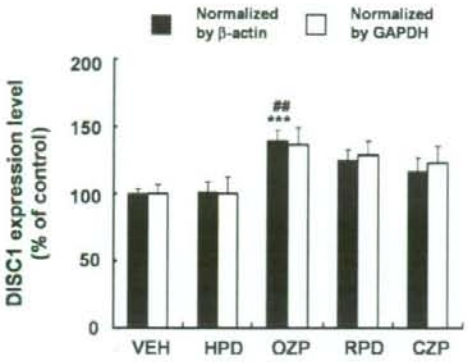

Fig. 2. Relative expression levels of DISC1 in hippocampus in clinical dose. DISC1 mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9). $^{***}p < 0.001$, compared with the control group. $\#\#p < 0.01$, compared with the haloperidol treated group

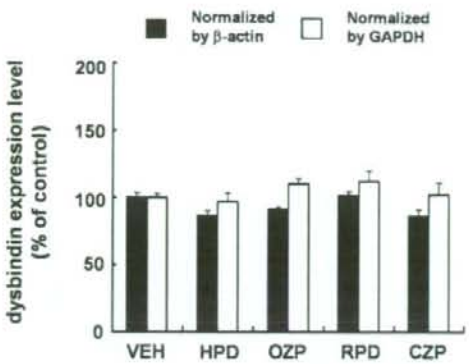

Fig. 3. Relative expression levels of dysbindin in frontal cortex in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9)

demonstrated significant effects of drug treatments (normalized by β-actin, $F_{4, 53} = 6.09$, $p < 0.001$, or GAPDH, $F_{4, 53} = 2.82$, $p = 0.034$). In post hoc analysis, DISC1 expression levels normalized by β-actin were significantly increased by the atypical antipsychotic, olanzapine, compared with control (39%, $p = 0.0006$) or haloperidol (29%, $p = 0.0054$) and similar trend was observed in risperidone compared with control (25%, $p = 0.079$). On the other hand, a slight increase of DISC1 expression was also found when normalizing by GAPDH (olanzapine vs control: 37%, $p = 0.094$; olanzapine vs haloperidol: 29%, $p = 0.23$; risperidone vs control: 29%, $p = 0.39$), which did not reach statistical significance. No effect of haloperidol or clozapine treatment was found in either normalization. These findings suggest that the mRNA expression levels of the DISC1 gene are increased by the chronic administration of some atypical antipsychotics in frontal cortex and possibly in hippocampus.

The expression levels of dysbindin mRNA normalized by β-actin and GAPDH in frontal cortex and hippocampus of mice administered treatment with a typical antipsychotic or atypical antipsychotics at the clinical dose are shown in Figs. 3 and 4. Dysbindin gene expression normalized by either β-actin or GAPDH in frontal cortex or hippocampus did not significantly differ between the treatment groups (frontal cortex: GAPDH, $F_{4, 53} = 1.45$, $p = 0.23$; hippocampus: β-actin, $F_{4, 53} = 0.64$, $p = 0.64$, GAPDH, $F_{4, 53} = 0.46$, $p = 0.77$), except for that in frontal cortex normalized by β-actin ($F_{4, 53} = 3.68$, $p = 0.01$). However, post hoc analysis demonstrated no significant difference in dysbindin expression in frontal cortex normalized by β-actin in any of the drug treatments, although there were trends towards slightly decreased expression of dysbindin in mice

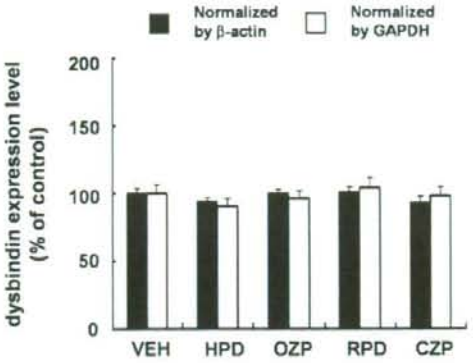

Fig. 4. Relative expression levels of dysbindin in hippocampus in clinical dose. Dysbindin mRNA expression levels normalized by β-actin or GAPDH in control mice (treated with vehicle: VEH) and mice treated with haloperidol (HPD), olanzapine (OZP) risperidone (RPD), or clozapine (CZP) are shown. Expression levels were calculated by comparison to percentage of average of those of control mice. Data are the means ± SEM from 19 control mice or mice treated with HPD (n = 10), OZP (n = 10), RPD (n = 10) or CZP (n = 9)

treated with haloperidol, compared with control (14%, $p = 0.074$) and in mice treated with risperidone (16%, $p = 0.094$). These data suggest that administration of typical and atypical antipsychotics do not have a consistent influence on mRNA expression levels of the dysbindin gene in frontal cortex or in hippocampus.

Discussion

In this study, we have measured mRNA expression levels of two susceptibility genes for schizophrenia, DISC1 and dysbindin, in frontal cortex and hippocampus using a real-time quantitative RT-PCR in mice treated chronically with typical or atypical antipsychotics. We found preliminary evidence that the expression levels of DISC1 may be altered by treatment with the atypical agents in frontal cortex and possibly in hippocampus and that the expression levels of dysbindin may not be changed under these conditions. Upregulation of DISC1 mRNA in frontal cortex by olanzapine and risperidone was observed in both normalizations by β-actin and GAPDH, however, that in hippocampus by olanzapine was found only in normalization by β-actin. As DISC1 has been shown to interact with actin (Miyoshi et al., 2003), it is possible that the DISC1 mRNA expression level normalized by β-actin in hippocampus may be somehow affected by the interaction. Upregulation of DISC1 mRNA in hippocampus by atypical antipsychotics appears to be marginal while that in frontal cortex is more apparent. As DISC1 expression is dominant in hippocampus compared with frontal cortex (Miyoshi et al., 2003), there is a possibility that this differential expression of DISC1 might affect the degree of the upregulation of DISC1 mRNA by the atypical antipsychotics.

Specifically, there was an increase of DISC1 expression levels after treatment with olanzapine and risperidone and possibly with clozapine in a simulated clinical dose in frontal cortex. As consistent results were obtained from normalization of the DISC1 expression by two house keeping genes, our findings would seem to be robust at least in comparison to results that might have been based on using only one control gene. However, it should be noted that there were some effects of antipsychotics on housekeeping gene expression, though largely nonsignificant. It is conceivable that some of the effect on our measures of DISC1 expression could be exaggerated by these effects on our control genes, as significant effects of drug treatments on the raw expression levels of DISC1 (non-normalized) were not observed in either frontal cortex or hippocampus (data not shown). Our data raise the possibility that DISC1 may be involved in the treatment of schizophrenia. However, as our study did not include the measurement of DISC1 proteins, or expression in other brain regions, or of treatment with other psychotropic drugs, further work is necessary to clarify whether

changes in DISC1 mRNA impact on protein expression and are specific for brain regions and psychotropic drugs. It also should be noted that we measured expression only of the common transcript for both of these genes. It is not currently known whether schizophrenia involves alternate processing of these genes into disease related transcripts or isoforms and we cannot rule out that treatment may impact on variable splicing or processing of these genes.

A balanced translocation in the DISC1 gene segregates with schizophrenia and other major psychiatric illnesses in a Scottish family (Millar et al., 2000). However, little is known about how the translocation affects the expression and/or function of the DISC1 gene. DISC1 protein expression in lymphoblasts derived from the family member with the translocation was observed to be decreased but the mutant truncated form of DISC1, which should be produced by the translocation, was not found (James et al., 2004). It is unknown whether the expression of DISC1 in brains of the family members is altered or not, however, this observation in peripheral cells suggested that the translocation might decrease the expression of DISC1. Alternatively, mutant truncated form of DISC1, which has been shown to play a role in inhibiting neurite outgrowth (Ozeki et al., 2003), might down-regulate the DISC1 protein expression and/or function. These findings suggest that reduced expression of DISC1 in brain might be expected in schizophrenic brain if DISC1 is involved in the pathogenesis of schizophrenia. On the other hand, gross expression levels of DISC1 protein have not been found to be changed in frontal cortex in patients with schizophrenia (Sawamura et al., 2005) and expression levels of DISC1 mRNA tended to be increased in hippocampus of schizophrenia patients (Lipska et al., 2004). Our data suggest that increased expression of DISC1 mRNA may be, at least in part, related to treatment with some atypical antipsychotics.

Evidence that dysbindin is associated with schizophrenia is now quite strong, although no functional mutation in dysbindin gene has yet been identified. Recent postmortem studies have found decreased expression of dysbindin mRNA and protein in hippocampus and frontal cortex in schizophrenic patients (McClintock et al., 2003; Talbot et al., 2004; Weickert et al., 2004). In contrast to our data with DISC1, we found no consistent pattern of altered dysbindin expression in hippocampus and frontal cortex following antipsychotic treatment.

Knowledge about protein functions of DISC1 and dysbindin is insufficient, however, we discuss a possibility how these genes affect the mechanisms of schizophrenia. As DISC1 has a prominent role in the neurite extension and its expression is developmentally regulated (Ozeki et al., 2003), upregulation of DISC1 could support the maturation of dendritic spine, which is believed to be affected in schizophrenia. As dysbindin promotes glutamate release in neuronal culture (Numakawa et al., 2004), reduced expression of dysbindin in schizophrenic brain could be relevant to glutamatergic dysfunction, which has been implicated in the pathophysiology of schizophrenia.

In summary, our findings offer preliminary evidence that altered expression of DISC1 may be caused by certain antipsychotic drugs, suggesting a role for DISC1 in therapeutic actions of these drugs. Additional studies are warranted to examine DISC1 and dysbindin expression, including western blotting analysis, in situ hybridization, immunohistochemistry, and the effect of other psychotropic drugs.

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THE CELLULAR AND BEHAVIORAL CONSEQUENCES OF INTERLEUKIN-1 ALPHA PENETRATION THROUGH THE BLOOD–BRAIN BARRIER OF NEONATAL RATS: A CRITICAL PERIOD FOR EFFICACY

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Abstract—Proinflammatory cytokines circulating in the periphery of early postnatal animals exert marked influences on their subsequent cognitive and behavioral traits and are therefore implicated in developmental psychiatric diseases such as schizophrenia. Here we examined the relationship between the permeability of the blood–brain barrier to interleukin-1 alpha (IL-1α) in neonatal and juvenile rats and their later behavioral performance. Following s.c. injection of IL-1α into rat neonates, IL-1α immunoreactivity was first detected in the choroid plexus, brain microvessels, and olfactory cortex, and later diffused to many brain regions such as neocortex and hippocampus. In agreement, IL-1α administration to the periphery resulted in a marked increase in brain IL-1α content of neonates. Repeatedly injecting IL-1α to neonates triggered astrocyte proliferation and microglial activation, followed by behavioral abnormalities in startle response and putative prepulse inhibition at the adult stage. Analysis of covariance with a covariate of startle amplitude suggested that IL-1α administration may influence prepulse inhibition. However, adult rats treated with IL-1α as neonates exhibited normal learning ability as measured by contextual fear conditioning, two-way passive shock avoidance, and a radial maze task and had no apparent sign of structural abnormality in the brain. In comparison, when IL-1α was administered to juveniles, the blood–brain barrier permeation was limited. The increases in brain IL-1α content and immunoreactivity were less pronounced following IL-1α administration and behavioral abnormalities were not manifested at the adult stage. During early development, therefore, circulating IL-1α efficiently crosses the blood–brain barrier to induce inflammatory reactions in the brain and influences later behavioral traits.

Key words: BBB, cytokine, IL-1, prepulse inhibition, sensorimotor gating, schizophrenia.

Obstetric complications as well as maternal and perinatal viral infection have been proposed to increase the risk of schizophrenia (O'Callaghan et al., 1991, 1992; Cannon et al., 2002). This hypothesis has been examined by challenging rodent dams or neonates with viral infection, hypoxia and asphyxia (Borrell et al., 2002; Fatemi et al., 2002a; Zuckerman et al., 2003; Shi et al., 2003). Infecting pregnant mice with influenza virus results in inflammatory reactions in the fetal body and brain although the virus is not detectable in the fetus (Shi et al., 2005). Non-vital viral and bacterial components, polyinosinic-polycytidylic acid and bacterial lipopolysaccharides, can produce similar maternal immune responses and the glial reaction in fetal brain without apparent neurodegeneration, and later induce various abnormal behaviors of offspring that are sensitive to antipsychotic medication (Urakubo et al., 2001; Fatemi et al., 2002b, 2005; Gayle et al., 2004; Meyer et al., 2006a). Thus, immune inflammatory responses, rather than viral cytotoxicity, play a central role in perturbing brain development including aspects that contribute to later neurobehavioral traits.

The fetal and neonatal immune inflammatory reactions following viral challenge or hypoxia include induction of various proinflammatory cytokines that are implicated in the etiology or neuropathology of schizophrenia (Nawa and Takei, 2006). Maternal challenge with polyinosinic-polycytidylic acid and neonatal exposure to Borna virus strongly induce interleukin (IL)-1β in fetal body and tumor necrosis factor (TNF)α in neonates, respectively (Hornig et al., 1999; Watanabe et al., 2003; Meyer et al., 2006b,c). These proinflammatory cytokines are markedly induced in human fetal amniotic fluids following obstetric complications or abnormal pregnancy (Romero et al., 1990; Halgunset et al., 1994). Thus embryos and newborns can be exposed to high concentrations of proinflammatory cytokines depending on the severity of labor and the term of pregnancy (Tsunoda et al., 1990; Sarandakou et al., 1998). While cytokine-mediated intra-placental inflammation has been implicated in abnormal brain development and impaired brain function later in life (Muller and Acken-

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Abbreviations: ANCOVA, analysis of covariance; BBB, blood–brain barrier; CS, conditioned stimulus; EDTA, ethylenediaminetetraacetic acid; ED-1, ectodysplasin 1; EGF, epidermal growth factor; EIA, enzyme immunoassay; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; IL-1, interleukin-1; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; PND, postnatal day; PPI, prepulse inhibition; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; US, unconditioned stimulus.

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heil, 1998; Nawa et al., 2000), it is not clear how cytokines in the periphery penetrate the blood–brain barrier (BBB) and act on immature neurons or glial cells in the brain.

IL-1 is a proinflammatory cytokine that mediates stress and inflammatory responses in the immune, endocrine, and nervous systems (Minami et al., 1991, 1992; Rothwell and Luheshi, 2000). Infection, hypoxia and tissue injury all induce the production of IL-1α and/or IL-1β in the periphery, both of which bind to and activate the same receptor (Rothwell and Luheshi, 2000). In this context, IL-1 is one of the proinflammatory cytokines that are commonly involved in schizophrenia risk events such as maternal viral infection and obstetric complications (Ito, Nawa and Takei, 2006). Although IL-1α and IL-1β have limited access to the CNS in adults (McLay et al., 2000; Banks et al., 2002–2003), the perinatal or early postnatal impact of IL-1 in the periphery appears to be more dynamic and prolonged (Tohmi et al., 2004; Tsuda et al., 2006). In previous studies, we s.c. treated rat pups with similar high doses of the proinflammatory cytokines, epidermal growth factor (EGF), IL-1α, IL-2, IL-6, interferon γ, and leukemia inhibitory factor, and found that, among the cytokines tested, only EGF and IL-1α exhibit the severe and persistent influence on prepulse inhibition (PPI), social interaction, exploratory behaviors and acoustic responses (Futamura et al., 2003; Watanabe et al., 2004; Tohmi et al., 2004; Tsuda et al., 2006). In addition, genetic and brain imaging studies on schizophrenia also indicate a tight link between IL-1 and this illness. Nucleotide polymorphism of the IL-1 gene complex is associated with a schizophrenia risk, potentially contributing to ventricular enlargement of schizophrenia patients (Katila et al., 1999; Papiol et al., 2005). Despite evidence implicating IL-1 as one of the factors that can modulate neurobehavioral development and are implicated in schizophrenia etiology or pathology, molecular and cellular influences of circulating IL-1α in developing brain are largely unknown.

In the present study, we s.c. administered human recombinant IL-1α to rats at neonatal and juvenile stages. Permeation of IL-1α was estimated by immunohistochemistry and enzyme immunoassay (EIA) for human IL-1α in the acute phase and following repeated IL-1α injections by glial responses in a subchronic phase. To assess the consequences of IL-1α penetrance through neonatal BBB on later brain function, we examined the behavioral performance of these rats as adults. The permeability of the BBB to IL-1α was compared at neonatal and juvenile ages and correlated with the behavioral deficits elicited by administration of this cytokine.

EXPERIMENTAL PROCEDURES

Animals and cytokine treatment

Newborn Sprague–Dawley rats (all male, 12 litters total; SLC, Shizuoka, Japan) were obtained at postnatal day (PND) 2. We minimized the number of animals used and their suffering. Litters were designated born on PND1 and culled to 10 male pups by the vendor. Rats were housed with their dam in polypropylene cages (58L×28W×24H cm) in a temperature-controlled colony room maintained on a 12-h light/dark cycle (light on 08:00 h). Recombinant human IL-1α (1.0 μg/g body weight; Dainippon Pharmaceuticals, Osaka, Japan) or vehicle (phosphate-buffered saline; PBS) was administered s.c. to rats daily at the nape of the neck during PNDs PND2 to PND10 or PND14 to PND22. In our preliminary study, lower doses of recombinant human IL-1α (0.3, 0.1, and 0.01 μg/g body weight) failed to affect behavioral traits (Fig. 1 in a supplement). Thus, we gave neonatal rats the dose of 1.0 μg IL-1α/g body that caused mild weight loss but not lethality (Tohmi et al., 2004). At PND22-25, pups were weaned and separated into cage (three to four animals per cage). Two litters of rats were assigned to each behavioral test and not reused in other behavioral tests. Tests were performed during the night cycle (20:00–02:00 h). In a second set of experiments, IL-1α (1.0 μg/g) or PBS was injected s.c. into rat pups (PND2 or PND14) to examine acute effects on intracellular signaling. Food and water were available *ad libitum* except for rats tested with the radial maze task. These animals were limited to 10 g of food per day during the maze task. All animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Use and Care Committee of Niigata University.

EIA

Postnatal rats (PND2 and PND14) were given an s.c. injection of human recombinant IL-1α (1.0 μg/g body weight). One h or 4 h after injection rats were subjected to hypothermia and then transcardially perfused with cold PBS (10 ml for PND2 and 30 ml for PND14) to wash out IL-1α remaining in blood vessels. To estimate the amount of human IL-1α penetrating the BBB, the frontal cortex including the anterior cingulate was taken and homogenized in 10 volumes of homogenization buffer containing 1% Triton X-100 and protease inhibitors [aprotinin (200 kallikrein U/ml), 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 1 mM benzamidine (all, Sigma Chemical Co., St. Louis, MO, USA), and 1 mM EDTA] (Nawa et al., 1995). Brain homogenates were centrifuged at 14,000×*g* for 30 min at 4 °C and the supernatants were stored at −80 °C until use. Protein concentrations in the samples were determined using a Micro BCA kit (Pierce, Rockland, IL, USA) with bovine serum albumin as a standard. IL-1α levels were measured by a sandwich EIA kit for this cytokine (Cayman Chemical, Ann Arbor, MI, USA), as described in a manufacturer's protocol. The secondary antibody directed against human IL-1α was conjugated to acetylcholinesterase. The acetylcholinesterase activity retained in each well was measured by incubation with Ellman's reagent. The amount of the resulting yellow product was monitored by a plate reader with 450 nm light. EIA had a minimum dynamic range of 5–250 pg/well.

Immunoblot analysis

To evaluate the subchronic influence of IL-1α on neuroinflammatory markers, protein samples were prepared from IL-1α-treated animals 24 h after the last of multiple cytokine injections. Protein (5 μg or 50 μg/lane) was subjected to 8% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with antibodies directed against Iba1 neural cell adhesion molecule (NCAM, 1:5000, Sigma), glial fibrillary acidic protein (GFAP, 1:5000, Dako, Tokyo, Japan), D1 dopamine receptor (1:1000), D2 dopamine receptor (1:1000), AMPA receptor subunit GluR1 (1:1000), type 1 NMDA receptor (NR1, 1:1000), glutamic acid decarboxylase 67 (GAD; 1:500), tyrosine hydroxylase (TH; 1:1000), synaptophysin (1:1000), and synapsin I (1:800) (all from Chemicon, Temecula, CA, USA). Primary antibodies were detected with appropriate secondary antibodies and chemiluminescence (Amersham-Pharmacia, Tokyo, Japan).

Brain histology

To visualize brain distributions of IL-1α following s.c. injection, neonatal (PND2) and juvenile rats (PND14) received an s.c. injection of IL-1α (1.0 μg/g body weight) or PBS. One h or 4 h after injection, rats were transcardially perfused with 4% paraformaldehyde and in a 0.1 M phosphate-buffered solution (pH 7.4). For neuropathological examination, alternatively, IL-1α-treated and vehicle-treated rats (PND11 or PND60) were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in a 0.1 M phosphate-buffered solution (pH 7.4) by transcardial perfusion. Serial sections (4 and 10 μm thick) were cut from paraffin-embedded or frozen tissues, stained with hematoxylin and eosin or Kluver-Barrera stain. Alternatively, brain sections were incubated with antibodies directed against human IL-1α (1:100, Chemicon), GFAP (1:500, Dako) and ectodysplasin 1 (ED-1, 1:50, Serotec, Oxford, UK) subsequently stained using the ABC method (Vector Laboratories, Burlingame, CA, USA).

Measurement of acoustic startle and PPI

Startle amplitude and PPI responses were assessed in a startle chamber (SR-LAB Startle Response System, San Diego Instrument, San Diego, CA, USA) with 120-dB acoustic stimuli, a single 100-ms prepulse interval, and three different prepulse intensities [5, 10, and 15 dB above background noise (white noise, 70 dB)]. Depending on the animal's mass, the sensitivity of the startle chamber was adjusted to obtain a linear dynamic range by setting the amplitude of the vibrator to 250 units for 4 week-old rats and 125 units for 8 week-old rats (arbitrary unit measure of this machine). This twofold difference in sensitivity was normalized after data acquisition. PPI was determined with the following two calculations: The percentage reduction in startle amplitudes following prepulse stimuli (% PPI) was calculated as: 100−[(startle response on prepulse–pulse stimulus trials–no stimulus trials)/(pulse-alone trials−no stimulus trials)×100] (Braff and Geyer, 1990; Swerdlow and Geyer, 1998). The absolute reduction from pulse-alone startle amplitudes (absolute PPI) was calculated as: pulse-alone trials−startle response on prepulse-pulse stimulus trials (Grillon et al., 1992).

Active-avoidance learning

Rats were given 10 sessions of two-way active-avoidance conditioning (10 trials/day). Active-avoidance testing was conducted in a two-way automated shuttle box (Muromachi-kiki, Tokyo, Japan). The conditioned stimulus (CS) was an 80-dB tone for 5 s. The unconditioned stimulus (US), a 5-s positive half-wave constant current of 0.6-mA intensity, was initiated if the animal failed to make an escape response (crossing to the other side of the shuttle box). The inter-trial interval was variable (20–40 s; Futamura et al., 2003).

Contextual conditioning

Rats were habituated in a test chamber (30L×30W×90H cm box; Muromachi-kiki) for 5 min and exposed to 0.8-mA electric shocks (2 s, twice). One day after conditioning, rats were returned to the chamber. The time spent freezing (i.e. no movements except those necessary for respiration) was counted at 1-min intervals for 3 min. Freezing behavior was recorded by a video camera during all sessions (Ohno et al., 2001).

Radial arm maze task

The radial arm maze contained eight arms (48×12 cm) extending radially from a central area (32 cm in diameter) with a 5-cm edge around the apparatus (Neuroscience Inc., Tokyo, Japan). Each animal was subjected to a reference and working memory task for 15 days (three sessions/day) during which the same four arms were baited for each daily training session (Mizuno et al., 2000). The other four arms were never baited. The training trial continued until four baits had been consumed or until 5 min had elapsed. The number of reference memory errors (entering an arm that was not baited) was counted.

Statistical analysis

Results were expressed as means±S.E.M. Startle response, PPI, and learning measures were analyzed using ANOVA with repeated measures, followed by a Tukey post hoc test for groups having similar deviations or a Games-Howell post hoc test for groups having different deviations. In repeated measures ANOVA, a between subject factor was treatment (IL-1 and PBS) and a within subject factor was either test session or prepulse intensity of absolute or percentage reduction of startle amplitudes. A Pearson's correlation test between pulse-alone startle responses and PPI levels was performed, followed by analysis of covariance (ANCOVA) with a subject factor of treatment and a covariate of an absolute amount of pulse-alone startle. To quantify immunoreactivity on blots, the densitometry of bands (arbitrary units) was performed and subjected to two-way ANOVA with subject factors of treatment and age or univariate analysis of Student's *t*-test for data at a single age. *N* values represent the number of animals. A *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS software (version 11.5; SPSS Japan Inc., Tokyo, Japan).

RESULTS

IL-1α crosses the BBB in neonates

To assess the permeability of the BBB to IL-1α in neonatal and juvenile rats, we examined brain distributions of human IL-1α following peripheral administration of this cytokine (Figs. 1–3). One h after s.c. administration of IL-1α, immunoreactivity for human IL-1α was predominantly distributed in the choroids plexus of the ventricles and microvessels in the brain (Fig. 1b–d). A significant numbers of cells in the olfactory cortex also became immunopositive for IL-1α at this time point. In contrast, there was little IL-1α immunoreactivity in the brain of vehicle-injected animals (Fig. 1a). Four h after IL-1α injection, immunoreactivity for IL-1α disappeared from the ventricles and microvessels (Fig. 2a) and moved to cell surfaces in the neocortical regions (Fig. 2b–d) as well as in the hippocampus and hypothalamus (data not shown). In particular, there was strong IL-1α immunoreactivity in cingulate cortex and olfactory cortex (Fig. 2c). Following peripheral administration of human IL-1α into juvenile rats, penetration of IL-1α to the brain was limited. IL-1α immunoreactivity in the brain was modest or negligible in IL-1α-treated juvenile rats, compared with that in of IL-1α-treated neonates (Fig. 3).

To confirm that the permeability of the BBB decreases during development, we similarly administered IL-1α to the periphery of neonatal (PND2) and juvenile rats (PND14) and measured IL-1α content in the frontal cortex including cingulate cortex 1 h and 4 h after injection (Fig. 4). To remove human IL-1α remaining in bloodstream of the brain, rats were perfused with excess amounts of PBS before tissue dissection. ANOVA with subject factors of treatment (IL-1α and PBS), time (1 h and 4 h) and age (PND2 and PND14) revealed significant main effects of IL-1α treatment ($F(1,24)=43.8$, $P<0.001$) and age

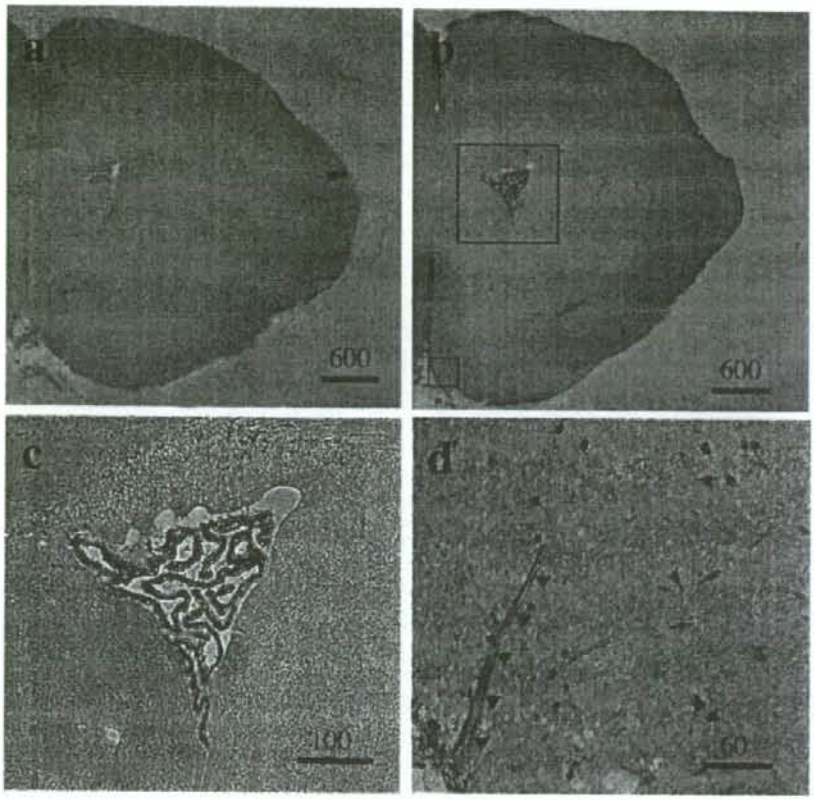

Fig. 1. IL-1α immunoreactivity in neonatal rat brain 1 h after s.c. injection. Sixty min after PBS (a) or human IL-1α (b–d) was s.c. injected into neonatal rats (PND2), brains were fixed and immunostained with an antibody directed against anti-human IL-1α. The higher magnification images of c (the lateral ventricle) and d (the olfactory cortex) correspond to the areas indicated by the open boxes in b, respectively. Arrowheads mark microvessels carrying IL-1α immunoreactivity. The numbers indicate the length of scale bars (μm). Note: the choroid plexus in other brain sections exhibited similar immunoreactivity for IL-1α (data not shown).

($F(1,24)=11.8$, $P=0.002$). A post hoc analysis indicated that human IL-1α content in neonatal rats (PND2) was higher than that in juvenile rats (PND14) at both time points. Thus, these results confirmed that peripheral IL-1α permeates the brain through the BBB at the neonatal stage of rats more efficiently than in the juvenile stage.

Neuroinflammatory responses in the brain following repeated IL-1α treatment

The neuropathological consequences of repeated IL-1 injections were examined at PND11 when 24 h passed after the last IL-1α injection was performed. When neonatal rats received daily injections of IL-1α nine times, its effects were widespread to various regions of the brain. Cells positive for GFAP (an astrocyte marker) and ED-1 (a microglia marker) were present not only in the cingulate cortex and olfactory cortex (Fig. 5e–h) but also in other brain regions such as neocortex and hippocampus (Fig. 2 in a supplement). Expression of these glial markers was not a consequence of cell death as histological examination revealed few signs of neurodegeneration (i.e. cytoplasmic shrinkage, vacuolization, chromatin condensation, etc.) in hematoxylin and eosin or Kluver-Barrera staining (Fig. 5a–d). The increase in immunoreactivity for GFAP and ED1 presumably reflects glial responses to IL-1α rather than to secondary glial reactions following neurodegeneration (Bonni et al., 1997; Proescholdt et al., 2002). This widespread glial activation in early postnatal rats indicates that peripherally administered IL-1α promotes inflammatory reactions in various types of glial cells of the CNS, although the IL-1 effect on neuronal degeneration was undetectable.

Neurochemical alterations after neonatal IL-1α treatment

We quantified the subchronic effects of repeated peripheral IL-1α injections on developing neurons and glial cells in three brain regions of rat pups: the frontal cortex (including the cingulate cortex), the striatum and the hippocampus. In agreement to the histological examination, immunoblotting also revealed the glial responses to IL-1α:

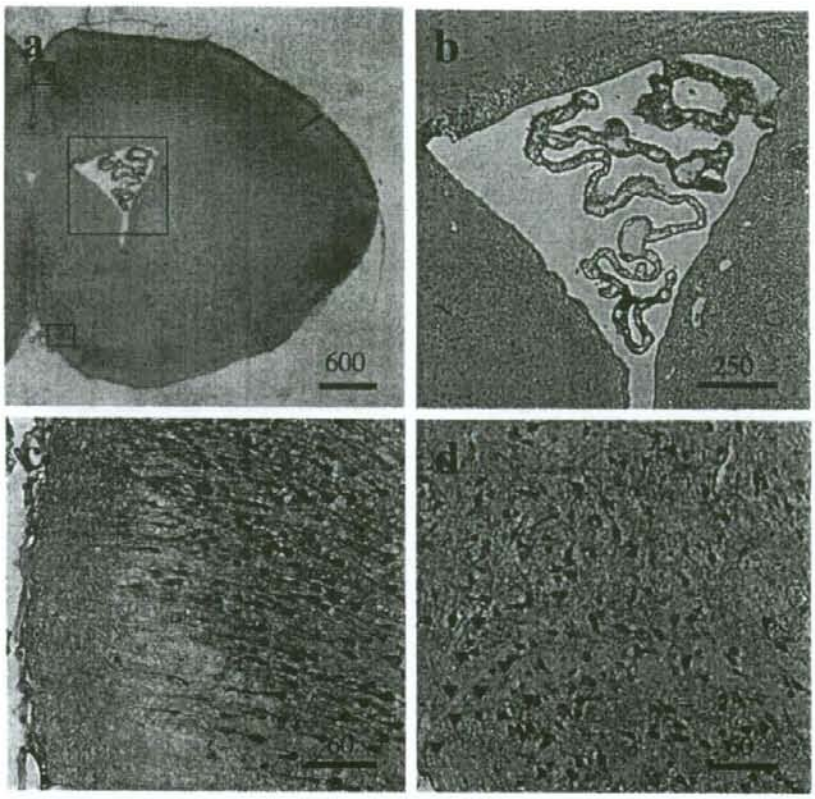

Fig. 2. Diffusion of IL-1α immunoreactivity into neonatal rat brain 4 h after s.c. injection. Four h after human IL-1α was s.c. injected into neonatal rats (PND2), distributions of IL-1α immunoreactivity were examined in the brain (a–d). The higher magnification images of b (the lateral ventricle), c (the cingulate cortex) and d (the olfactory cortex) correspond to the areas indicated by the open boxes in a, respectively. Arrowheads mark microvessels that lost IL-1α immunoreactivity found in Fig. 1d. The numbers indicate the length of scale bars (μm). Note: Diffusion of IL-1α immunoreactivity to the hypothalamus was also found (data not shown).

GFAP levels were increased ($P<0.001$, $n=5$) in the frontal cortex following IL-1α-treatments. In the same brain region, protein levels for TH, a rate-limiting enzyme for dopamine synthesis, increased 20% ($P=0.017$, $n=5$) whereas GAD67 levels decreased 19% following neonatal IL-1α treatment ($P=0.039$, $n=5$) (Fig. 6). There was no detectable change in TH or GAD67 in the striatum and hippocampus (data not shown). In parallel, levels for dopamine receptors (D1 and D2) were significantly decreased ($P<0.001$ for D1, $P=0.048$ for D2, $n=5$) in frontal cortex. A similar decrease in the expression of dopamine receptors was also detected in the striatum and hippocampus (data not shown). In contrast, the levels of neuronal markers (NCAM) as well as post-synaptic markers GluR1 and NR1 and pre-synaptic markers synaptophysin and synapsin I, were indistinguishable between groups in all brain regions examined ($P=0.111–0.921$, $n=5$). At the adult stage when IL-1α-treated rats grew up, we did not detect significant changes in any of the neuronal and astroglial markers examined, suggesting the temporal limitation of the neuroinflammatory reactions following neonatal treatment with IL-1α (data not shown).

Abnormal sensorimotor gating following neonatal IL-1α administration

IL-1α or saline (vehicle) was administered daily to littermates of neonatal rats (PND2) for 9 days as described above. Startle responses to a 120-dB tone in the presence and absence of the prepulse stimuli (none, 75-dB, 80-dB, and 85-dB tones) were monitored at 4 and 8 weeks of age ($n=15$ each). The effects of neonatal IL-1α administration on sensorimotor gating were mathematically evaluated as the percentage and the absolute reduction in startle magnitudes obtained with prepulse stimuli compared with trials without these prepulses (abbreviated to % PPI and absolute PPI, respectively) (Grillon et al., 1992; Cadenhead et al., 1993; Swerdlow et al., 2000), as the pulse-alone startle was affected by IL-1α treatment (see below).

In young rats at 4 weeks of age, the absolute startle magnitudes were analyzed by repeated measures ANOVA

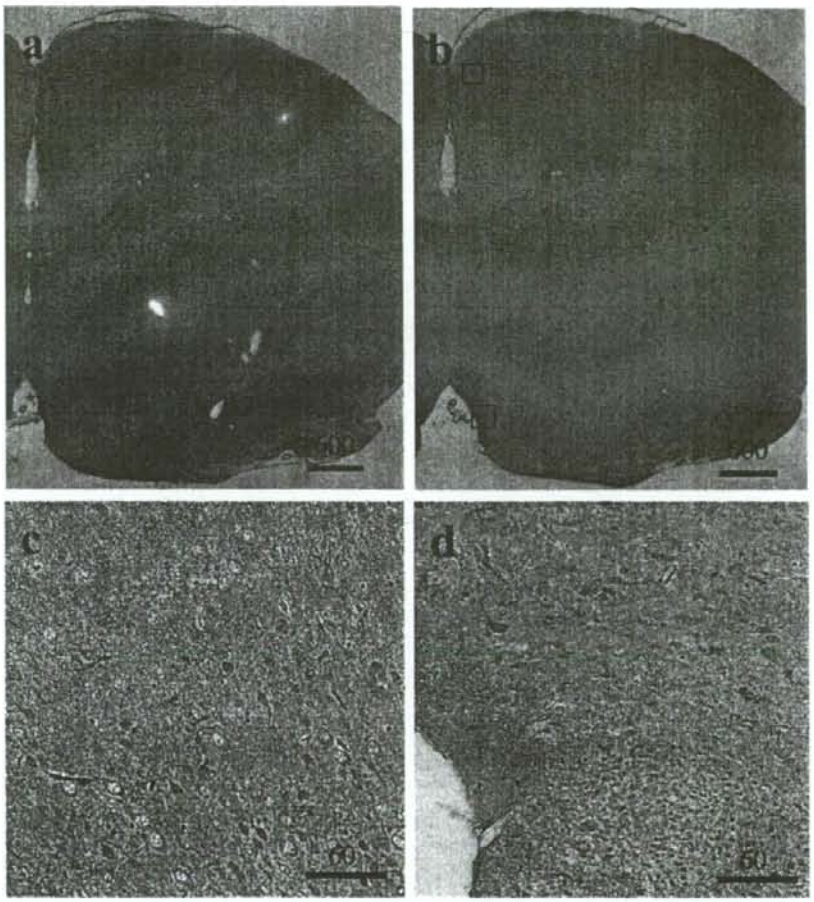

Fig. 3. Limited diffusion of IL-1α immunoreactivity into juvenile rat brain following s.c. injection. Human IL-1α was s.c. injected into juvenile rats (PND14), and distributions of IL-1α immunoreactivity were examined in the brain 1 h (a) and 4 h after injection (b–d). The higher magnification images of c (the cingulate cortex) and d (the olfactory cortex) correspond to the areas indicated by the open boxes in b, respectively. The numbers indicate the length of scale bars (μm).

with a between subject factor of treatment (IL-1α and vehicle) and a within subject factor of prepulse amplitude (none, 75, 80, and 85 dB) (Fig. 7a). There was no significant main effect of IL-1α treatment on the magnitude of the startle response ($F(1,28)=0.33$, $P=0.57$, repeated measures ANOVA) without interaction ($F(3,26)=1.17$, $P=0.39$, repeated measures ANOVA). Absolute PPI was not significantly altered by IL-1α treatment ($F(1,28)=0.36$, $P=0.55$, repeated measures ANOVA) without interaction between IL-1α treatment and prepulse ($F(2,27)=1.82$, $P=0.18$, repeated measures ANOVA) (Fig. 7b). In addition, IL-1α had no significant effect on % PPI levels ($F(1,28)=0.97$, $P=0.33$, repeated measures ANOVA) without interaction ($F(3,27)=2.29$, $P=0.12$, repeated measures ANOVA) (Fig. 7c). Thus, we conclude that neonatal treatment with IL-1α does not influence sensorimotor gating at the juvenile stage.

When we measured the startle amplitudes of these same rats in the presence and absence of prepulse stimuli at 8 weeks of age, the rats showed significant changes in absolute startle amplitudes (Fig. 7d). There was a main effect of IL-1α treatment ($F(1,28)=16.5$, $P<0.001$) and an interaction of IL-1α treatment and prepulse ($F(3,26)=4.31$, $P=0.0135$). Post hoc analysis detected significant increases in absolute startle amplitudes at all prepulse levels, as well as in the pulse-alone condition. To examine whether these differences only reflect the change in pulse-alone startle, we re-evaluated the data by calculating absolute PPI and % PPI. Repeated measures ANOVA for absolute PPI revealed no significant main effect of IL-1α treatment ($F(1,28)=1.93$, $P=0.18$) but an interaction between IL-1α treatment and prepulse ($F(2,27)=6.66$, $P=0.004$) (Fig. 7e). However, subsequent post hoc analysis failed to detect a significant difference between IL-1α- and

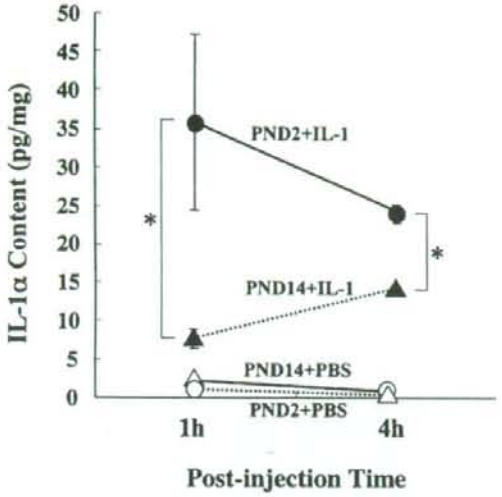

Fig. 4. Brain IL-1α content following s.c. administration to neonatal and juvenile rats. IL-1α or PBS was s.c. injected into neonatal rats (PND2, $n=4$) and juvenile rats (PND14, $n=4$). One and 4 h after injection, IL-1α remaining in blood vessels of the brain was washed out by cardiac perfusion of PBS and then the frontal cortex was taken. Protein content of human IL-1α was measured by an EIA kit. Human IL-1α levels were normalized by protein concentrations of tissue extracts. Post hoc; * $P<0.05$, compared between PND2 and PND14.

vehicle-treated groups at each prepulse level. When we calculated % PPI, we found significant effects of IL-1α treatment (Fig. 7f). Ratio normalization with absolute pulse-alone startle decreased % PPI of the IL-1α group ($F(1,28)=11.2$, $P=0.002$, repeated measures ANOVA) without an interaction of IL-1α treatment×prepulse ($F(3,27)=1.04$, $P=0.36$, repeated measures ANOVA). The explanation of the results from absolute PPI and % PPI is controversial with the given increase in pulse-alone startle (Swerdlow et al., 2000).

To examine how the increase in pulse-alone responses might affect values of absolute PPI and % PPI, data of individual animals were re-analyzed with the Pearson's correlation analysis and ANCOVA (Fig. 8) (Cadenhead et al., 1993). When the absolute magnitude of PPI for an 80-dB prepulse was plotted against the magnitude of the pulse-alone startle for each animal (Fig. 8a), there were strong linear correlations ($r=0.99$, $P<0.001$ for vehicle group and $r=0.91$, $P<0.001$ for IL-1α group). There was a significant difference in the intercepts of the regression equations for vehicle and IL-1α groups ($F(1,27)=-2.78$, $P=0.010$) but not in their slopes. Thus we performed ANCOVA with the pulse-alone values as covariates. The group main effect of IL-1α treatment on absolute PPI was significant ($F(1,27)=9.88$, $P=0.004$, ANCOVA) toward the direction of reducing absolute PPI. Similar statistical results were obtained for the other prepulse intensities as well ($F(1,27)=4.83$, $P=0.037$ for 75 dB; $F(1,27)=4.56$, $P=0.041$ for 85 dB, both ANCOVA).

The data of % PPI for an 80-dB prepulse were also plotted against the absolute magnitude of the pulse-alone startle (Fig. 8b). In the vehicle group, there was a correlation between pulse-alone startle and % PPI ($r=0.52$, $P=0.048$). There was a marginal trend toward a positive correlation between pulse-alone startle and % PPI in the IL-1α group ($r=0.43$, $P=0.10$). Similar results were obtained for the other prepulse magnitudes (data not shown). These results suggest that neonatal IL-1α treatment impairs acoustic startle responses and potentially sensorimotor gating at post-pubertal stages, assuming that the ANCOVA results are biologically meaningful. The putative abnormality in PPI scores was detected even at least 9 months of age in a separate group of animals (data not shown).

Delayed IL-1α treatment at the juvenile stage and its influences

To test whether the neonatal administration of IL-1α was critical for the later emergence of sensorimotor gating deficits, IL-1α treatment was delayed for 12 days and performed at the juvenile stage (PND14-22). Levels of startle responses and sensorimotor gating were estimated with the absolute startle scale at 4 and 8 weeks of age (Fig. 9a–d). At 4 weeks of age, the absolute magnitude of startle was analyzed by repeated measures ANOVA with a between subject factor of treatment (IL-1α and vehicle) and a within subject factor of prepulse magnitude (none, 75, 80, and 85 dB) (Fig. 9a). There was significant main effects of IL-1α treatment ($F(1,18)=8.61$, $P=0.009$, repeated measures ANOVA) without factorial interactions ($F(2,17)=1.47$, $P=0.26$, repeated measures ANOVA).

The effects of IL-1α were evaluated further with values of absolute PPI and % PPI. Absolute PPI levels for an 80-dB prepulse were significantly decreased by IL-1α treatment ($F(1,18)=4.89$, $P=0.04$, repeated measures ANOVA) (Fig. 9c) whereas % PPI levels were not significantly altered ($F(1,18)=1.24$, $P=0.28$, repeated measures ANOVA) (Fig. 9b). As there were significant positive correlations between absolute PPI levels and pulse-alone startle in both groups, the effects of IL-1α on absolute PPI were re-evaluated by ANCOVA with the pulse-alone values as covariates (Fig. 9d). ANCOVA revealed no significant effect of IL-1α on absolute PPI ($F(2,17)=0.024$, $P=0.88$). Thus, the decrease in absolute PPI was superficial and may be attributed to the decrease in pulse-alone startle. Similar statistical results were obtained for the other prepulse magnitudes as well ($F(1,17)=0.16$, $P=0.69$ for 75 dB; $F(1,17)=1.30$, $P=0.27$ for 85 dB, both ANCOVA).

At week 8 of age, the startle responses, absolute PPI, and % PPI of these rats were re-examined (Fig. 10). Repeated measures ANOVA for absolute startle amplitudes, absolute PPI and % PPI did not detect any effects of IL-1α treatment ($F(1,18)=1.24$, $P=0.28$; $F(1,18)=0.56$, $P=0.46$; $F(2,17)=1.47$, $P=0.26$, respectively) without interactions. Thus, we conclude that the long-term effects of IL-1α on sensorimotor gating are less pronounced or negligible when this cytokine is administered to juvenile rats.

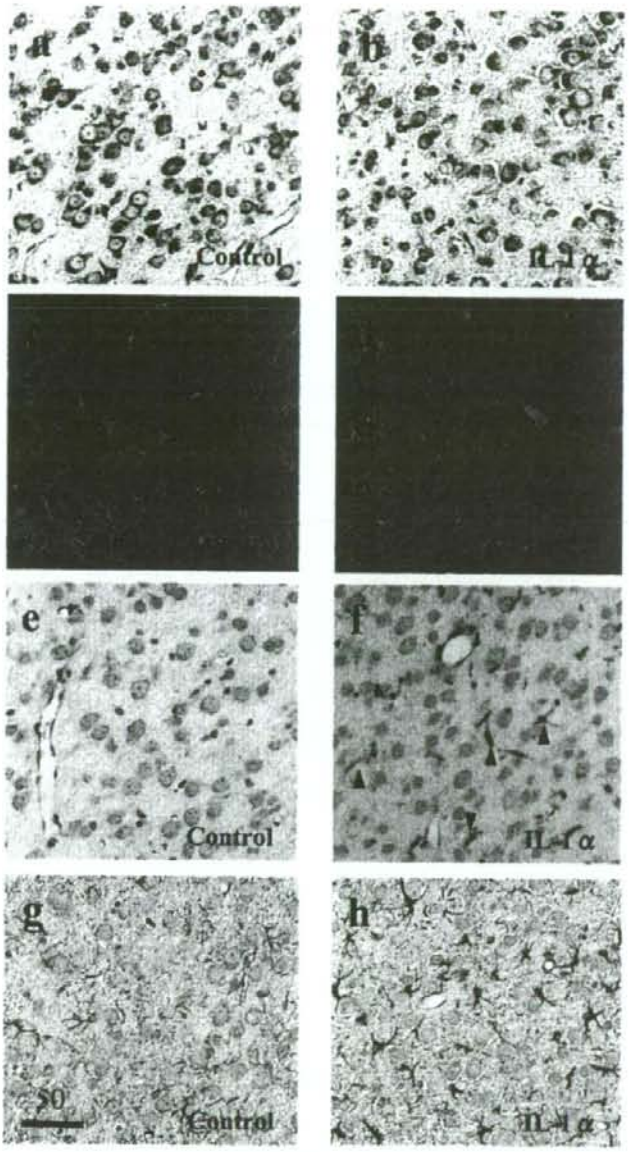

Fig. 5. Neuropathological alterations following repeated IL-1α injections to rat neonates. IL-1α or PBS was daily injected (s.c.) into neonatal rats from PND2 to PND10. Brain sections including the striatum and cingulate cortex were prepared from vehicle-treated (a, c, e, g) and IL-1α-treated (b, d, f, h) rats at PND11 (24 h after the last IL-1α administration) and examined for the Kluver-Barrera stain (KB; a, b), hematoxylin/eosin staining (HE; c, d), the astroglial marker, GFAP (e, f), and the microglial marker, ED-1 (g, h). Arrowheads indicate ED-1 immunoreactivity. Note; There were few signs of apoptosis or necrosis (cytoplasmic shrinkage, vacuolization, chromatin condensation) in both IL-1α-treated and vehicle-treated rats. Scale bar=50 μm.

Rats treated with IL-1α display normal learning ability

To determine if the changes in PPI and acoustic startle response induced by IL-1α treatment resulted from a general impairment of neural function, the learning ability of adult rats treated with IL-1α as neonates was examined with three learning paradigms; an active-avoidance test, contextual fear conditioning, and an eight-arm radial maze task. The active-avoidance test was performed in a two-way shuttle chamber with a buzzer sound (CS) and electric shock (US). There were no differences in the ability of vehicle- and IL-1α-treated rats (n=9 each) to avoid an

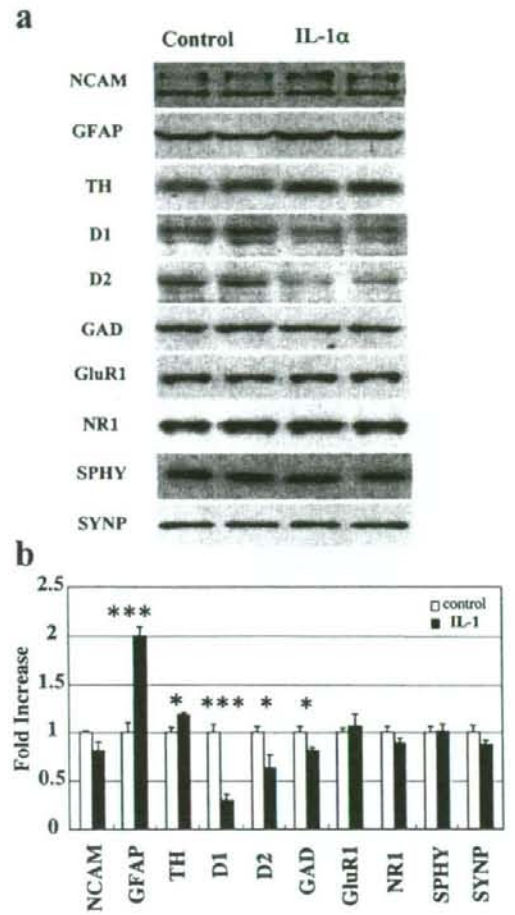

Fig. 6. Effects of IL-1α treatment on neurochemical markers in the frontal cortex. The subchronic effect of IL-1α on the expression of neuronal and glial markers was examined in the frontal cortex of rat neonates by immunoblotting. Neonatal rats were similarly treated with IL-1α or PBS from PND2 to PND10 and brain tissues were dissected out at PND11 when 24 h passes after the last injection were performed. Immunoblots were probed with the antibodies for a general neuronal marker, NCAM and an astroglial marker, GFAP. Neuronal markers specific for dopaminergic, GABAergic and glutamatergic neurons were also examined; TH, dopamine receptor 1 (D1), dopamine receptor 2 (D2), GAD67, AMPA-type glutamate receptor 1 (GluR1), NMDA-type glutamate receptor 1 (NR1), synaptophysin (SPHY), and synapsin I (SYNP) were compared between PBS-treated and IL-1α-treated rat pups (PND11; $n=5$ animals each) by immunoblot analysis. (a) Two representative lanes in each group for all markers are shown for display. (b) Immunoreactivity on immunoblots was measured by densitometric analysis. Black bars represent ratios of immunoreactivity in IL-1α-treated rats to that in control rats (mean±S.E.M.). * $P<0.05$, *** $P<0.001$.

electric shock after a tone stimulus during active-avoidance training ($F(1,16)=0.67$, $P=0.43$, repeated measures ANOVA) or in their ability to escape from electric shock ($F(1,16)=0.044$, $P=0.84$, repeated measures ANOVA; Fig. 11a). There was no factorial interaction between treatment and training session. In the contextual conditioning test, an electric shock was given only in the test chamber. In this paradigm, freezing times between groups were indistinguishable before ($F(1,28)=1.03$, $P=0.320$, ANOVA) and after conditioning ($F(1,28)=0.761$, $P=0.391$, ANOVA; Fig. 11b). In the eight-arm radial maze task, rats were trained to learn the location of four baited arms. The IL-1α-treated rats displayed normal rates of acquisition in spatial memory ($F(1,18)=0.796$, $P=0.384$, repeated measures ANOVA). There was no factorial interaction between IL-1α treatment and training session (Fig. 11c). Thus, the gross learning ability of adult rats treated with IL-1α-treated earlier in life was normal.

Neuropathological examination of adult rats treated with IL-1α as neonates

To examine whether the observed behavioral changes accompanied neuropathological alterations, we examined histologically the brains of IL-1α-treated rats, at the adult age (>PND60) when they exhibited the most pronounced neurobehavioral impairments. There was no indication of neuronal degeneration or structural alterations in any of the regions examined (Fig. 12a–h). Cortical structures were normal (Fig. 12a, b), and hippocampal pyramidal layers were intact in rats treated with IL-1α as neonates (Fig. 12c, d). The striatum of IL-1α-treated rats exhibited a normal organization (Fig. 12e, f). There were no significant differences in the molecular and granular layers of the cerebellum between IL-1α- and vehicle-treated animals (Fig. 12 g, h). These histological observations agree with the results from immunoblotting that there were no apparent decreases in any neuronal markers at the adult stage of the IL-1α-treated animals.

DISCUSSION

In neonatal rats, peripheral IL-1α efficiently penetrated the BBB and induced inflammatory reactions in early postnatal brain. The neuropathological changes triggered by IL-1α were followed by abnormalities in startle responses and putative PPI at post-pubertal ages well after the initial inflammatory responses in the brain had subsided. In contrast to these behavioral abnormalities, the gross learning ability of IL-1α-treated rats was normal. Although there was an apparent effect of neonatal exposure to IL-1α on acoustic startle reaction, the abnormality in PPI is controversial and presumptive with the present results. The reason why we judge that the present data reflect PPI abnormality is because the following reports indicate dissociation between PPI and startle amplitudes in this animal model. An antipsychotic drug, clozapine, decreases PPI levels without affecting pulse-alone startle (Tohmi et al., 2004). In C57BL/6 mice, neonatal IL-1α challenge alters PPI but not pulse-alone startle at the adult stage (Tsuda et al., 2006). In a critical sense, however, we cannot fully rule out the possibility that the putative PPI deficit of IL-1α-treated rats might be in fact a reflection of their increased startle magnitude. The confirmation of the argument about

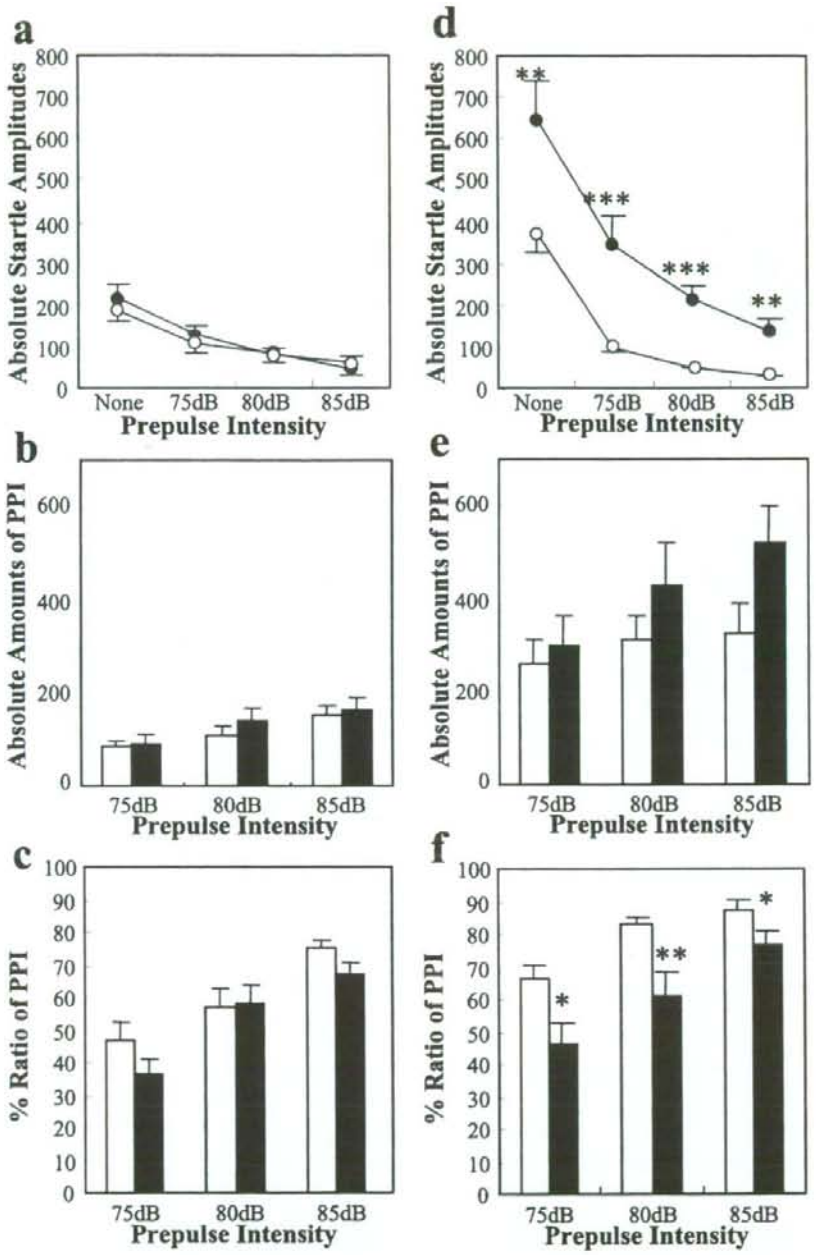

Fig. 7. Deficits in acoustic startle and PPI after neonatal IL-1α treatment. (a, d) Absolute amplitudes of the 120-dB acoustic startle of rats treated with vehicle (open circle, $n=15$) and IL-1α (closed circle, $n=15$) as neonates were monitored in the absence and presence of prepulse stimuli (none, 75, 80, and 85 dB) at 4 weeks of age (a) and 8 weeks of age (d). (b, e) Absolute magnitude of PPI was calculated as the absolute decrease from pulse-alone startle to prepulse-preceding startle and compared between vehicle-treated (open bars) and IL-1α-treated (closed bars) animals at 4 (b) and 8 (e) weeks of age. (c, f) The percentage of the prepulse-preceding startle to pulse-alone startle (% PPI) was calculated and compared between groups at postnatal weeks 4 (c) and 8 (f). Absolute startle amplitudes are presented with an arbitrary unit of the acoustic startle machine made by SR-LAB Startle Response System (San Diego Instrument). Data represent means±S.E.M. (%). Post hoc; * $P<0.05$, compared with vehicle-treated animals at the same age.

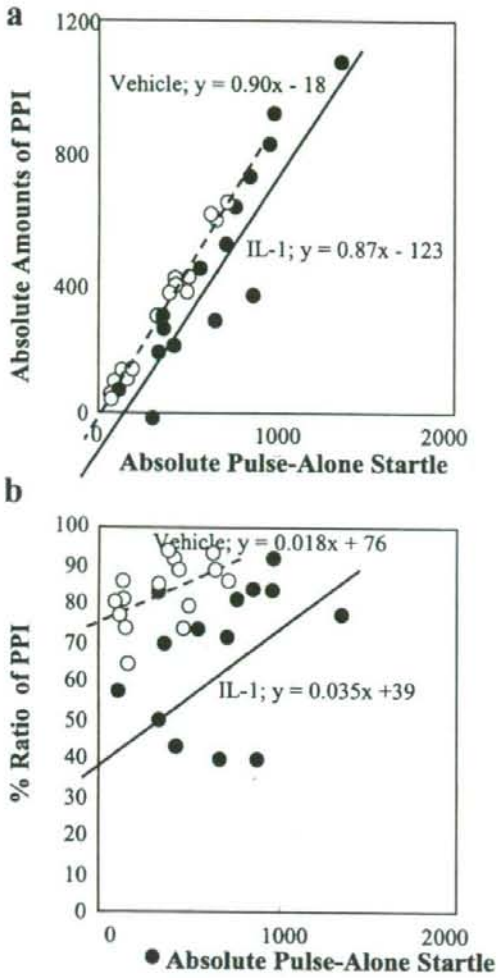

Fig. 8. Correlation analyses between absolute PPI and pulse-alone startle and between % PPI and pulse-alone startle. (a) Absolute magnitudes of PPI were plotted against the 120-dB pulse-alone acoustic startle of 8 week-old rats treated with vehicle (open circle, $n=15$) and IL-1α (closed circle, $n=15$) as neonates. The data correspond to Fig. 7e. The slopes of the regression equations for vehicle and IL-1α groups were similar ($F(1,26)=0.034$, $P=0.85$), but their intercepts were significantly different ($F(1,27)=-2.78$, $P=0.010$). (b) Percentage of PPI was calculated from the data of the same adult rats treated with vehicle (open circle, $n=15$) and IL-1α (closed circle, $n=15$) as neonates and plotted against the pulse-alone startle. The data correspond to Fig. 7f. Note: There was no trend toward a decrease in the percentage of PPI versus absolute pulse-alone startle.

PPI deficit will require more elaborate biological analyses in future.

Both our previous and present studies failed to detect neurodegeneration following IL-1α treatment in neonates (Tohmi et al., 2004) and adults (present data), despite evidence that IL-1α may contribute to cytotoxic processes (Licinio and Wong, 1999; Rothwell and Luheshi, 2000). Thus, the immature brain is not fully protected from such inflammatory cytokines generated in response to peripheral immune and inflammatory stress until the BBB is fully established. Acute administration of a variety of proinflammatory cytokines, such as IL-1, IL-2, IL-6, and TNFα, to adult animals is known to produce a set of depressive behaviors, namely sickness behavior (Anisman and Merali, 2003; Dantzer and Kelley, 2007). Sickness behaviors are different in quality and persistence from the behavioral impairments induced by neonatal IL-1α treatment, however (Tohmi et al., 2004). For example, there was no decrease in locomotor activity of rats treated with IL-1α as neonates. In addition, s.c. administration of all these inflammatory cytokines to neonates, even when the BBB is still leaky, does not produce such behavioral abnormalities at their adult stage. Among the proinflammatory cytokines examined (IL-1α, IL-2, IL-6, TNFα, and interferon γ), only IL-1α produces chronic behavioral impairments. These observations suggest that the biological activity of this particular cytokine has a crucial impact in determining the specificity and persistency of behavioral influences (Tohmi et al., 2004; Nawa and Takei, 2006).

The postnatal permeability of the BBB to IL-1α appeared to be correlated with the later emergence of PPI abnormality in adult. IL-1α administration during early development induced inflammatory reactions in the brain and led to the behavioral changes. S.c. injection of recombinant IL-1α into neonates immediately increased brain content of this cytokine, suggesting that IL-1α efficiently penetrates the neonatal BBB (McLay et al., 2000). In contrast, IL-1α administration to juvenile rats resulted in the modest increase in IL-1α content in the brain and did not produce later neurobehavioral impairments. These observations are consistent with a previous report that the BBB is immature and leaky at neonatal stages but fully develops around PND10 in rats (Kleshcheva, 1988). The leakiness of the BBB may not be a sole reason why IL-1 treatment in neonatal rats produced the behavioral alterations. It is possible that developmental plasticity of neonatal brain neurons and glia may additionally be required for the later mergence of the behavioral abnormality.

Among the neurochemical markers we examined, the increased TH and decreased dopamine receptor levels in the frontal cortex were noteworthy. Dopamine signaling may be involved in the PPI impairment we observed as deficits in sensorimotor gating are often correlated with dopaminergic dysfunction (Murphy et al., 1996; Jentsch et al., 1997; Swerdlow and Geyer, 1998). IL-1 is a potent differentiation factor for immature midbrain dopaminergic neurons and their precursors (Ling et al., 1998; Ho and Blum, 1997; Li et al., 2003) and this cytokine also increase the expression of GTP cyclose and enhance the production of BH4, rate limiting co-factor for TH (Pluss et al., 1996). Thus, neonatal IL-1α treatment might accelerate aberrant development of dopaminergic neurons or trigger abnormal synapse formation of these neurons that persists into adulthood (Ho and Blum, 1998; Kim et al., 2002). Alternatively, glial cells, which were activated by IL-1α, might produce the other cytokines or inflammatory mediators that perturb development of dopaminergic neurons or GABAergic

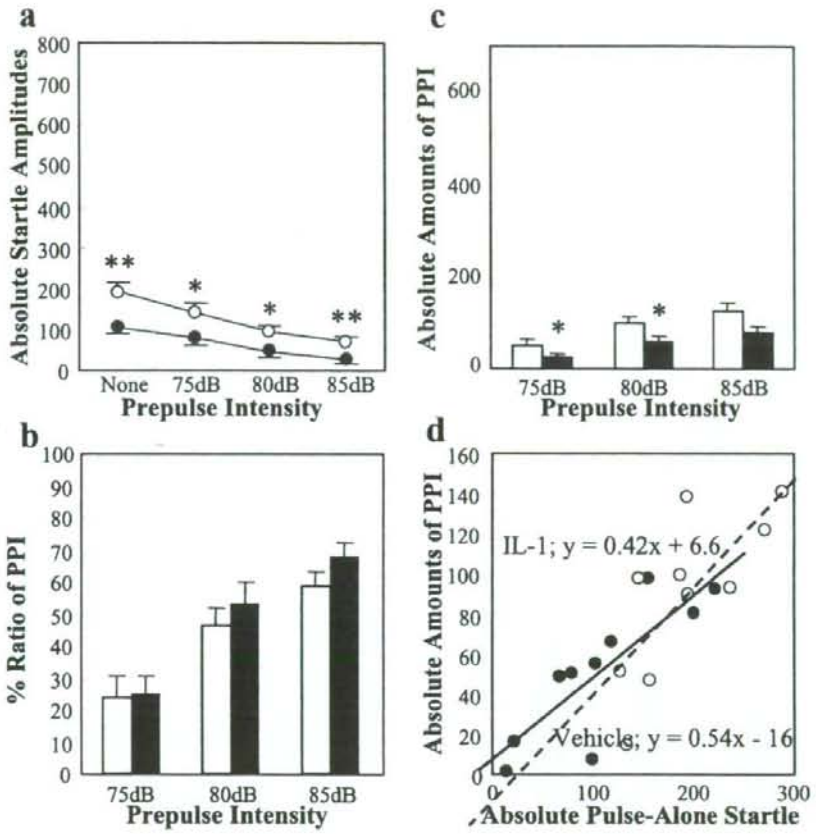

Fig. 9. Acoustic startle and PPI of 4 week-old rats following delayed IL-1α treatment. Juvenile rats (PND14) s.c. received the same dose of IL-1α or vehicle for 9 days. Compared with the neonatal treatment with IL-1α, the treatment was delayed 2 weeks. (a) Absolute amplitudes of the 120-dB acoustic startle of rats treated with vehicle (open circle, $n=10$) and IL-1α (closed circle, $n=10$) were measured in the absence and presence of prepulse stimuli (none, 75, 80, and 85 dB) at 4 weeks of age. (b) Percentage PPI was calculated and compared between the animals treated with vehicle and IL-1α. (c) Absolute magnitudes of PPI were calculated as an absolute decrease from pulse-alone startle to prepulse-preceding startle and compared between the animals treated with vehicle (open bar) and IL-1α (closed bar). Data represent means±S.E.M. Post hoc; * $P<0.05$, compared with vehicle-treated animals at the same age. (d) Correlation analyzed between absolute PPI and pulse-alone startle at 4 weeks of age. The data correspond to those in Fig. 9c. Correlation of the data for vehicle-treated group (open circle) was significant ($r=0.78$, $P=0.007$) and that for IL-1α-treated group (closed circle) was also significant ($r=0.79$, $P=0.007$). There was no significant difference in regression lines for IL-1α-treated and vehicle-treated groups ($F(2,17)=0.024$, $P=0.88$). The slopes and intercepts of the regression equations were not significantly different between vehicle-treated and IL-1α-treated groups ($F(1,16)=0.75$, $P=0.40$ for slope, $F(1,17)=0.010$, $P=0.92$ for intercept).

neurons. Another dopaminergic differentiation factor, EGF, is also known to increase TH levels in the neonatal brain and induce similar behavioral abnormalities in adult rats (Futamura et al., 2003). In this context, it is noteworthy that EGF and IL-1α exert common effects on physical development of rats and mice, such as accelerating eyelid opening and tooth eruption (Futamura et al., 2003; Tsuda et al., 2005). Therefore, a similar pathologic mechanism may underlie the cognitive and behavioral impairments induced by neonatal EGF and IL-1α treatment, although the responsible pathological nature of the rats treated with these cytokines as neonates remains to be characterized.

Abnormal inflammatory cytokine signals are often detected in patients with various psychiatric diseases including schizophrenia (Licinio et al., 1993; Katila et al., 1994; Mittleman et al., 1997; Lin et al., 1998; Toyooka et al., 2003). Although it is unclear whether elevated cytokine levels in these patients result from their pathologic condition or have etiologic implications, prior epidemiologic investigations propose the involvement of inflammatory cytokines in abnormal brain development or later behavioral/cognitive impairment (Nawa et al., 2000; Nawa and Takei, 2006). Viral infection or abnormal parturition can increase the levels of endogenous inflammatory cytokines by greater than 100-fold in amniotic fluid (Romero et al., 1990; Tsunoda et al., 1990; Halgunset et al., 1994) and are risk factors for schizophrenia (O'Callaghan et al., 1991, 1992; Fatemi et al., 2002a; Shi et al., 2003). Maternal immune responses are transmitted through the placental barrier and induce IL-1β and other cytokines in embryos (Cai et

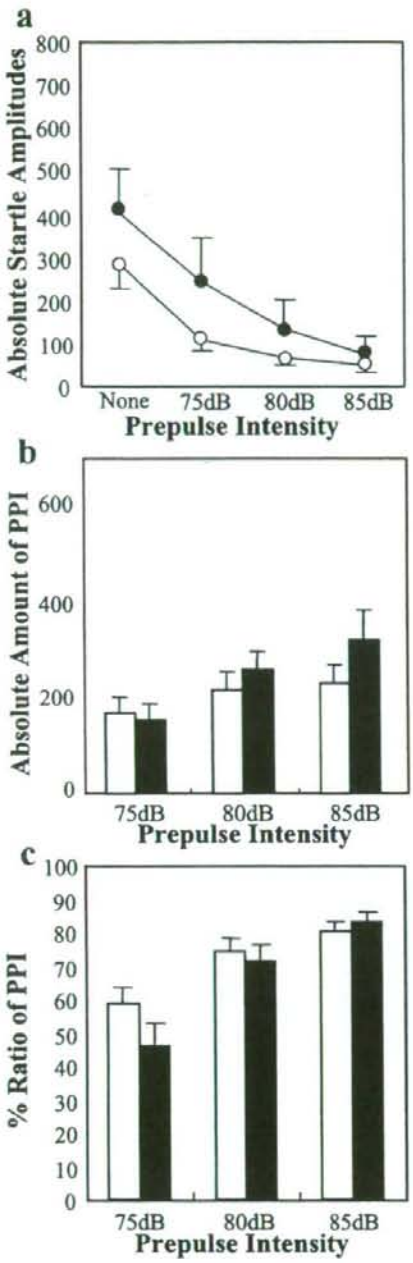

Fig. 10. Effects of delayed IL-1α treatment on startle responses as adults. The rats used in Fig. 9 were subjected to the same startle tests at 8 weeks of age. (a) Absolute amplitudes of the 120-dB acoustic startle of rats treated with vehicle (open circle, $n=10$) and IL-1α (closed circle, $n=10$) were monitored in the absence and presence of prepulse stimuli (none, 75, 80, and 85 dB). (b) Absolute magnitudes of PPI were calculated and compared between vehicle-treated (open bar) and IL-1α-treated (closed bar) animals. (c) Percentage PPI was calculated and compared between vehicle- and IL-1α-treated animals. Data represent means±S.E.M.

al., 2000; Zuckerman et al., 2003; Meyer et al., 2006c). It is therefore possible that after maternal inflammation, infection, or obstetric complication, fetuses are exposed to concentrations of IL-1 or other inflammatory cytokines high enough to perturb brain development (Cai et al., 2000;

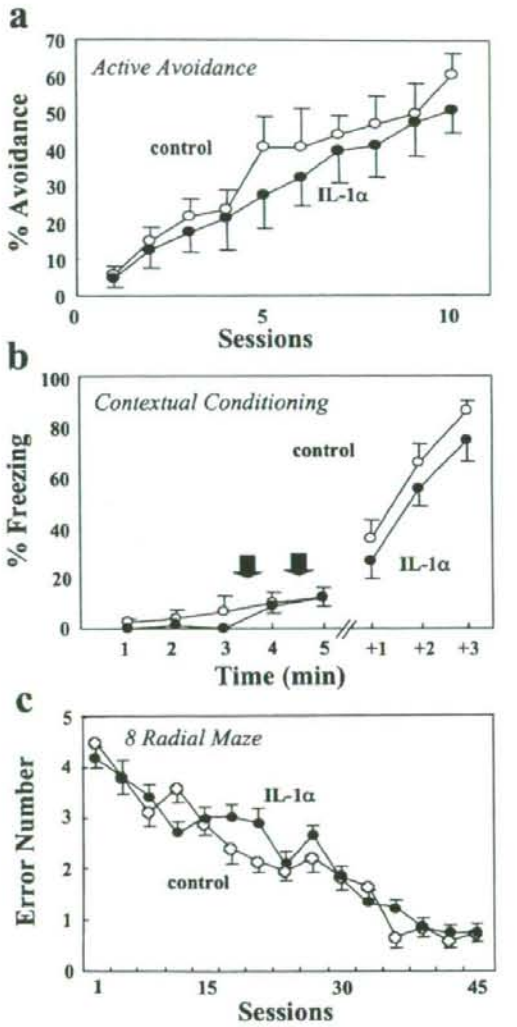

Fig. 11. Learning ability of IL-1α-treated rats. Learning in rats treated as neonates with IL-1α was assessed by active-avoidance response, contextual conditioning, and a radial arm maze task from PND56 to PND70. (a) The mean value (±S.E.M.) of percent avoidance response in active-avoidance test for control (open circle, $n=9$) and IL-1α-treated rats (closed circle, $n=9$) was determined at PND56-65. (b) Mean percentage (±S.E.M.) of freezing time of vehicle-treated (open circle, $n=15$) and IL-1α-treated rats (closed circle, $n=15$) was scored for 3 min before and 1 day after conditioning stimuli (arrows). (c) Spatial reference was scored in the eight-arm radial maze task. The number of errors was averaged over three sessions and compared between control (open circle, $n=10$) and IL-1α-treated rats (closed circle, $n=10$).

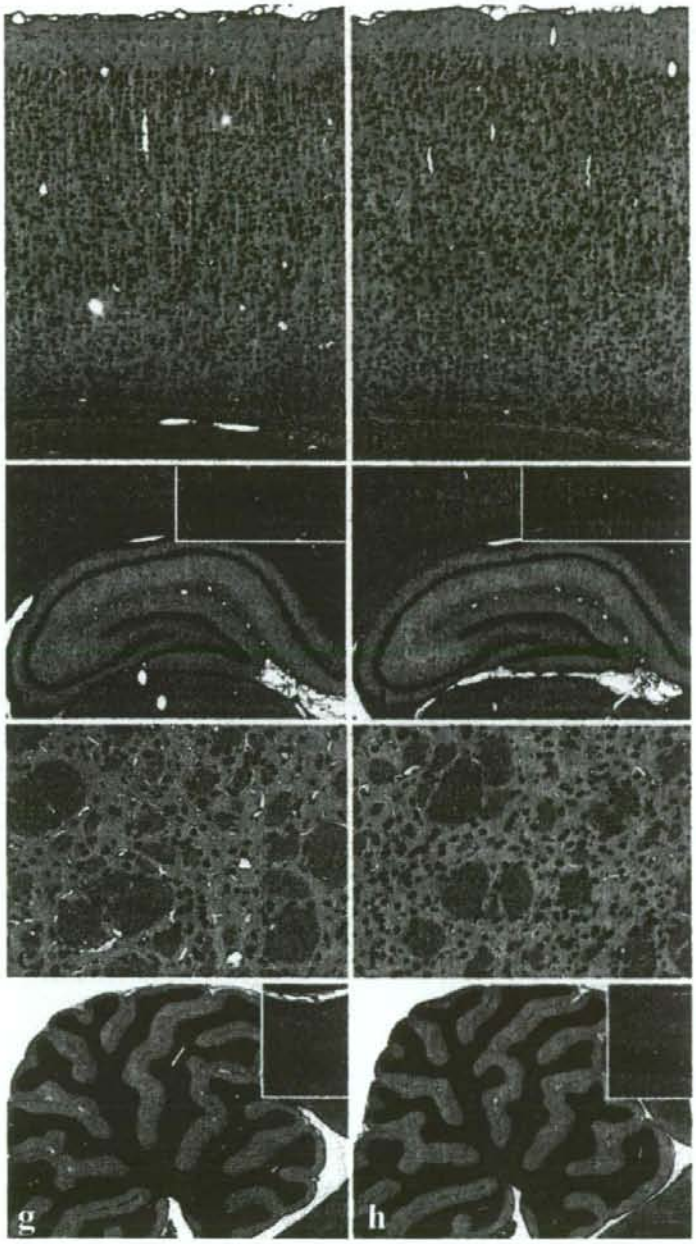

Fig. 12. Absence of gross neuropathological abnormality in the adult brain of IL-1α-treated animals. Sections from the cortex (a, b), hippocampus (c, d), striatum (e, f) and cerebellum (g, h) of rats treated with PBS (control; a, c, e, g) or IL-1α as neonates (b, d, f, h) were examined at PND60 with the Kluver-Barrera stain. Hematoxylin/eosin staining of the CA1 pyramidal layer and Purkinje layer is shown in the inset. Staining revealed no apparent differences in sections from three animals. Scale bars = 1.0 mm in c, d, g, h and 0.1 mm in a, b, e, f.

Borrell et al., 2002; Fatemi et al., 2002b; Zuckerman et al., 2003). A similar mechanism may contribute to the neurobehavioral impairments in other animal models of schizophrenia as brain injury and psychostimulants also induce IL-1 (Ho and Blum, 1998; Touzani et al., 1999; Jankowsky and Patterson, 2001; Wang et al., 2001; Lipska et al., 2002). In this context, it is noteworthy that reductions in dopamine receptors and GAD67, which were observed in the present

IL-1 model, have been implicated in the neuropathology associated with schizophrenia (Akbarian et al., 1995; Okubo et al., 1997; Hakak et al., 2001). Although we failed to detect significant difference in these phenotypic markers at the adult stage, we cannot rule out the possibility that such neurochemical abnormality might persist in limited brain region(s) until adulthood.

A postmortem study indicates higher vulnerability of IL-1-mediated neuroinflammatory reactions in schizophrenia patients (Toyooka et al., 2003) and genetic investigations point out the association of the IL-1 gene complex and the risk of this illness (Katila et al., 1999; Meisenzahl et al., 2001; Zanardini et al., 2003). Both results suggest a potential biological link between IL-1-triggered neuroinflammatory processes and schizophrenia etiology or pathology. A variety of cytokines interacts with IL-1α in such neuropathological conditions (Hornig et al., 1999; Jankowsky and Patterson, 2001; Watanabe et al., 2003; Meyer et al., 2006b,c). It is possible that IL-1 affects the production and signaling of neurotrophic molecules such as BDNF and indirectly influences neurobehavioral development as well as psychopathological traits (Tong et al., 2007; Angelucci et al., 2005). In this context, neuropathological functions and neurobehavioral impact of other cytokines in prenatal and postnatal immune-inflammatory processes remain to be characterized. The present animal experiments indicate that, when immune inflammatory reactions occur and induce IL-1α at embryonic or early postnatal stages, this cytokine reaches immature brain and impairs later development of sensorimotor gating. In humans, under analogous circumstances, IL-1 and other inflammatory cytokine may contribute to the risk of developing schizophrenia or other psychobehavioral impairments.

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