic-resistant behavioral change with the relatively selective blocking of the NMDA receptor (Johnson and Jones; 1990).

Total RNA was prepared from the neocortex using an RNeasy Midi Kit (Qiagen, GmbH, Hilden, Germany) and converted to single-stranded cDNA by reverse transcriptase (Superscript Preamplification system, GIBCO BRL, Rockville, MD) with random hexamer primers. In every experimental group, equal amounts of the resulting cDNAs of the individual rats were pooled. The pooled samples were serially diluted in TE buffer (pH 8.0) and used as a template for PCR using a rhodamine-labelled 12-mer arbitrary primer pair (5′-CAGGTGTGGGTT-3′ and 5′-GGCGAGGGAGGA-3′), which was randomly selected from the commercial ready-made set of primers (Bex Co. Ltd., Tokyo, Japan). This type of primer pair was chosen in order to detect the known and unknown transcripts both with and without the poly-A tail. The PCR parameters were 94 °C for 2 min, 40 °C for 5 min, 72 °C for 5 min for the first cycle, 94 °C for 30 s, 40 °C for 2 min, 72 °C for 1 min with 34 cycles, and then 72 °C for 5 min for extension. The PCR products were separated in a 5% denaturing polyacrylamide gel including 7 M urea and 1× TBE (90 mM Tris-borate and 2 mM EDTA). The gel was stained with SYBR Green I (Molecular Probes), and the display pattern of the cDNA bands was visualized and analyzed by an FMBIO II Multi-View fluorescence image analyzer (Hitachi, Tokyo, Japan). The cDNA bands that showed higher intensity in the PCP-treated group than in the saline-injected controls at PD 50 in the adult period were cut out from the gel. The extracted DNA fragments were re-amplified by a second PCR with the same primer set. The amplified DNA fragments were cloned using a TA cloning vector (pGEM-T Easy vector system, Promega, Madison, WI), and sequenced (ABI Prism 377, PE Biosystems, Foster City, CA). To determine the full length structure from the isolated DNA fragments, we performed 5′ and 3′ rapid amplification of the cDNA ends (RACE)-PCR with an aliquot of the oligo-dT selected RNA (1 μg) prepared from the neocortex using a SMART RACE cDNA amplification kit (Clontech, La Jolla, CA) according to the manufacturer's protocol.

2.5. Quantitative RT-PCR

Quantitative measurements of the RNA levels in the different samples were achieved by a competitive RT-PCR method (Becker-Andre and Hahlbrock, 1989; Tsuchida et al., 2001; Fujiyama et al., 2003; Kajii et al., 2003). Each homologous competitor fragment was generated by introducing the respective restriction site using the overlap extension PCR-based site-directed mutagenesis (Higuchi et al., 1988). For the competitive PCR of the long-form *SAP97* mRNA and short-form *SAP97* mRNA, the following primers were synthesized by Bex (Tokyo, Japan). N* or underlining indicates the mutated base or the sequence including the respective restriction site, respectively: *SAP97 long*, restriction enzyme, Xho I; *SAP97 long* sense 1 (5′-CTC-ACC-CAT-AAA-GCC-CAC-AGA-AG-3′, nucleotides 922–944), *SAP97 long* antisense 1 (5′-GTT-TCC-AAC-ACC-TCG*-AGC-AAT-G-3′, nucleotides 1444–1465), *SAP97 long* sense 2 (5′-GCA-TTG-CTC*-GAG-GTG-TTG-GAA-3′, nucleotides 1443–1463), and *SAP97 long* antisense 2 (5′-CCA-AGT-ATG-AAG-ACG-GGC-TAA-CA-3′, nucleotides 1738–1760); *SAP97 short*, restriction enzyme, Xho I, *SAP97 short* sense 1 (5′-ATC-TCA-CCC-ATA-AAG-GCA-AAT-C-3′, nucleotides 920–934 flanked with 1034–1040), *SAP97 short* antisense 1 (5′-GTT-TCC-AAC-ACC-TCG*-AGC-AAT-G-3′, nucleotides 1444–1465), *SAP97 short* sense 2 (5′-GCA-TTG-CTC*-GAG-GTG-TTG-GAA-3′, nucleotides 1443–1463), and *SAP97 short* antisense 2 (5′-CCA-AGT-ATG-AAG-ACG-GGC-TAA-C-3′, nucleotides 1739–1760). The amplified DNA fragments were cloned using a TA cloning vector (pGEM-T Easy vector system, Promega, Madison, WI).

In the competitive RT-PCR assay, the total RNA samples from the individual animals were analyzed separately and identical aliquots of cDNA were spiked with a dilution series of a competitor template that was identical to the target except for a point mutation generating a restriction site and was amplified by the same PCR primers as the target template (sense 1 and antisense 2 primers for each gene). The PCR parameters were 94 °C for 3 min, 50 °C for 2 min, 72 °C for 5 min for the first cycle, 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s with 30 cycles, and then 72 °C for 5 min for complete extension. Because the PCR products form a hetero-double strand composed of a target and a competitor strand that are not cut by the restriction enzyme when PCR amplification has reached its saturation ("plateau phase") (Becker-Andre and Hahlbrock, 1989), we carefully chose the number of PCR cycles so that the reaction is in the "exponential phase" where essentially every DNA molecule is amplified and forms homodimeric DNA species according to the method of Becker-Andre and Hahlbrock (1989). The resulting PCR products of the target and mutated sequence were identified by restriction analysis. The digested PCR products were separated by electrophoresis on 3% NuSive agarose gel in 1× TAE (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA). The gel was stained with 0.25 μg/ml of ethidium bromide for 30 min. The resulting DNA bands were visualized by ultraviolet irradiation for 500 ms and quantitatively analyzed by a Lumi-Imager chemiluminescence detector. The amount of the target was estimated as the copy number of the competitor sequence per one microgram of total RNA that leads to a 1:1 ratio of the target to competitor amplicons. The reliability of the competitive RT-PCR analysis was established by comparing its results with those obtained by our quantitative RT-PCR co-amplifying another endogenous template, 28S ribosomal RNA, as the internal standard in the study of neocortical expression of another developmentally-regulated shizophrenomimetic-responsive gene *mrt1* (Kajii et al., 2003). The accuracy of the present competitive RT-PCR assay appears to be supported by the similar values for the basal expression of SAP97 mRNAs (in the neocortex of saline-treated rats) and by the reproducible increases and its magnitudes in the amounts of SAP97 mRNAs after PCP injections among different experiments using the adult rats (see Figs. 3–7).

2.6. Statistics

For comparison between the two groups, the statistical significance of the data was evaluated using the two-tailed Student's *t*-test. Statistical differences among more than three groups were estimated by a one-way analysis of variance (ANOVA) followed by the Fisher PLSD (the homogeneous variance and the same number of animals for each experimental group) or the Kruskal–Wallis test followed by the Scheffé (the heterogeneous variance or the different number of animals for each experimental group) post hoc test for the quantitative analysis of the gene expression. In some developmental differences in the effects of PCP were also examined by a two-way ANOVA followed by the Scheffé post hoc test. We confirmed by Barlett test that the data sets analyzed with the two-way ANOVA had the heterogeneous variance.

2.7. Nucleotide sequences

The DDBJ/GenBank/EMBL accession number for primary nucleotide sequence of rat *SAP97* is NM_012788.

3. Results

3.1. Detection of *SAP97* as a developmentally-regulated PCP-responsive gene

From the expressional signal intensity data obtained from the RAP-PCR experiments using the rhodamine-labelled 12-mer arbitrary primer set following PCP or saline treatment in the infant and adult mice, we screened four cDNA bands that are upregulated (intensified) 1 h after PCP treatment (5 mg/kg) in the adult, but not infant, period (Fig. 1). The sequence analyses revealed that these bands corresponded to the transcripts of genes for VGF, myelin-associated oligodendrocytic basic protein (MOBP), glutamate receptor interacting protein (GRIP) and 97 kDa synapse-associated protein (*SAP97*: *DLG1*). Because PCP appeared to induce the most prominent changes in the apparent signal intensity of the cDNA bands of the *SAP97*-like transcript (Fig. 1), we presently characterized the structural, developmental and pharmacological properties of the transcript. In the filtering procedure for the candidate gene using the RNA fingerprinting, the *SAP97* transcripts were detected as the PCR products of 503 bp and have been shown to encode PDZ domains containing proteins (Fig. 2). *SAP97* was originally isolated from the post-synaptic density (Müller et al., 1995) and has been recently shown to interact with subunits of the alpha-amino-3-hydroxyl-5-isoxazolepropionate (AMPA), kainate and NMDA type glutamate receptors and other synaptic proteins by their various domains (Rumbaugh et al., 2003; Mehta et al., 2001; Müller et al., 1995; Regalado et al., 2006; Wang et al., 2005). The *SAP97* transcripts consist of at least two types of splicing variants

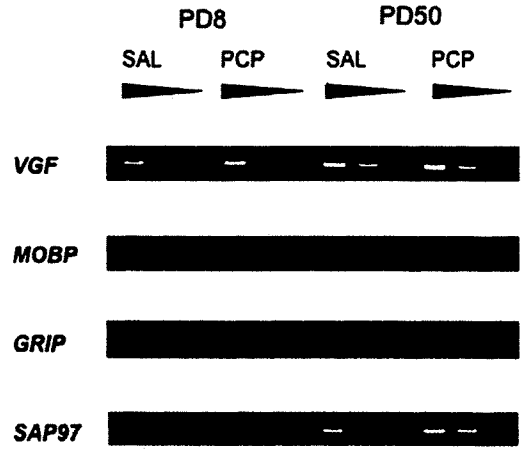

Figure 1 Screening of developmentally-regulated PCP-responsive transcripts in the rat neocortex by RNA fingerprinting. Detection of the cDNA bands for developmentally-regulated and PCP-responsive transcripts by RNA fingerprinting using arbitrarily-primed PCR from the neocortex of the adult (PD 50) and infant (PD 8) rats treated with saline or PCP. Increasing amounts of the neocortical cDNA template (2.5–10 µl) were amplified in each group. Each cDNA band contains the A6021 sequence of each transcript that is differentially regulated by PCP and development. Abbreviations: *VGF* (not an acronym: Salton et al., 1991); *MOBP*, myelin-associated oligodendrocytic basic protein (Yamamoto et al., 1994); *GRIP*, glutamate receptor interacting protein (Dong et al., 1997); *SAP97*, synapse-associated protein 97 (Müller et al., 1995).

with different open reading frames, which are designated as the long- and short-form mRNAs (Fig. 2). The predicted amino acid sequence for analysis of the cloned *SAP97* cDNA indicates that the rat *SAP97* gene encodes at least two types of the putative scaffold protein *SAP97* with three PDZ domains, a LIN-2/7 (L27) domain, an Src homology 3 (SH3) domain and GK-domain (Müller et al., 1995).

The effects of various drugs and postnatal development on the cerebral *SAP97* gene expression were examined by a variant-specific and quantitative RT-PCR. Variant-specific PCR amplification was performed using the primer sets shown in Fig. 2. As indicated in Fig. 2, the upper specific primers for the respective *SAP97* gene transcripts, which recognize the splicing boundaries, were designed to enable the selective amplification of the long-form specific 839-bp and the short-form specific 742-bp PCR products, respectively, in combination with the common lower primers. The mRNA expression levels were quantified by a competitive RT-PCR method described in the Experimental procedures section.

3.2. Developmental changes in basal and PCP-induced expression of *SAP97* transcripts in the neocortex of the rats

In the neocortical tissues of the developing rats, PCP produced an augmentation in the amount of the long and short forms of the *SAP97* mRNAs at PD 50, but not at PD 8 (Fig. 3). These results, obtained by quantitative assays, confirmed the differential responses of the *SAP97* transcripts to PCP between PD 8 and 50 as revealed by the RNA fingerprinting analysis.

Unlike the response to PCP, the long and short forms of the *SAP97* transcripts exhibit different ontogenic patterns in their amounts following saline injection in the neocortex (Fig. 3), which are considered as nearly basal levels. In the saline-treated rats, there were no significant changes in the neocortical contents of the long-form *SAP97* mRNA between PD 8 and PD 50, while the short-form *SAP97* mRNA at PD 50 showed a much higher expression level than that at PD 8.

3.3. Effects of acute administration of various drugs on expression of *SAP97* transcripts in the neocortex of adult rats

After the acute administration of PCP (5 mg/kg, s.c.), the contents of the long (Fig. 4A) and short (Fig. 4B) form *SAP97* mRNAs in the neocortex of 50-day-old rats rapidly increased, peaked at 1 h, and returned to the saline-treated control levels within 2-h post-injection. Therefore, we routinely measured the *SAP97* mRNA 1 h after the acute injection of PCP. However, the acute administration of another schizophrenomimetic methamphetamine (4.8 mg/kg, s.c.) that is an indirect dopamine agonist did not affect the long (Fig. 4A) and short (Fig. 4B) form *SAP97* mRNA expressions 1, 3, 6 and 24 h thereafter.

In the adult animals, the elevating effect of the acute subcutaneous administration of 10 mg/kg of PCP on the neocortical levels of the two types of *SAP97* transcripts exceeded that of 5 mg/kg PCP although this dose-related increase is not statistically significant (Fig. 5A,B). This rank order potency is similar to that which causes the NMDA receptor blocking-related consequences such as abnormal behavior consisting of hyperactivity, stereotypy and ataxia (Tanii et al., 1991, 1994) and facilitation of the cortical dopamine neurotransmission (Umino et al., 1998).

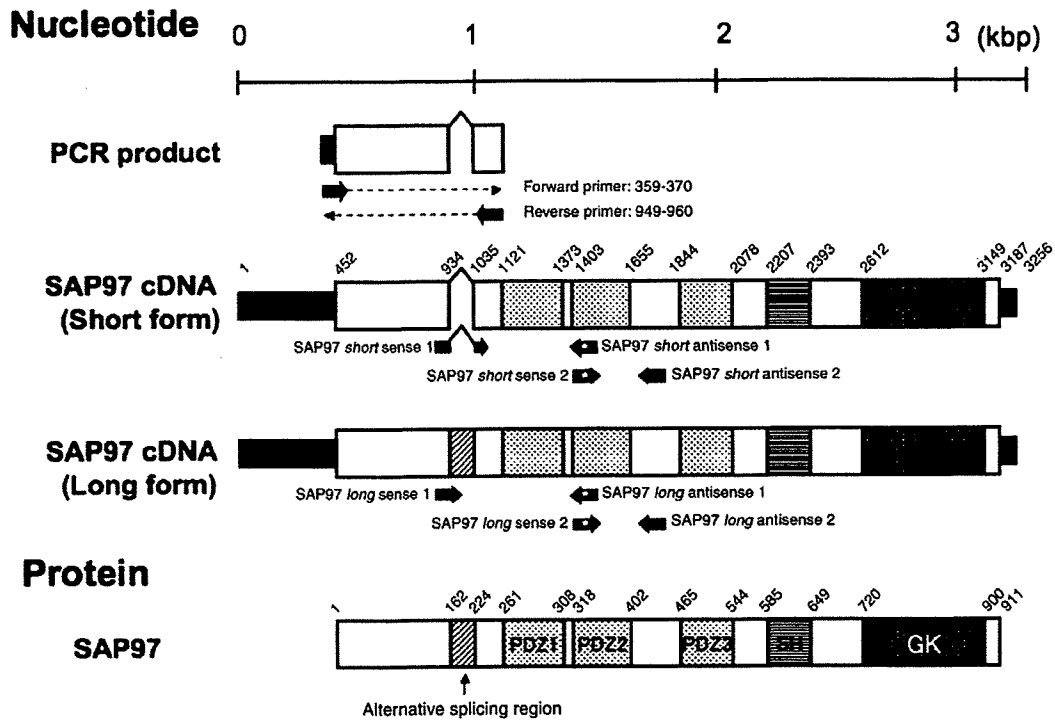

Figure 2 Schematic representation of structures of *SAP97* cDNAs for the RAP-PCR product, the long and short forms of mRNA, and of the *SAP97* proteins. The broader boxes illustrate the predicted coding regions. The alphabet letters in the boxes denote the functional domains found in the *SAP97* proteins. Each primer is shown as a closed small arrow with its name as described in the Experimental procedures section. An asterisk included in an arrow represents the mutated base for the restriction site for Xho I. The 5'-primer of the primer set for the long- or short-form mRNA is designed to recognize the splicing boundary so that this primer set will specifically amplify the long- or short-form *SAP97* mRNA-specific fragment of 839 bp or 742 bp, respectively. The figures above the boxes indicate the nucleotide or amino acid number of the deduced *SAP97* cDNAs or proteins, respectively.

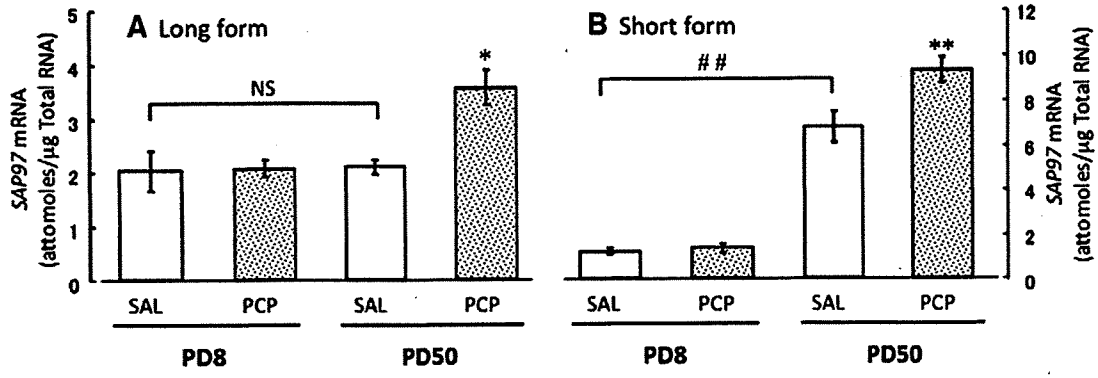

Figure 3 Developmental changes in PCP-induced expression of *SAP97* transcripts in the neocortex of the rats. An increase in the levels of the long (A) and short (B) form *SAP97* transcripts was observed 1 h after the s.c. injection of 5 mg/kg of PCP in the neocortex of the 50-day-old, but not 8-day-old animals. The results are the means with S.E.M. of data obtained from 4 to 5 (long form) or 5 (short form) animals. $^{*}P<0.05$, $^{**}P<0.02$ vs. respective saline-treated controls (SAL) (Schffé test). $\#\#P<0.01$ between the two groups linked by a solid line (Schffé test). Percentages of respective control values (SAL): short form, adult rats (PD 50), saline 100±7%, PCP 169±15%; infant rats (PD 8), saline 100±18%, PCP 102±8%; long form, adult rats (PD 50), saline 100±10%, PCP 138±8%; infant rats (PD 8), saline 100±11%, PCP 112±17%. Two-way ANOVA: short form, postnatal days effect, $F1, 15=7.684$, $P<0.02$; PCP treatment effect, $F1, 15=7.083$, $P<0.02$; postnatal days×PCP treatment effect, $F1, 15=6.432$, $P<0.03$; long form, postnatal days effect, $F1, 15=208.814$, $P<0.0001$; PCP treatment effect, $F1, 15=8.518$, $P<0.02$; postnatal days×PCP treatment effect, $F1, 15=6.778$, $P<0.02$.

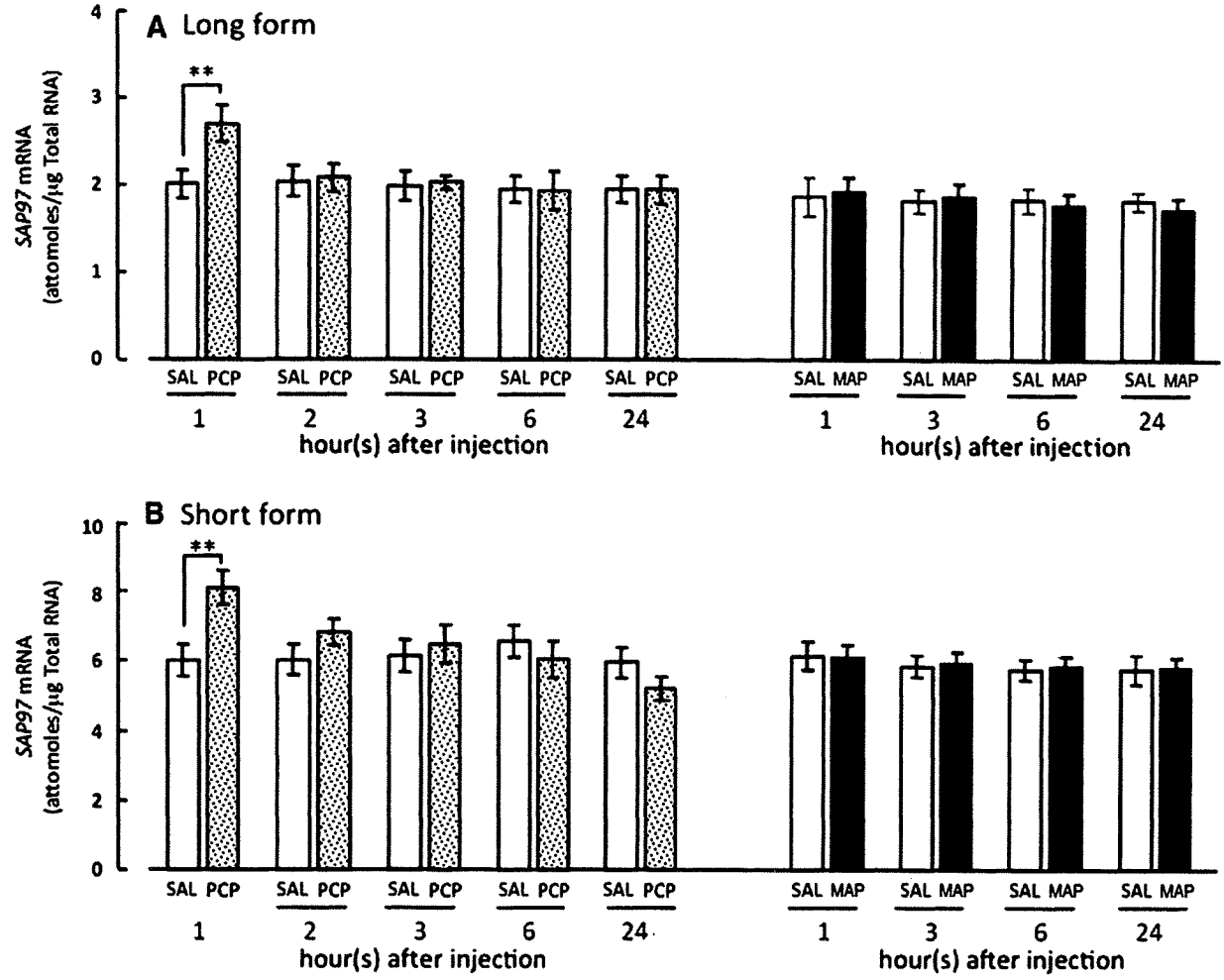

Figure 4 Time course of effects of acute injection of PCP and MAP on neocortical *SAP97* gene expression. Results are the means with S.E.M. of data obtained from 7 to 10 animals. (A) The long-form *SAP97* mRNA expression: The acute PCP-induced increase in copies of the long-form *SAP97* mRNA peaked at 1 h and disappeared within 2 h after the injection, while the acute MAP injection failed to change the *SAP97* gene expression throughout the total examined hours. $^{**}P<0.01$ vs. respective saline-treated controls. (B) The short-form *SAP97* mRNA expression: The acute PCP-induced increase in copies of the short-form *SAP97* mRNA peaked at 1 h and disappeared within 2 h after the injection, while the acute MAP injection failed to change the *SAP97* gene expression throughout the total examined hours. $^{**}P<0.01$ vs. respective saline-treated controls (SAL).

Furthermore, the neocortical expression of the long- (Fig. 6A) and short-form (Fig. 6B) *SAP97* mRNAs was upregulated by a non-competitive NMDA receptor antagonist, dizocilpine (DIZ, 0.5 and 1 mg/kg, s.c.), but not by an indirect dopamine agonist, cocaine (COC, 30 mg/kg, s.c.), an anesthetic with abuse potential, pentobarbital (PB, 40 mg/kg, intraperitoneally (i.p.)), a selective D1 dopamine receptor antagonist, SCH23390 (SCH, 0.5 mg/kg, s. c), and a D2 receptor-preferring antagonist, haloperidol (HAL, 1 mg/kg, s.c).

3.4. Effects of the pretreatment with haloperidol on PCP-induced increase in *SAP97* transcripts in the neocortex of adult rats

Because a part of the biochemical and behavioral effects of the NMDA antagonist, PCP, has been shown to be mediated by the secondarily augmented cerebral dopamine neurotransmission and considered to underlie the antipsychotic-sensitive schizophrenia-like positive symptoms, the effects of a typical antipsychotic, haloperidol, on the PCP-induced increase in the *SAP97* mRNA in the neocortex were examined. The pretreatment with haloperidol (HAL, 1.0 mg/kg, i.p.) failed to antagonize the ability of PCP (5.0 mg/kg, s.c.) to upregulate the neocortical expression of the long (Fig. 7A) and short (Fig. 7B) form *SAP97* transcripts. Haloperidol by itself caused no significant changes in the gene expression. One mg/kg was selected for the haloperidol injection as it is well established that this dose completely eliminates the abnormal behavior and alterations in various gene expressions following the acute administration of dopamine agonists such as methamphetamine (Sakurai et al., 2004; Shintomi, 1975; Shirayama et al., 2000).

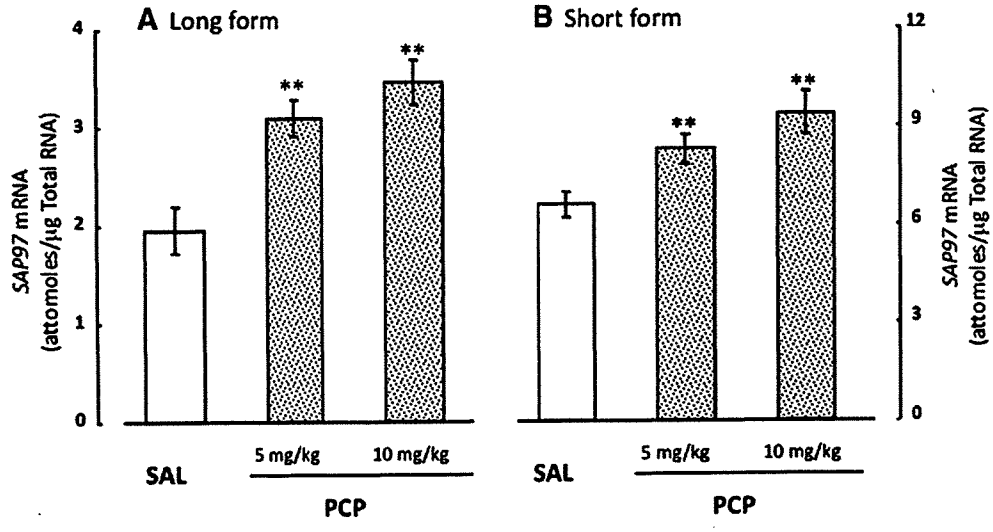

Figure 5 Effects of acute injection of PCP at different doses on neocortical *SAP97* expression. The levels of the long (A) and short (B) form *SAP97* transcripts in the neocortex increased 1 h after the s.c. injection of 5 and 10 mg/kg of PCP. The results are the means with S.E.M. of data obtained from 5 animals. $^{**}P<0.01$ vs. saline-treated controls (SAL).

4. Discussion

In the present study, we revealed by an RNA fingerprinting technique that the *SAP97* gene is developmentally regulated and PCP-inducible in the rat neocortex in that the systemic administration of the schizophrenomimetic PCP causes a prominent increase in the amounts of the two variants, i.e., the long and short forms, of the neocortical *SAP97* transcripts in the young adult rat without affecting those in the infant rat. In the adult rats, a similar upregulation of the *SAP97* gene was observed after the s.c. injection of another psychotomimetic acting as an antagonist for the NMDA receptor, dizocilpine, but not the dopamine agonists, i.e., methamphetamine and cocaine. We further found that the augmenting effects of PCP on the cortical *SAP97* expression was insensitive to the D2 family dopamine receptor antagonist, haloperidol. These pharmacological profiles of the *SAP97* responses suggest that the *SAP97* transcripts and/or proteins could be involved in the non-dopaminergic abnormal behavior that is recognized as a model of the typical antipsychotic-resistant symptoms of schizophrenia.

The two types of the *SAP97* mRNAs, the long and short forms, show different patterns of developmental changes in the basal expression (Fig. 3), but share the development-dependent nature of the PCP-induced expression. The

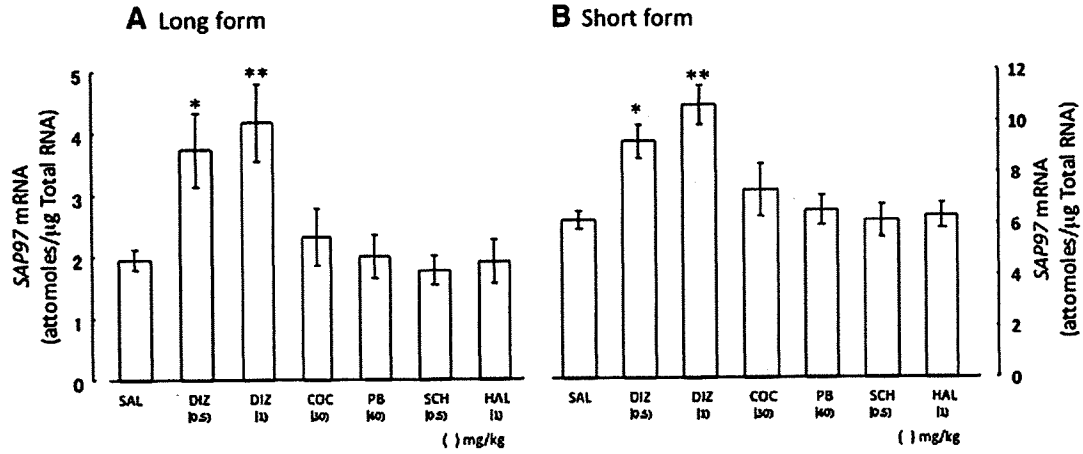

Figure 6 Effects of acute injection of various neuroactive drugs on neocortical *SAP97* expression. The levels of the long (A) and short (B) form *SAP97* transcripts in the neocortex were quantified by the competitive RT-PCR method 1 h after the s.c. administration of saline ($n=22$), dizocilpine (DIZ, 0.5 mg/kg, $n=8$, 1.0 mg/kg, $n=8$), the indirect dopamine agonist, cocaine (COC, $n=6$), D1 antagonist, SCH23390 (SCH, $n=6$), anesthetic, pentobarbital (PB, $n=8$), and D2 receptor-preferring antagonist, haloperidol (HAL, $n=8$). Results are the means with S.E.M. of data obtained from 6 to 22 animals. $^{*}P<0.05$. $^{**}P<0.01$ vs. saline-treated controls (SAL).

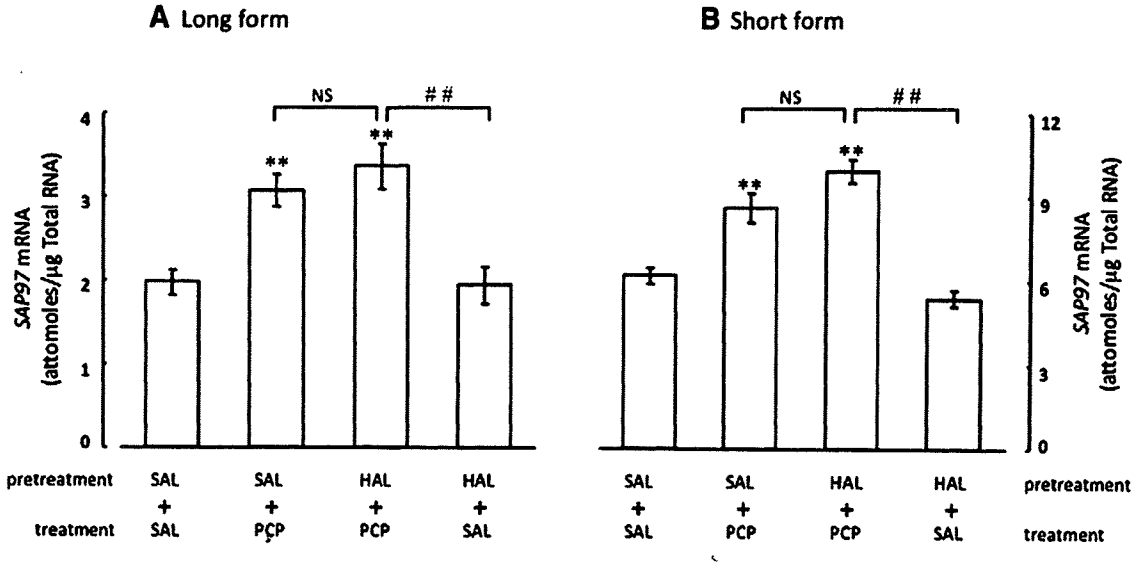

Figure 7 Effects of a typical antipsychotic, haloperidol, on PCP-induced upregulation of *SAP97* expression in the neocortex. The levels of the long (A) and short (B) form *SAP97* transcripts in the neocortex were quantified by the competitive RT-PCR method 1 h after the s.c. administration of PCP. The pretreatment by the D2 preferring antagonist, haloperidol (HAL), 30 min before the PCP injection did not affect the increasing effects of PCP on the amounts of the long and short forms of the *SAP97* transcript. Results are the means with S.E.M. of data obtained from 8 animals. $^{**}P<0.01$ vs. saline-pretreated saline-injected controls (SAL+SAL). $\#\#P<0.01$ between saline-pretreated PCP-injected (SAL+PCP) animals and saline-pretreated saline-injected (SAL+SAL) controls. NS, no significant difference between haloperidol-pretreated PCP-injected (HAL+PCP) and SAL+PCP animals.

ontogenic differences in the brain *SAP97* induction might depend on them during the time course of the pharmacodynamics of PCP or the general responses of the brain. However, this possibility seems to be contrary to the observations that (1) a similar time course of the acute PCP-induced increase in the *c-fos* gene expression was seen in the various brain areas of the rat at PD 8 and 50 (Sato et al., 1997), and (2) PCP caused no change in the 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 (Takebayashi et al., 2009). Moreover, it is unlikely that the *SAP97* gene upregulation could be solely associated with a general signaling pathway to addiction, because other drugs of potential abuse, MAP, cocaine and pentobarbital, do not affect the *SAP97* expression.

The PCP-induced increase in the adult neocortical contents of the long and short forms of the *SAP97* mRNA is more likely to be related to the blocking of the NMDA receptor. Thus, a selective NMDA antagonist, dizocilpine, mimicked the elevating effects of PCP on the levels of the two variants of the *SAP97* mRNAs. These augmentations could be due to the secondarily accelerated cortical dopaminergic transmission following the systemic administration of the NMDA receptor antagonists (Hata et al., 1990; Nishijima et al., 1994, 1996; Tanii et al., 1990; Umino et al., 1998). However, this possibility appears to be denied by the present observations that the systemic administration of the indirect dopamine agonists, methamphetamine and cocaine, with the actions on the noradrenaline and serotonin reuptake and/or release failed to affect the *SAP97* expression in the neocortex of the 50-day-old rats 1 or 1–24 h thereafter. The dopamine-independency is further supported by the insensitivity of the PCP-induced *SAP97* upregulation to the pretreatment with 1 mg/kg of haloperidol that sufficiently antagonizes the behavioral and biochemical changes by overactivation of the D2 dopamine receptor (Sakurai et al., 2004; Shintomi, 1975; Shirayama et al., 2000).

The early and transient induction of the neocortical *SAP97* by the small to moderate and psychotomimetic dose (5 mg/kg) of PCP (Fig. 3) seems to differ from the previously reported changes in the cerebral *SAP97* expression by the much higher and anesthetic doses of the NMDA receptor antagonists (Linden et al., 2001), because 1) the PCP and dizocilpine injection at 15 and 5 mg/kg, respectively, caused a significant increase in the *in situ* hybridization signal of the *SAP97* mRNAs in the entorhinal cortex, but not in the neocortical areas at 4 h post-injection (Linden et al., 2001) when the acute administration of 5 mg/kg of PCP no longer altered the *SAP97* expression in the neocortex in the present study, and 2) there was a significant reduction in the *SAP97* mRNA expression in the superficial layers of the parietal cortex (the neocortical areas) 4 h after the single application of 5 mg/kg of dizocilpine (Linden et al., 2001). These relatively delayed effects by the high amounts of the NMDA antagonists on the *SAP97* expression might be associated with the irreversible toxicities of these drugs. Indeed, neuronal necrosis was observed in the posterior cingulate/retrosplenial cortex after the single administration of dizocilpine of 5 mg/kg and more, but not by 1 mg/kg or less (Fix et al., 1993). It should be noted that the vulnerability to the NMDA receptor-related neuronal damage was found to be age-dependent, having an onset at approximately adolescence (45 days of age) and becoming

maximum in the early adulthood of the rat (Farber et al., 1995), because, for the study of the pathophysiology of the schizophrenia-type psychosis, it would be highly relevant to clarify the exact relationship between this vulnerability and the psychotomimetic actions of the NMDA receptor antagonists.

The upregulation of the neocortical *SAP97* gene transcription by PCP and dizocilpine at the doses causing no apparent neuronal degeneration in the present study may be related to the functional changes in the NMDA receptor-linked signal cascades that underlie the behavioral modifications by these NMDA antagonists. The direct connection between the *SAP97* and NMDA receptor is evidenced by the binding of the first and second PDZ domains of SAP97 with the cytoplasmic C terminus of the NR2A and NR2B subunits of the NMDA receptor (Niethammer et al., 1996; Klocker et al., 2002; Wang et al., 2005). Through these bindings, *SAP97* has been considered to play important roles in the proper trafficking and cell surface maintenance of the NMDA receptor (Lynch and Guttmann, 2001; Mori et al., 1998; Sprengel et al., 1998) and the associated synaptic functions (Regalado et al., 2006). Remarkable alterations in the regional distribution of the brain NR2A and NR2B subunits during the rodent lactation period (Watanabe et al., 1992; Portera-Cailliau et al., 1996; Wenzel et al., 1997), therefore, could explain the differences in the PCP-induced *SAP97* gene expression between the infant and adult periods of the rat. However, there has so far been no evidence for developmental changes in the binding features of the *SAP97* proteins to the NMDA receptor subunits. Because *SAP97* also binds to the AMPA- and kainate-type glutamate receptor that mutually interacts with the NMDA receptor (Mathé et al., 1998; O'Brien et al., 1998), it cannot be excluded that PCP and dizocilpine might secondarily influence the control of the AMPA or kainate receptor-associated *SAP97* after blocking the NMDA receptor. This possibility appears to be supported by the inhibition by an AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione, of the ability of dizocilpine to produce hyperlocomotion and accumbal dopamine release (Mathé et al., 1998).

The developmentally-regulated, NMDA receptor-connected, dopamine agonist-unrelated and haloperidol-insensitive nature of the PCP-induced upregulation of the neocortical *SAP97* gene expression appears to be similar to those of the abnormal behavior and psychosis caused by a variety of NMDA antagonists as a model of schizophrenia (Javitt and Zukin, 1991), suggesting that *SAP97* and its protein could compose the molecular cascades that are dysregulated in schizophrenia. This view is likely to be in line with the observations by Toyooka et al. (2002) that there was a marked reduction in the amounts of SAP97 protein in the prefrontal cortex (neocortex), but not in the hippocampus (allocortex), of the postmortem brains from schizophrenic patients and that repeated treatment with high doses of haloperidol (1.5 mg/kg/day, i.p.) for 14 days failed to affect the frontal cortical and hippocampal levels of *SAP97*. The region-selective changes in the SAP97 protein expression could be related to the subsequent report by Dracheva et al. (2005) that *SAP97* mRNA levels were not significantly altered in the dorsolateral prefrontal cortex and the occipital cortex. Furthermore, we have recently shown that some SNPs and two- and three-SNP haplotypes of the human *SAP97* gene are significantly associated with male schizophrenia (Sato et al., 2008), although the regions of the human genome in which *SAP97* is located (3q29) have not been suggested to be associated with an altered risk of schizophrenia. Taken together, the dysfunction of *SAP97* could be involved in the pathophysiology of schizophrenia by causing a disorganization of the NMDA, AMPA and kainate receptor system that has been well described in the postmortem schizophrenic brains (Nishikawa et al., 1983; Deakin et al., 1989; Kerwin et al., 1988; Meador-Woodruff and Healy, 2000). However, this view appears to be challenged by the observation by Dracheva et al. (2005) that there was no difference in SAP97 mRNA expression between elderly schizophrenic patients and controls in the dorsolateral prefrontal cortex and the occipital cortex.

It is also interesting to further investigate the possible relationship between schizophrenia and the VGF, GRIP or MOBP gene each of which was detected as a candidate for the developmentally-regulated PCP-inducible genes by our finger printing method although the competitive RT-PCR analysis to characterize the expression of these transcripts was not performed in this study. In fact, the CSF (cerebrospinal fluid) contents of VGF protein, a nervous system-selective, nerve growth factor- and plasticity-related molecule (Levi et al., 2004), have been reported to be altered in the patients' prodromal for psychosis (Salton et al., 1991; Huang et al., 2007). The mRNA expression levels of an oligodendrocyte-related gene, MOBP (Yamamoto et al., 1994), were found to differ between schizophrenic patients with a history of substance abuse and controls in the dorsolateral prefrontal cortex white matter (Mitkus et al., 2008). Moreover, Dracheva et al. (2005) demonstrated in the studies using postmortem brains that the mRNA expression of GRIP, a synaptic PDZ domain-containing protein that interacts with AMPA receptors (Dong et al., 1997), was significantly elevated in the dorsolateral prefrontal cortex and occipital cortex in schizophrenics.

In conclusion, the present findings demonstrate the schizophrenomimetic PCP induction of the neocortical *SAP97* gene transcript and its development- and NMDA receptor-regulated properties. Further studies of the functional, neuroanatomical and developmental features of *SAP97* and their regulatory mechanisms will, thus, provide a useful clue for the molecular basis of the onset and the antipsychotic-resistant symptoms of schizophrenia or related mental disorders.

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Contributors

S. Hiraoka mainly performed the present experiments, wrote their protocols and the preliminary draft of the manuscript and figures. Y. Kajii developed the method for the mRNA arbitrarily-primed PCR

used in this study. Y. Kuroda and A. Umino achieved a part of this series of experiments and A. Umino also undertook the statistical analysis of the data. T. Nishikawa conceived, designed and guided this project, and wrote the final version of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

We do not have a financial interest/arrangement of affiliation with any organization that could be perceived as a real or apparent conflict of interest of the subject of this paper.

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ARTICLE

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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 D_2-preferring 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.

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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).

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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 [*CCN1* = 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 (Andreasen *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

PCP 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. 2*b* 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 manifacturer'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*

Table 1. Primer sequences for the real-time and semi-quantitative RT–PCR

Official symbol of gene	Genbank accession no.	Bases spanned	Sense primer (5′ to 3′)	Antisense primer (3′ to 5′)
Real-time quantitative PCR				
Lmod2	AB331240	1545–1645	5′-AATGGAAGAAAGCTCCCGG-3′	5′-CCACCCTCCGTAGCTGTCTTAT-3′
Tmod1	NM_013044	2330–2437	5′-GACAGTGATGGCTCAAAAGCTG-3′	5′-CGATGCCACTCTAGTTACCCTG-3′
Tmod2	BF567833	116–243	5′-CGTTTCCGTTAACCATATCCG-3′	5′-TGTACAAGCCACCAAGCGTGT-3′
GAPDH	NM_017008	1457–1714	5′-ACATCATCCCTGCATCCACT-3′	5′-GGGAGTTGCTGTTGAACTCA-3′
Semi-quantitative RT–PCR				
Lmod2	AB331240	19–1668	5′-ATGTCTACATTTGGCTACAGAAGGGG-3′	5′-CTATCTCAGAGCTTCGGGAACTTC-3′

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 (*Lmod2*), for 26 cycles (*Tmod1*), for 25 cycles (*Tmod2*), and for 22 cycles (*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.

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 *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 *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.

Internal standard housekeeping gene for Northern blot and RT–PCR analyses

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 *Lmod2* gene by RT–PCR techniques.

In-situ *hybridization*

In-situ hybridization histochemistry was performed on coronal or sagittal brain sections according to the methods of Kiyama's research group. For *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 *Lmod2* mRNA signals following the pre-hybridization and hybridization procedures (see legend of Fig. 4*c–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

Gene name	Genbank accession no.	PD 50		PD 8	
		Log_2 ratio (% of control)	Change p value	Log_2 ratio (% of control)	Change p value
Fos: FBJ murine osteosarcoma viral oncogene homolog	BF415939	2.1 (428)	0.00002	0.2 (115)	0.35
Klf2_predicted: Kruppel-like factor 2 (lung) (predicted)	BF288243	1.6 (303)	0.00002	0.2 (115)	0.5
Nr4a3: Nuclear receptor subfamily 4, group A, member 3	NM_031628.1	1.4 (264)	0.00002	0.1 (107)	0.5
Klf4: Kruppel-like factor 4 (gut)	NM_053713.1	1.4 (264)	0.00002	−0.3 (77)	0.5
LOC296935: Similar to leiomodin 2 (cardiac)	AI453854	1.1 (214)	0.000214	Absent	

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 [α-^{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 μg/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. 4*b* 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-fold (200% 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.

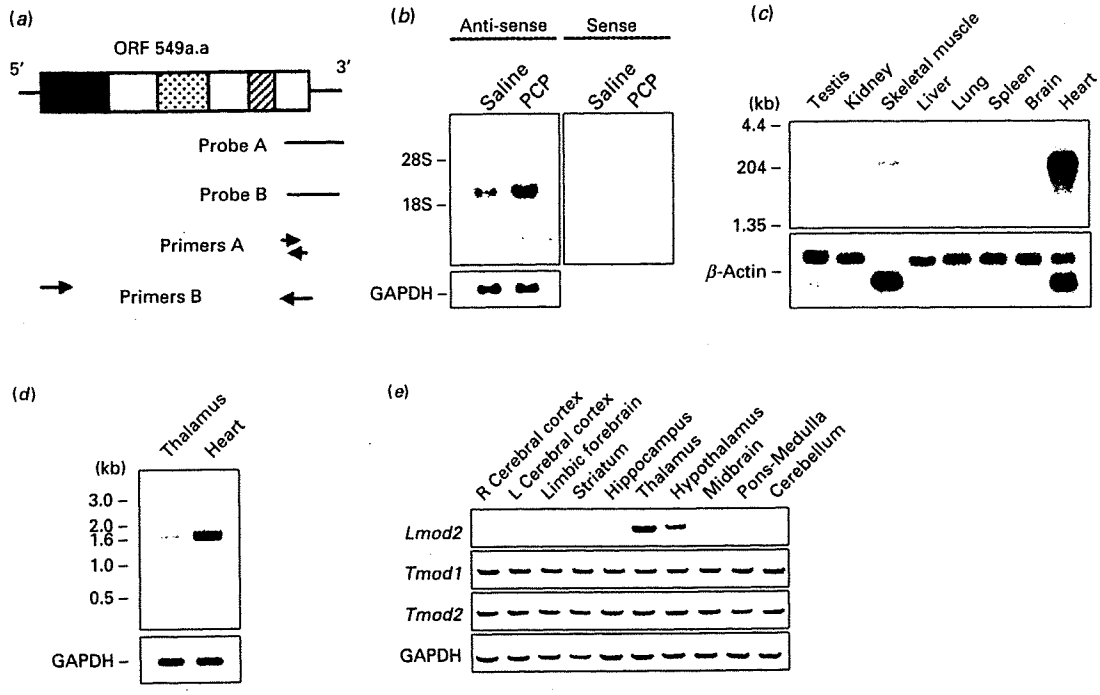

Fig. 1. Cloning and expression of rat *Lmod2*. (*a*) Schematic representation of the structure of rat *Lmod2* cDNA and the nucleotide sequences encoding the open reading frame (ORF). Domains and motifs: closed box, possible tropomyosin-binding domain; dotted box, leucine-rich repeats; hatched box, polyproline motif. Bars and arrows underneath indicate the positions of probes and primers used for the following purposes of experiments: probe A, microarray hybridization (probe name: 1377610_at, ID: RAG230_2_10158); probe B, Northern hybridization and *in-situ* hybridization; primer set A, real-time RT–PCR; primer set B, isolation and determination of the ORF of rat *Lmod2*. (*b*) Northern blot analyses of the poly(A)$^+$ RNAs (2 μg/lane) from the thalamus of the adult rat treated with saline or PCP (7.5 mg/kg s.c.). Hybridization with a selective probe to *Lmod2* mRNA against the sequence of nt 1549-1883 [probe B in panel (*a*)] recognized a single band in each lane. (*c*) Tissue distribution of *Lmod2* mRNA analysed by Northern blot of poly(A)$^+$ RNAs (2 μg/lane) from various organs of the adult rat with probe B in panel (*b*). The signal detected in the rat heart and skeletal muscles was the same size as that detected in the thalamus. (*d*) Semi-quantitative RT–PCR analysis of basal *Lmod2* mRNA expression in the thalamus and heart of the adult rat. The same PCR products, which were amplified by primer B in panel (*a*), were detected at 1.6 kb in both the thalamus and heart. (*e*) Semi-quantitative RT–PCR analysis of the basal expression of *Lmod2*, *Tmod1*, *Tmod2* and GAPDH mRNAs in the discrete brain areas of the adult rat. The positions of primer sets used for the amplification of cDNA are described in detail in Table 1.

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. 1*a*). 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. 1*a*), 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 reserved 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).

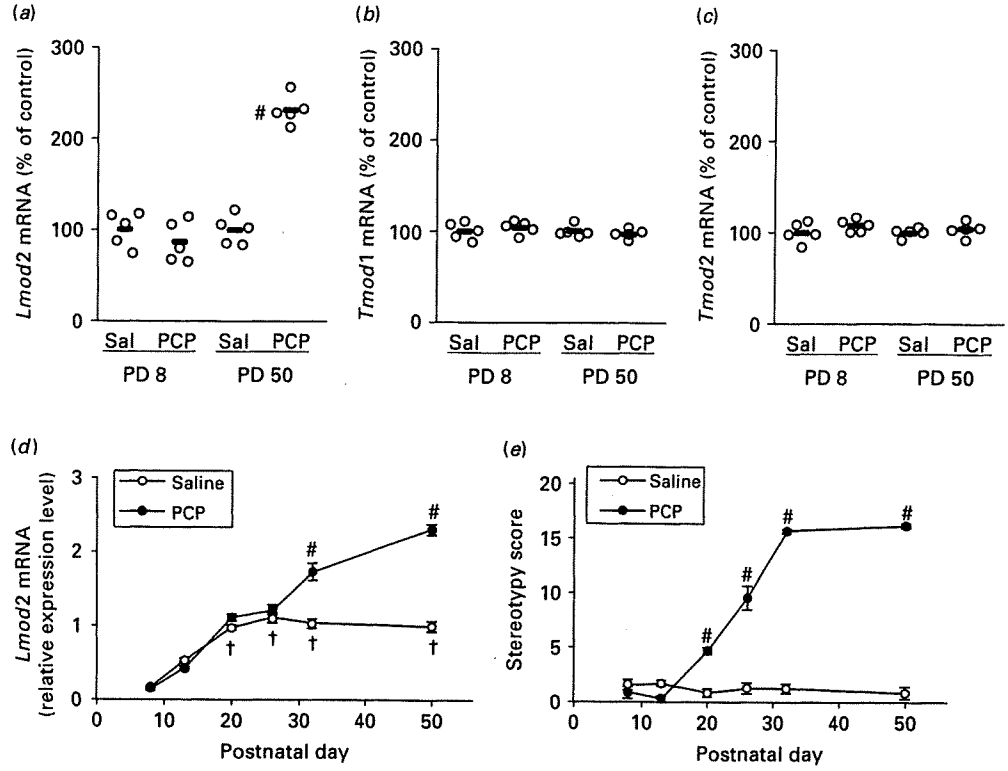

Fig. 2. Effects of acute PCP injection on expression of *Lmod2*, *Tmod1* and *Tmod2* mRNAs in the thalamus and behaviour in developing rats. Relative expression levels of *Lmod2*, *Tmod1* and *Tmod2* mRNAs (*Lmod2*, *Tmod1* or *Tmod2* to GAPDH mRNA ratio) were determined by the real-time RT–PCR method 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: $F_{1,16}=76.878$, $p<0.0001$; PCP treatment effect: $F_{1,16}=51.066$, $p<0.0001$; postnatal days × PCP treatment effect: $F_{1,16}=77.456$, $p<0.0001$). (*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: $F_{1,16}=0.981$, $p=0.34$; PCP treatment effect: $F_{1,16}=0.047$, $p=0.83$; postnatal days × PCP treatment effect: $F_{1,16}=0.944$, $p=0.35$). (*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: $F_{1,16}=0.241$, $p=0.63$; PCP treatment effect: $F_{1,16}=2.546$, $p=0.13$; postnatal days × PCP treatment effect: $F_{1,16}=0.228$, $P=0.64$). (*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 2 μg 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. 1*b*). 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. 1*c*). 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. 1*a*), 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. 1*d*). 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. 1*e*).

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. 2*a*, 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. 2*b*) and *Tmod2* (Fig. 2*c*), 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. 2*a*) 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. 2*d*).

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. 2*e*). 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. 3*a*). 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. 3*a*, *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. 4*a*). 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. 4*b*, left panel). Acute PCP treatment (7.5 mg/kg s.c.) augmented thalamic *Lmod2* expression without apparent influence on its basal distribution pattern (Fig. 4*b*, 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. 4*c*, *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

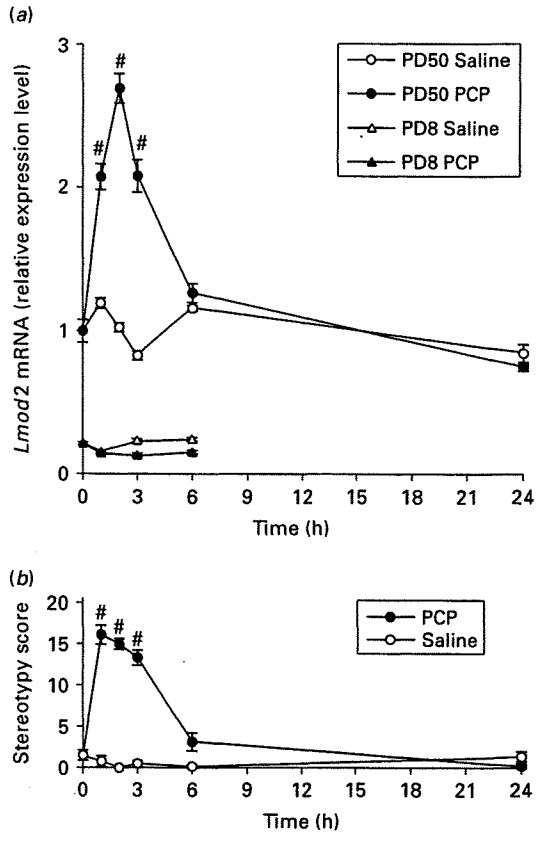

Fig. 3. Time-course of thalamic *Lmod2* mRNA expression and behavioural changes after acute PCP injection in adult or infant rats. (*a*) Relative expression levels of *Lmod2* mRNA in the thalamus of adult (PD 50) and infant (PD 8) rats (*Lmod2*:GAPDH mRNA ratio) were assayed by real-time RT–PCR at various times after acute PCP administration (7.5 mg/kg s.c.). The expression levels at time 0 were determined in the animals prior to the treatment. 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). (*b*) Stereotypy scores after acute PCP administration. The cumulative behavioural scores as the summation of each 10-min score are evaluated by the rating scale described in the legend of Fig. 2*b* and shown as the means with S.E.M. of the data obtained from six rats in each group ($^{\#}p<0.01$ *vs.* respective saline-treated controls).

feature with a characteristic large round-shaped nucleus (Fig. 4*e*). A more sensitive *in-situ* hybridization detection system with a ^{35}S-labelled anti-sense RNA probe revealed that strong hybridization signals of *Lmod2* mRNA were predominantly observed in the rostral areas of the thalamus including the anterior thalamic complex [i.e. the anteromedial (AM), anteroventral (AV), and inter-anteromedial (IAM) nucleus], and the ventral anterior-lateral complex (Fig. 4*f–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. 4*m*).

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

Because *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 *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 *Lmod2* mRNA seen following PCP administration (7.5 mg/kg s.c.) (Fig. 5*a*). An indirect dopamine agonist, methamphetamine (MAP, 4.8 mg/kg s.c.) produced a smaller significant increase in *Lmod2* mRNA levels than PCP and MK-801 (Fig. 5*a*).

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 *Lmod2* mRNA. Pretreatment with haloperidol (1.0 mg/kg i.p.) 30 min before PCP injection partially antagonized the ability of PCP to augment *Lmod2* mRNA expression (Fig. 5*b*). Hal, by itself, showed no significant effect on *Lmod2* mRNA expression.

Discussion

By using a DNA microarray technique, we show that *Lmod2* is a developmentally regulated and PCP-regulated gene in the rat thalamus in that a schizophrenomimetic respectively causes no and prominent up-regulation of thalamic *Lmod2* gene expression in infant (PD 8) and young adult (PD 50) rats. The significant increase in thalamic *Lmod2* mRNA expression after PCP administration is observed only after PD 32. We further demonstrate that the basal and PCP-induced expression of *Lmod2* is confined to the anterior and lateral nuclei of the thalamus in the brain. The up-regulation of thalamic *Lmod2* transcripts in the

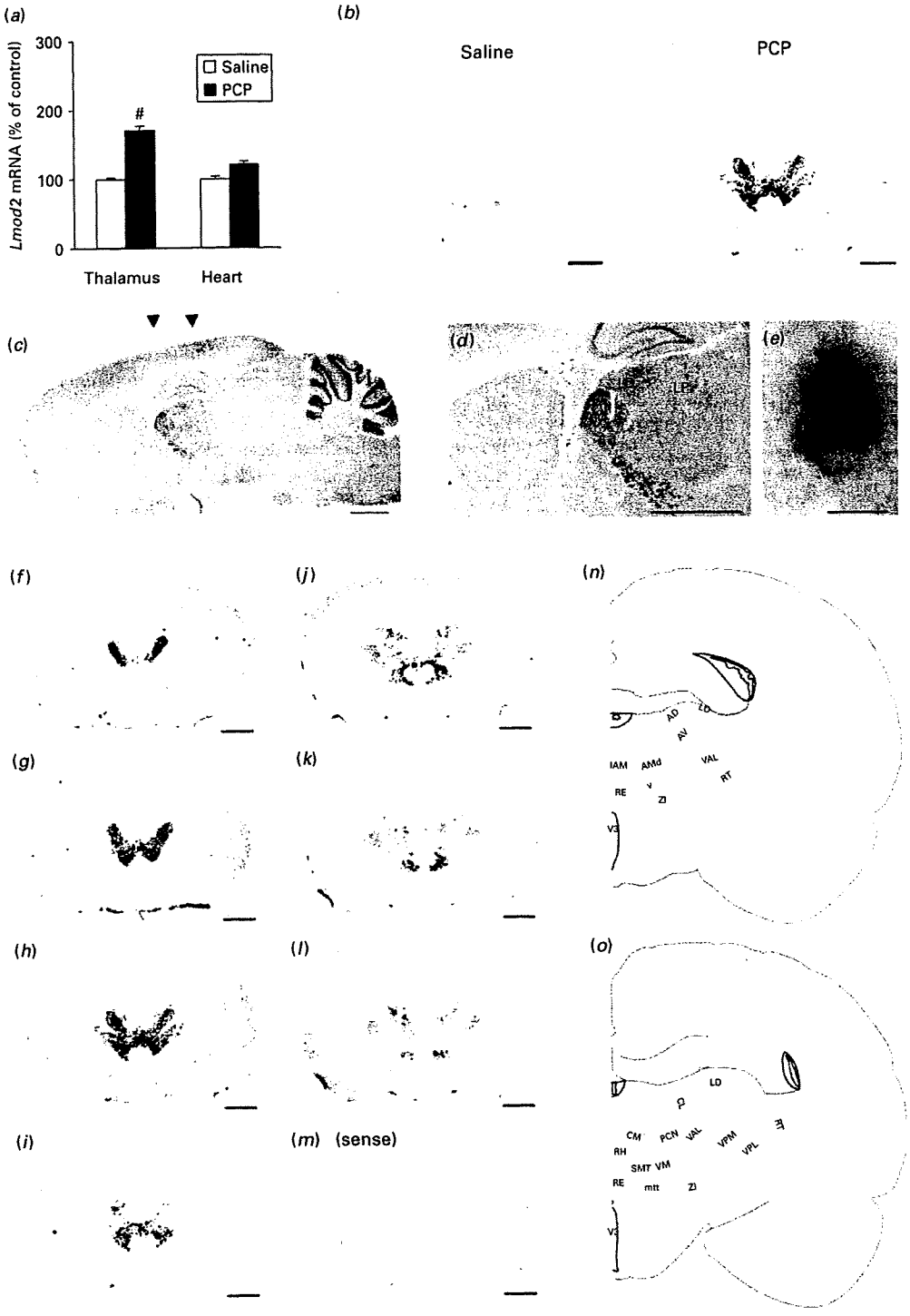

Fig. 4. Effects of acute PCP injection on *Lmod2* mRNA expression in the heart and brain regions of adult rats. (*a*) Relative expression levels of *Lmod2* mRNAs (*Lmod2*:GAPDH mRNA ratio) were determined by the real-time RT–PCR method in the thalamus and heart of the adult (PD 50) rat 60 min after administration 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