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Neonatal polyI:C treatment in mice results in schizophrenia-like behavioral and neurochemical abnormalities in adulthood

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ABSTRACT

It has been reported that viral infection in the first and second trimesters of pregnancy in humans increases the risk of subsequently developing schizophrenia. To develop a mouse model of immune activation during the early postnatal period, neonatal ICR mice were repeatedly injected with polyriboinosinic–polyribocytidilic acid (polyI:C; an inducer of strong innate immune responses) for 5 days (postnatal day 2–6) which may correspond, in terms of brain development, to the early second trimester in human. Cognitive and emotional behavior as well as the extracellular level of glutamate in the hippocampus were analyzed at the age of 10–12 weeks old. PolyI:C-treated mice showed anxiety-like behavior, impairment of object recognition memory and social behavior, and sensorimotor gating deficits, as compared to the saline-treated control group. Depolarization-evoked glutamate release in the hippocampus was impaired in polyI:C-treated mice compared to saline-treated control mice. Furthermore, to investigate the effect of neonatal immune activation on the expression levels of schizophrenia-related genes, we analyzed mRNA levels in the hippocampus 2 and 24 h after polyI:C treatment. No significant differences or only transient and marginal changes were observed between polyI:C-treated and saline-treated control mice in the expression levels of schizophrenia-related genes in the hippocampus.

1. Introduction

Schizophrenia is a chronic mental disorder characterized by psychosis (e.g., hallucinations and delusions), flattened emotions and impaired cognitive function, which affects about 1% of the general population. Although the disease etiology remains poorly understood, various hypotheses, including dopamine hyperfunction, glutamatergic hypofunction, GABAergic deficits, stress vulnerability and impaired neurodevelopment have been proposed as the etiology/pathophysiology of schizophrenia (Nawa and Takei, 2006; Tan et al., 2008). Among them, the neurodevelopmental hypothesis of schizophrenia supported by clinical, neuroimaging, neuropathologic, and genetic studies, is a more fundamental theory that does not conflict with other hypotheses (Sawa and Snyder, 2002; Ross et al., 2006).

The estimated heritability of schizophrenia is approximately 80% (Burmeister et al., 2008), and recent studies have identified possible candidate susceptibility genes for schizophrenia, such as *dysbindin*, *neuregulin1* and *disrupted-in-schizophrenia 1* (*DISC1*) (Harrison and Weinberger, 2005; Gogos and Gerber, 2006; Ross et al., 2006). Not only genetic factors, but also environmental factors are important in the etiology of mental disorders, and are believed to interact in most cases of schizophrenia (Caspi and

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Moffitt, 2006; Burmeister et al., 2008). One of the most significant environmental risk factors for schizophrenia is maternal viral infection in the first (Brown et al., 2000, 2004) and second (Mednick et al., 1988; Clarke et al., 2006) trimester of pregnancy, which could affect brain development by releasing stress hormones, producing hypoxia, hyperthermia, or malnutrition, or by triggering proinflammatory cytokine responses in the mother, the placenta, or the fetus (Patterson, 2007).

Polyriboinosinic–polyribocytidilic acid (polyI:C) is a synthetic analogue of double-stranded RNA that leads to the pronounced but time-limited production of pro-inflammatory cytokines after administration to mammalian organisms through the activation of toll-like receptor 3 (Wang et al., 2004). Maternal immune activation by polyI:C exposure in rodents is known to precipitate a wide spectrum of behavioral, cognitive and pharmacological abnormalities in adult offspring (Shi et al., 2003; Zuckerman et al., 2003; Meyer et al., 2005, 2006; Ozawa et al., 2006; Cameron et al., 2007; Smith et al., 2007).

There are some concerns about the prenatal polyI:C treatment model of schizophrenia. First, prenatal treatment with polyI:C in pregnant dams is reported to increase the rate of abortion. Some of the dams administrated polyI:C could not bear live pups due to abortion (Ozawa et al., 2006; our unpublished observation). Second, it is controversial to match pregnancy stages between rodents and humans. Glial proliferation and migration, as well as establishment of the blood–brain barrier, peak during the early postnatal period in rodents (Clancy et al., 2001; Nawa and Takei, 2006; Mouri et al., 2007), but such a critical developmental stage occurs *in utero*, more specifically in the second trimester of pregnancy in humans (Adinolfi et al., 1976; Nawa and Takei, 2006). The correspondence of fetal development progression in different species can be compared using database-driven websites. The programs are based on statistical algorithms that integrate hundreds of empirically derived developing neuronal events in ten mammalian species, including rats, mice and humans (http://translatingtime.net/; see also Clancy et al., 2007).

In the present study, we sought to develop a novel mouse model in which immune activation is induced during the brain development matching the stage of fetus in the second trimester of pregnancy in humans. Accordingly, neonatal ICR mice were repeatedly injected with polyI:C for 5 days from postnatal day 2–6, which correspond to post-conception day 128–158 for cortical events and 93–115 for limbic events of brain development in humans (http://translatingtime.net). Cognitive and emotional behaviors as well as glutamatergic neurotransmission in the hippocampus were analyzed in the adult mice. Furthermore, we analyzed changes in the gene expression in the hippocampus of polyI:C-treated mice using DNA microarray and real-time reverse transcription (RT)-PCR.

2. Experimental procedures

2.1. Animals

ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and maintained under standard specific pathogen-free environmental conditions. Pregnant females were monitored for the parturition date, which was taken as postnatal day (PD) 0. They were housed under a standard 12-h light/dark cycle (lights on at 9:00) at a constant temperature of 23 ± 1 °C, with free access to food and water throughout the experiments. We used male mice exclusively to minimize any potential variability due to sex-specific effects in behavioral performance. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Treatment

All litters were randomly divided into saline and polyI:C-treated groups. From PD 2 to 6, mice were injected s.c. daily with either pyrogen-free saline or polyI:C (Sigma–Aldrich, St. Louis, MO) at a dose of 5 mg/kg. The time needed for polyI:C injection in each mouse was less than 1 min, which minimized the influence of separation of neonates from the mothers on maternal behaviors directed towards the pups. Animals were weaned at PD 21, and divided by gender at PD 28. Both groups were derived from at least 3 different litters to preclude possible differences in individual maternal behavior as a mitigating factor in any subsequent long-lasting changes induced in the offspring.

2.3. Nissl staining

Mice were deeply anesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, post-fixed with the same fixative and cryoprotected with 30% sucrose containing PBS. Twenty micrometers thick coronal brain sections were cut on a cryostat and mounted on slides. Nissl staining was done according to standard procedures (Takuma et al., 2007). Nissl stained sections were analyzed using a light microscope (Axio Imager; Zeiss, Jene, Germany).

2.4. Behavioral analysis

Behavioral analysis of polyI:C-treated mice was carried out at the age of 10–12 weeks old.

2.5. Locomotor activity under a novel environment

Each mouse was placed in a standard transparent rectangular rodent cage (25 cm × 30 cm × 18 cm) under moderately light conditions (15 lx). Locomotor activity was then measured for 120 min using an infrared sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed over the cage (Kamei et al., 2006).

To investigate the effect of neonatal treatment with polyI:C on the sensitivity to MK-801 (Sigma–Aldrich, St. Louis, MO) or methamphetamine hydrochloride (METH; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), each mouse was allowed a 120-min habituation period before MK-801 (0.1–0.3 mg/kg, i.p.) or METH (2 mg/kg, i.p.) treatment. Locomotor activity was then measured for 180 min immediately after MK-801 or METH treatment.

2.6. Open field test

Mice were placed in the center of the arena and were allowed to explore the open field (diameter: 60 cm, height: 35 cm) for the following 5 min under moderately light conditions (80 lx), while their activity was measured automatically using the Ethovision automated tracking program (Brainscience Idea Co., Ltd., Osaka, Japan) (Lee et al., 2005; Wang et al., 2007). The open field was divided into an inner circle (diameter: 40 cm), and an outer area surrounding the inner circle. The movement of mice was measured via a camera mounted above the open field. Measurements included distance and time spent in the inner and outer sections.

2.7. Novel object recognition test

A novel object recognition test was carried out as described previously (Kamei et al., 2006; Nagai et al., 2007). Mice were individually habituated to an open-box (30 × 30 × 35 high cm) for 3 days. During the training session, two novel objects were placed in the open field and the animals were allowed to explore for 10 min under moderately light conditions (10 lx). The time spent exploring each object was recorded. During retention sessions, the animals were placed back into the same box 24 h after the training session, one of the familiar objects used during training was replaced by a novel object, and the mice were allowed to explore freely for 5 min. The preference index in the retention session, the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of time spent exploring the object that was replaced by a novel object in the retention session, to the total exploration time.

2.8. Social interaction test

We used the experimental paradigm described by Tremolizzo et al. (2005) to measure social behavior (e.g., social interaction, aggression and escape behavior). PolyI:C-treated or saline-treated control mice were individually housed in a home cage (29 cm × 18 cm × 12 cm) for 2 days before the trial. We used 10–15-week-old male ICR mice as intruders which had not shown aggressive behavior. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage under bright light conditions (75 lx). The duration of social interaction (close following, inspection, anogenital sniffing, and other social body contacts except aggressive behavior), aggression (attacking/biting and tail rattling) and escape behavior were analyzed. Four trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse.

2.9. Prepulse inhibition test

The prepulse inhibition (PPI) test was carried out as described previously (Takahashi et al., 2007; Arai et al., 2008). After the animals were placed in the chamber under moderately bright light conditions (180 lx) (San Diego Instruments, San Diego, CA), they were allowed to habituate for 10 min, during which 65 dB

background white noise was present. The animals then received 10 startle trials, 10 no-stimulus trials and 40 PPI trials. The inter-trial interval was between 10 and 20 s and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. PPI trials consisted of a prepulse (20 ms burst of white noise at 69, 73, 77 or 81 dB intensity) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the four prepulse trials (69, 73, 77 or 81 dB) was presented 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]\%$, in which PPx was the mean of the 10 PPI trials (PP69, PP73, PP75 or PP80) and P120 was the basal startle amplitude.

2.10. In vivo microdialysis

The *in vivo* microdialysis study was carried out in mice that had previously not been used for behavioral experiments. To measure extracellular glutamate release in the hippocampus, *in vivo* microdialysis was carried out as described previously (Murai et al., 2007). Mice at the age of 10–12 weeks old were anesthetized with pentobarbital Na (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA). A guide cannula (AG-4 EICOM Corp., Kyoto, Japan) was implanted into the hippocampus [AP: −3.3, ML: +2.8 from the bregma, DV: −2.5 mm from the skull] according to the atlas (Paxinos and Franklin, 2004). A dialysis probe (A-I-4-01; membrane length 1 mm, EICOM Corp.) was implanted into the hippocampus and Ringer solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1.0 μl/min, 2 days after implantation of the guide cannula. The dialysate was collected every 10 min and the amount of glutamate in the dialysate was determined using HPLC (HTEC-500, EICOM Corp.) with electrochemical detection. Three samples were taken to establish baseline levels of extracellular glutamate. For depolarization stimulation, 60 mM KCl-containing Ringer solution was delivered through the dialysis probe for 20 min in order to induce the K^+-evoked release of glutamate.

In some experiments, Ca^{2+}-free Ringer solution (149.3 mM NaCl and 4 mM KCl) with tetrodotoxin [TTX: (1 μM), Nacalai Tesque Co., Ltd., Kyoto, Japan)] was perfused for 60 min, following the measurement of basal glutamate levels.

2.11. DNA microarray

Neonatal mice were decapitated 2 or 24 h after the final treatment with polyI:C and their brains were removed. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). DNA microarray and expression profiling were carried out as described previously (Ibi et al., 2008). Purified total RNA was used for expression profiling with GeneChip mouse genome 430 2.0 arrays for DNA (Affymetrix, Santa Clara, CA), containing 45,101 probe sets, according to the manufacturer's protocol.

2.12. Quantitative analyses of schizophrenia susceptibility gene expression by real-time RT-PCR

Total RNA isolated from the hippocampus was converted into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Levels of mRNA expression were quantified using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in a volume of 25 μl with 500 ng cDNA and 500 nM primers in the Power SYBR Green Master Mix (Applied Biosystems). The primers are described in Table 1.

2.13. Statistical analysis

Data are expressed as the mean ± SE. Differences between two groups were analyzed by two-tailed Student's *t*-test. Differences in body weight gain, locomotor activity, social interaction test, PPI test and K^+-induced (60 mM) glutamate release in microdialysis were analyzed by repeated analysis of variance (ANOVA). Multiple group comparisons were made by one-way ANOVA, followed by Bonferroni's test when *F* ratios were significant ($p < 0.05$).

3. Results

3.1. General appearance of polyI:C-treated mice

No mice died following repeated neonatal treatment with polyI:C at a dose of 5 mg/kg for 5 days. There was no apparent difference in maternal behaviors directed towards the saline- and polyI:C-treated pups. Repeated treatment with polyI:C in neonatal mice slightly decreased the body weight gain (less than 10%) in adolescence, but the effect was not statistically significant (Fig. 1a). There was no difference in exploratory locomotor activity between saline- and polyI:C-treated mice in adult (Fig. 1b). Furthermore, Nissl staining showed no apparent abnormalities of anatomy in the frontal cortex and hippocampus of polyI:C-treated mice in adult (Fig. 1c and d).

Table 1
Primers for schizophrenia-related and other genes.

Target gene	Forward	Reverse
GAD67	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC
Reelin	CCCAGCCCAGACAGACAGTT	CCAGGTGATGCCATTGTTGA
BDNF	TAAATGAAGTTTATACAGTACAGTGGTTCTACA	AGTTGTGCGCAAATGACTGTTT
COMT	GGGCACCCCAGGACCTTAT	GTCTGGAAGGTAGCGGTCTTTC
14-3-3 ε	GCCGAGCGATACGACGAA	CCACGTCCATCCCTGCTACT
PRODH	TCCTGAGCGCGTGTATCG	AGCAAGAAGACGCGCTTGA
mGlu3	ACCACCGCGGTCAGCTT	AGAGCCCGTCACTGAATTCCT
PPP3CC	CGACCGAGCGCGTCAT	GTTAGCCGTCGGGTTGGA
RGS4	CCTGCGAACACAGTTCTTCACA	TGGCTTACCCTCTGGCAAGT
DISC1	AGCTTCTCGGAGCCATGTACA	TCCCCCTGGAGAGACTGAAA
ATF4	CATGGCGCTCTTCACGAAAT	GAGGAATGTGCTTAACTCGAAGGT
FEZ1	TGGTCCTATGAAGGGCTGAGA	CGGTCCAGCAGCTCTGTCA
Npas3	GAACTCCAAGTCCGACGAGAAG	CGGTCAGGCTCCGGATCT
NUDEL	AAGCCAGAGATTTAAGGCAAGAAC	ACTTGCGGGTCACTTCCTGTT
LIS1	CACATTGATTTCATATGTACGTTGCA	ATCTTCGGTGAACTCTTAACAATCTG
KIF5A	AGAACAACCTGGAACAGCTTACAA	CGAAGTCGTTTTTCCAATTTAGGA
KIF5B	ACATTCTGCCCAGATTGCAAA	TGTGCCCGGGTGAGTTG
IFITM3	GCCTATGCCTACTCCGTGAAGT	GCCTGGGCTCCAGTCACAT
NRG1	GGGACCAGCCATCTCATAAAGT	CGCCTCCATTCACACAGAAA
DTNBP1	AGAAGGCCCTGGAAATGGA	AACTTCTGCCGCTCCTTCAG
PDE4B	GACTTGTCACCAAAAGCGATGTC	CAGGTCATCGCCGTGTTG
TNF-α	GCCAGCCGATGGGTTGT	GCAGCCTTGTCCCTTGAAGA
β-Actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCA

GAD67 (glutamic acid decarboxylase 67), BDNF (brain-derived neurotrophic factor), COMT (catechol-O-methyltransferase), PRODH (proline dehydrogenase), PPP3CC (protein phosphatase 3, catalytic subunit, gamma isoform), RGS4 (regulator of G-protein signaling 4), DISC1 (disrupted-in-schizophrenia 1), ATF4 (activating transcription factor 4), FEZ1 (fasciculation and elongation protein zeta 1), NPAS3 (neuronal PAS domain protein 3), NUDEL (nuclear distribution gene E-like homolog 1), LIS1 (platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit), KIF5A (kinesin family member 5A), KIF5B (kinesin family member 5B), IFITM3 (interferon-induced transmembrane 3), NRG1 (neuregulin 1), DTNBP1 (dystrobrevin binding protein 1), and PDE4B (phosphodiesterase 4B, cAMP specific).

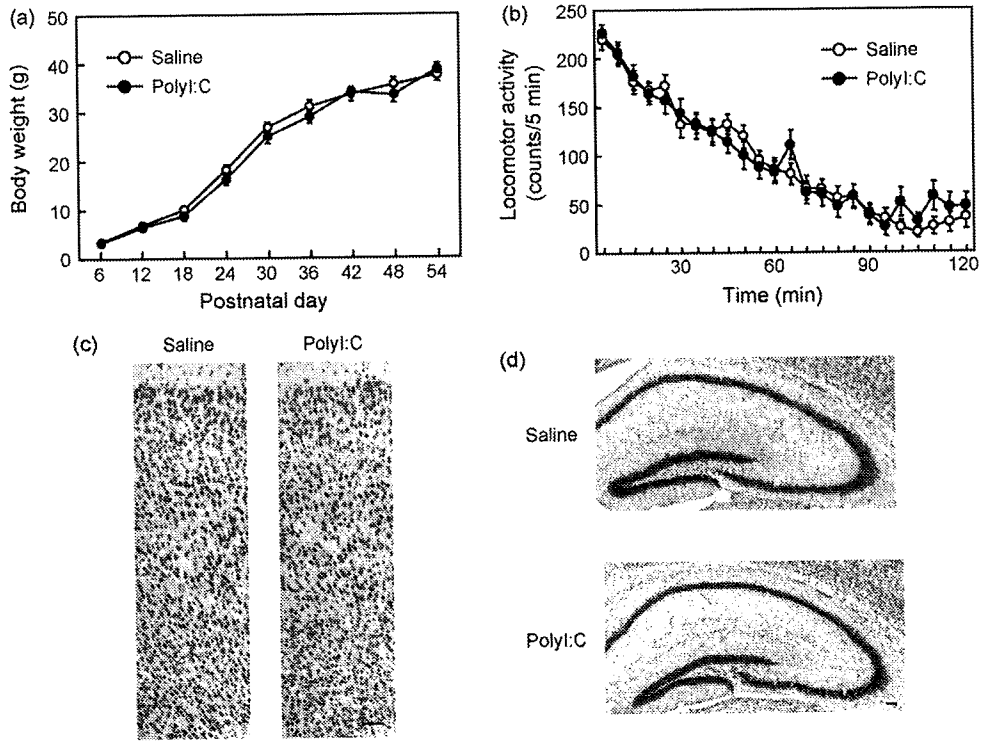

Fig. 1. General appearance and brain anatomy in polyI:C-treated mice. (a) Body weight gain after polyI:C treatment. (b) Exploratory locomotor activity under a novel environment. Gross brain anatomy in the (c) frontal cortex and (d) hippocampus. Scale bar: 200 μm. Values indicated the mean ± SE (a: $n = 11$–12, b: $n = 21$).

3.2. Effect of neonatal treatment with polyI:C on performance in the open field test

To investigate the effect of neonatal polyI:C treatment on general behavior in adult, an open field test was carried out, in which the conflict between the drive to explore a new environment and a natural aversion to illuminated open areas was used to examine both anxiety and motor activity (Wang et al., 2007). The time spent in the inner sector of the open field by the polyI:C-treated group was significantly less than by the saline-treated control group (Fig. 2a). In contrast, the time spent in the outer sector by the polyI:C-treated group was significantly more than by the control group (Fig. 2b). The distance traveled in the outer sector by the polyI:C-treated group was also significantly greater than by the control group ($p < 0.05$), although there was no difference in the inner sector between the groups (data not shown; $p > 0.05$). There was no difference between saline-treated and polyI:C-treated groups in the total distance traveled in the open field test, including inner and outer sectors (data not shown), suggesting that neonatal treatment with polyI:C does not affect motor function in adult. These results indicate that mice with neonatal polyI:C treatment avoid the inner sector and spend more time in the outer sector of the open field, suggesting increased anxiety of polyI:C-treated mice in adult.

3.3. Effect of neonatal treatment with polyI:C on performance in the novel object recognition test

During the training session, both polyI:C-treated and saline-treated control mice spent equal amounts of time exploring either of the two objects (Fig. 3b), and there was no biased exploratory preference in either group (Fig. 3a), suggesting no differences in motivation and curiosity about novel objects, and motor function between polyI:C-treated and saline-treated control mice. In the retention session, which was carried out 24 hr after the training session, the level of exploratory preference to the novel object in polyI:C-treated mice was significantly decreased compared to saline-treated control mice (Fig. 3b). Total exploration time in the retention

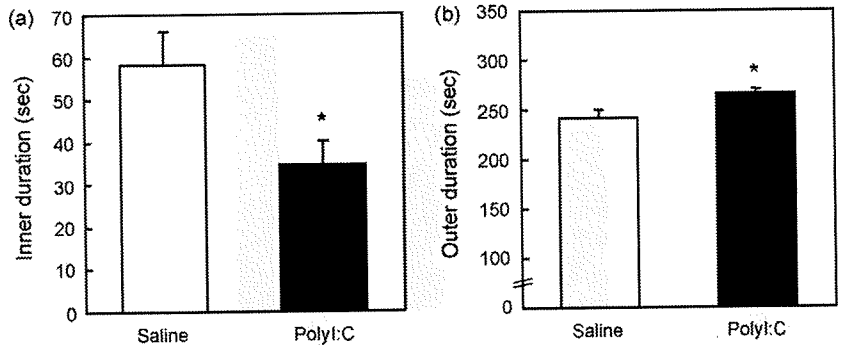

Fig. 2. Effect of neonatal polyI:C treatment on anxiety-like behavior in mice. Individual mice were allowed to explore the open field freely for 5 min. Time spent in (a) inner and (b) outer sectors. Values indicated the mean ± SE ($n = 7$–8). *$p < 0.05$ vs. saline-treated control group (two-tailed t-test).

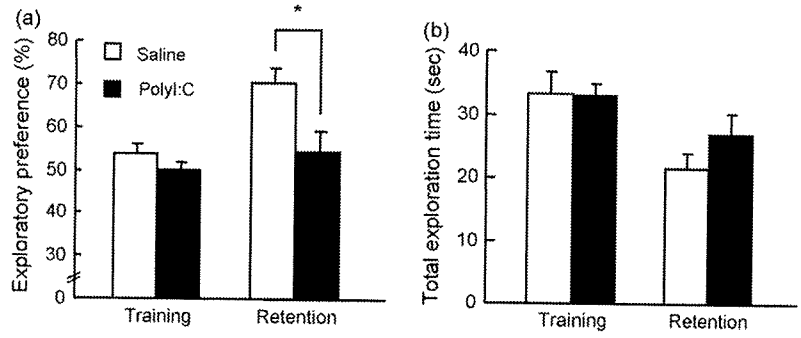

Fig. 3. Effect of neonatal polyI:C treatment on recognition memory in mice. (a) Exploratory preference. (b) Total exploration time. The retention session was carried out 24 h after the training session. Values indicated the mean ± SE ($n = 8$). *$p < 0.05$ vs. saline-treated control group (two-tailed t-test).

session did not differ between groups. These results suggest that polyI:C-treated mice have impaired recognition memory in adult.

3.4. Effect of neonatal treatment with polyI:C on performance in the PPI test

Repeated measures ANOVA of the data (73–77 dB) revealed significant effects of treatment [$F_{(1,14)} = 4.658$, $p < 0.05$] and prepulse intensity [$F_{(1,14)} = 14.644$, $p < 0.01$], but no interaction of treatment and prepulse intensity [$F_{(1,14)} = 0.061$, $p > 0.05$] (Fig. 4a). There was no difference in startle amplitude between control and polyI:C-treated groups (Fig. 4b). These results suggest that neonatal treatment with polyI:C induces PPI deficits of acoustic startle response in adulthood, suggesting an impairment of sensorimotor gating function.

3.5. Effect of neonatal treatment with polyI:C on performance in the social interaction test

In saline-treated control mice, repeated exposure to the same unfamiliar intruder mouse caused a gradual decrease in the social interaction time. In contrast, polyI:C-treated mice exhibited marked reduction of the social interaction time in trials 2–4, compared with the control group, although there was no difference in trial 1 between groups. Therefore, repeated measures ANOVA of the data (trials 2–4) revealed significant effects of treatment on social interaction [$F_{(1,10)} = 5.446$, $p < 0.05$] (Fig. 5a), but not escape [$F_{(1,10)} = 2.520$, $p = 0.146$] or aggressive behavior [$F_{(1,10)} = 0.590$, $p = 0.460$] (Fig. 5b and c), suggesting that mice receiving neonatal polyI:C treatment may possess the same level of curiosity as control mice (trial 1), but curiosity may be easily lost following repeated exposure (trials 2–4). Impaired social interaction in polyI:C-treated mice appears not to be due to the change in escape or aggressive behavior.

3.6. Effect of neonatal treatment with polyI:C on sensitivity to MK-801 or METH

Sensitivity to METH, an indirect dopaminergic agonist, or MK-801, a non-competitive N-methyl D-aspartate (NMDA) receptor blocker, is increased in individuals with schizophrenia (Lieberman et al., 1987; Duncan et al., 1999) as well as in several animal models of schizophrenia (Sakae et al., 2008). To investigate the sensitivity to METH or MK-801 in polyI:C-treated mice, locomotor activity was measured following the drug treatment. There were no differences between polyI:C-treated and saline-treated control mice in METH- or MK-801-induced hyperactivity. Repeated measures ANOVA of the data revealed no significant effects of treatment on METH-induced hyperactivity at 2 mg/kg [$F_{(1,16)} = 0.553$, $p = 0.468$], or MK-801-induced hyperactivity at 0.1 mg/kg [$F_{(1,15)} = 1.001$, $p = 0.333$] and 0.3 mg/kg [$F_{(1,16)} = 3.145$, $p = 0.0952$].

3.7. Effect of neonatal treatment with polyI:C on glutamate release in the hippocampus

Hypofunction of the glutamatergic system, such as impaired glutamate neurotransmission, has been proposed in the pathophysiology of schizophrenia (Javitt, 2004; McGuire et al., 2008). We focused on glutamate neurotransmission in the hippocampus

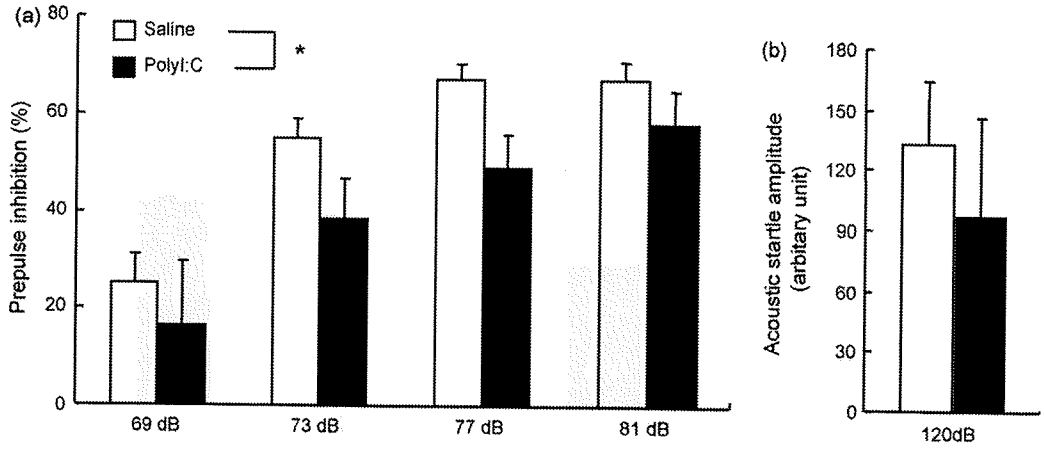

Fig. 4. Effect of neonatal polyI:C treatment on PPI in mice. (a) PPI (%) at four different prepulse intensities (69, 73, 77 and 81 dB). (b) Acoustic startle amplitude as measured in trials without prepulse. Values indicated the mean ± SE ($n = 8$). *$p < 0.05$ vs. saline-treated control group.

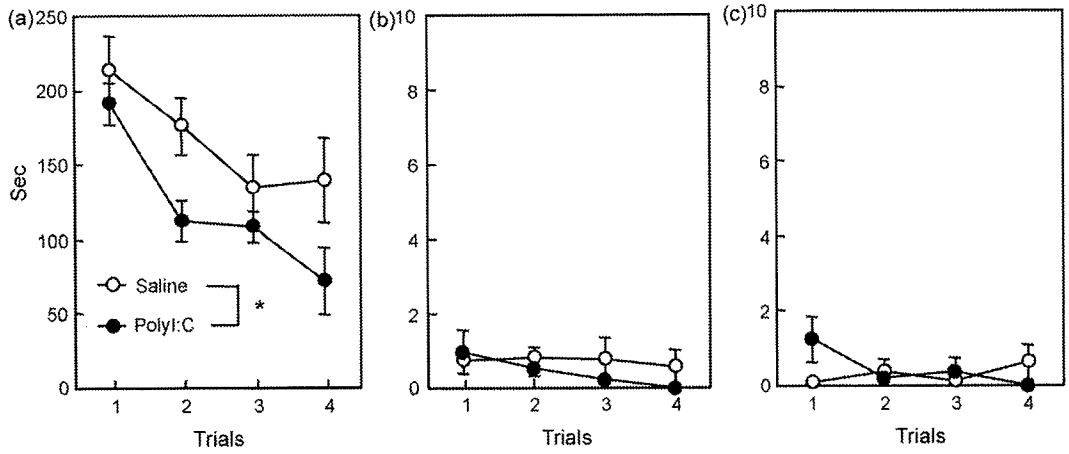

Fig. 5. Effect of neonatal polyI:C treatment on social behavior in mice. (a) Social interaction, (b) escape behavior and (c) aggressive behavior for 5 min in each trial. Each trial for 5 min was separated by a 30-min interval. Values indicated the mean ± SE ($n = 8$). $^{*}p < 0.05$ vs. saline-treated control group.

because hippocampal function is impaired in schizophrenia patients (Tamminga and Holcomb, 2005) and the hippocampus is developed and maturated during early neonatal period (Clancy et al., 2001).

PolyI:C-treated mice showed a significant increase in the basal level of glutamate in the hippocampus compared with the level in the saline-treated control group [saline-treated control ($n = 7$): 3.39 ± 0.68 pmol/10 μl, polyI:C-treated group ($n = 7$): 6.45 ± 1.01 pmol/10 μl]. Importantly, the elevated basal level of extracellular glutamate in the hippocampus of polyI:C-treated mice fell to the level in the control group by administering Ca^{2+}-free Ringer with TTX through the dialysis probe (Fig. 6a: $F_{(3,34)} = 7.134$, $p < 0.01$). The results suggest that increased basal glutamate levels in polyI:C-treated mice originally derived from neurons.

Following the measurement of basal glutamate levels, depolarization-evoked glutamate release in the hippocampus was evoked by delivering 60 mM KCl-containing Ringer through the dialysis probe for 20 min. The magnitude of high K^{+}-induced glutamate release in the hippocampus of polyI:C-treated mice was significantly decreased compared with the response in saline-treated control mice (Fig. 6b). Repeated measures ANOVA of the data revealed significant effects of group [$F_{(1,12)} = 9.697$, $p < 0.01$] and time [$F_{(3,36)} = 19.368$, $p < 0.01$], but no interaction (e.g., group × time) [$F_{(3,36)} = 2.109$, $p = 0.116$]. These results suggest that neonatal treatment with polyI:C impairs glutamatergic neurotransmission in the hippocampus in adult.

3.8. Changes in gene expression in the hippocampus 24 h after final polyI:C treatment

Scatter plots and hierarchical clustering analysis showed no obvious changes in global expression profiles between saline-treated control and polyI:C-treated mice; therefore, we analyzed changes in individual gene expression levels between polyI:C-treated and saline-treated control groups. Table 2 shows the genes whose expression ratio in the polyI:C-treated group was altered more than twofold from the level in the saline-treated control group. Student's *t*-test indicated that expression levels of some genes (*Srd5a2l*, *Creg2* and *Ptgds*) were significantly altered among the genes described in Table 2.

3.9. Effect of neonatal treatment with polyI:C on the expression of schizophrenia-related genes in the hippocampus

We analyzed the mRNA levels of schizophrenia-related genes in the hippocampus 2 and 24 h after the final treatment of polyI:C, using real-time RT-PCR. No marked differences or only transient and marginal changes were observed between polyI:C-treated and saline-treated control mice (Table 3). Among the genes examined, mRNA levels of *phosphatase 3, catalytic subunit, gamma isoform* (*PPP3CC*), *activating transcription factor 4* (*ATF4*), *kinesin family member 5B* (*KIF5B*) and *interferon-induced transmembrane protein 3* (*IFITM3*, also known as *interferon-inducible 1-8U*), were signifi-

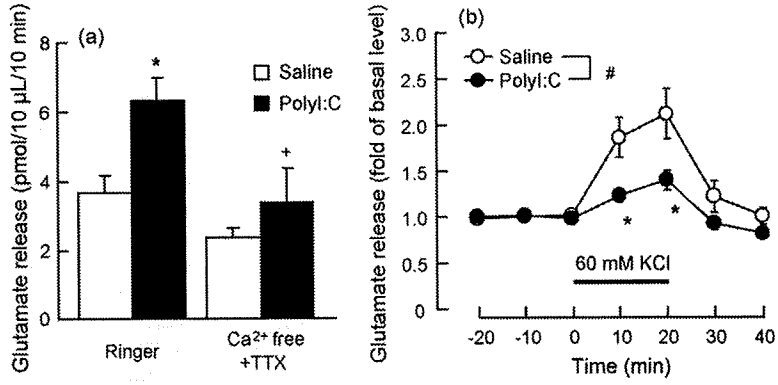

Fig. 6. Effect of neonatal polyI:C treatment on depolarization-evoked glutamate release in the hippocampus of mice. (a) Basal extracellular levels of glutamate and (b) K^{+}-induced (60 mM) glutamate release in the hippocampus of saline- or polyI:C-treated mice were determined by a microdialysis method. Each fraction was collected for 10 min. Values indicated the mean ± SE (a: $n = 6$–13, b: $n = 7$–8). (a) $^{*}p < 0.05$ vs. Ringer-perfused saline-treated control group. $^{+}p < 0.05$ vs. Ringer-perfused polyI:C-treated group. (b) $^{*}p < 0.05$ vs. saline-treated control group (two-tailed *t*-test). $^{\#}p < 0.05$ vs. saline-treated control group.

Table 2
Genes whose expression ratio in polyI:C-treated group was altered more than 2-fold from the level in saline-treated control group.

Gene title	Public ID	Gene symbol	Probe	Expression ratio	*p*-value
Histocompatibility 2, K1, K region	S70184	*H2-K1*	1427746_x_at	3.87	0.21
C-src tyrosine kinase	BB339034	*Csk*	1439744_at	3.47	0.07
Histocompatibility 2, K1, K region	L23495	*H2-K1*	1424948_x_at	3.11	0.16
Histocompatibility 2, K1, K region	BC011306	*H2-K1*	1425336_x_at	2.69	0.17
Beta-2 microglobulin	BF715219	*B2m*	1449289_a_at	2.65	0.10
P lysozyme structural	AV066625	*Lzp-s*	1436996_x_at	2.65	0.25
Beta-2 microglobulin	AI099111	*B2m*	1452428_a_at	2.6	0.05
Similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor	M34962	*LOC547343*	1451683_x_at	2.49	0.16
Lymphocyte antigen 6 complex, locus A	BC002070	*Ly6a*	1417185_at	2.42	0.20
Histocompatibility 2, D region	M69068	*H2-L*	1451931_x_at	2.4	0.12
Histocompatibility 2, D region locus 1	M86502	*H2-D1*	1425545_x_at	2.35	0.12
Histocompatibility 2, D region locus 1	L36068	*H2-D1*	1451784_x_at	2.27	0.12
Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	NM_012011	*Eif2s3y*	1417210_at	2.25	0.32
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1452077_at	2.17	0.33
Interferon induced transmembrane protein 3	BC010291	*Ifitm3*	1423754_at	2.16	0.12
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1426438_at	2.1	0.37
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1426439_at	2.03	0.44
RIKEN cDNA 1500015O10 gene	BB392676	*1500015O10Rik*	1460049_s_at	0.49	0.35
Steroid 5 alpha-reductase 2-like	BB825787	*Srd5a2l*	1439241_x_at	0.49	<0.05
Cellular repressor of E1A-stimulated genes 2	AV338277	*Creg2*	1436850_at	0.47	<0.05
Prostaglandin D2 synthase (brain)	AB006361	*Ptgds*	1423859_a_at	0.45	<0.05
Chloride intracellular channel 6	BQ176424	*Clic6*	1454866_s_at	0.44	0.41
Transthyretin	BC024702	*Ttr*	1451580_a_at	0.43	0.41
Folate receptor 1 (adult)	BG245669	*Folr1*	1450995_at	0.41	0.33
Inactive × specific transcripts	L04961	*Xist*	1427263_at	0.38	0.32
EST	BF537798	–	1438403_s_at	0.38	<0.05
Inactive × specific transcripts	L04961	*Xist*	1427262_at	0.38	0.31
Prostaglandin D2 synthase (brain)	AB006361	*Ptgds*	1423860_at	0.37	<0.05
Potassium voltage-gated channel, Isk-related subfamily, gene 2	NM_134110	*Kcne2*	1449421_a_at	0.37	0.38

Neonatal mice were sacrificed 24 h after final treatment with polyI:C for 5 days. Values indicated the mean of 3 mice.

cantly increased 2 h after the final treatment of polyI:C, compared to the saline-treated control group. At 24 h after polyI:C treatment, only the *IFITM3* mRNA level was significantly increased in the polyI:C-treated group compared to the saline-treated control group. In addition, the gene expression of tumor necrosis factor-α (TNF-α) was markedly increased in the polyI:C-treated group, suggesting a proinflammatory response in the hippocampus following polyI:C treatment.

Table 3
Expression levels of schizophrenia-related genes in the hippocampus after the final polyI:C treatment in neonatal mice.

	2 h Δ*Ct*				24 h Δ*Ct*			
	Saline	Poly I:C		*p*-value	Saline	Poly I:C		*p*-value
GAD67	4.198	4.108		0.613	4.096	3.975		0.466
Reelin	4.864	4.834		0.792	5.415	5.465		0.787
BDNF	5.024	5.130		0.709	5.308	5.454		0.366
COMT	5.675	5.628		0.597	5.086	5.143		0.588
14-3-3 ε	1.851	1.790		0.491	2.483	2.345		0.427
PRODH	12.674	12.348		0.181	12.849	12.930		0.627
mGlu3	0.090	0.158		0.952	6.893	6.890		0.983
PPP3CC	7.522	7.311	↑	< 0.05	7.982	8.053		0.611
RGS4	6.557	6.282		0.314	5.519	5.679		0.288
DISC1	13.511	13.164		0.073	12.124	12.135		0.937
ATF4	4.708	4.473	↑	< 0.05	5.071	5.059		0.942
FEZ1	3.820	3.624		0.117	3.023	3.076		0.696
Npas3	7.168	6.984		0.213	6.845	6.845		1.000
NUDEL	5.501	5.399		0.262	6.023	6.035		0.938
LIS1	2.048	1.965		0.316	1.313	1.454		0.342
KIF5A	3.644	3.572		0.688	4.338	4.510		0.353
KIF5B	5.506	5.306	↑	< 0.05	6.635	6.691		0.774
IFITM3	5.703	5.100	↑	< 0.01	6.784	5.893	↑	<0.001
NRG1	8.058	8.010		0.816	8.260	8.274		0.878
DTNBP1	5.691	5.588		0.211	4.571	4.612		0.747
PDE4B	7.070	6.948		0.306	6.834	7.003		0.157
TNF-α	13.507	13.048	↑	< 0.05	14.282	14.291		0.951

Neonatal mice were sacrificed 2 and 24 h after final treatment with polyI:C. Values indicate the mean of 8 mice. ↑: gene expression was significantly increased. Δ*Ct*: the *Ct* (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Δ*Ct* value is calculated by subtracting the number of cycles of the target gene from that of β-actin.

4. Discussion

We demonstrated in the present study that neonatal polyI:C treatment at 5 mg/kg for 5 days (PD 2–6) in ICR mice resulted in emotional and cognitive impairment, which was associated with the dysfunction of hippocampal glutamatergic neurotransmission in adulthood. Table 4 summarizes the differences of behavioral changes between prenatal and postnatal polyI:C injection models. Impairments of social interaction, memory and prepulse inhibition were evident in both prenatal and postnatal polyI:C injection models. PolyI:C treatment in pregnant dams caused an increase in sensitivity to METH and an impairment of latent inhibition in offspring, while neonatal treatment of polyI:C had no effect on these behaviors. The results suggest that there may be some commonality but also difference between the effects of neonatal and prenatal polyI:C treatment on phenotypic changes in adulthood (Meyer et al., 2005, 2008; Ozawa et al., 2006; Smith et al., 2007; Zuckerman et al., 2003; Zuckerman and Weiner, 2005).

Previous studies demonstrated that prenatal polyI:C treatment-induced cytokines, such as TNF-α and interleukin 1β, 6 and 10, in the serum of both mother and fetus, and polyI:C induced an increase in BBB permeability (Meyer et al., 2006; Chen et al., 2007). In the present study, we found that neonatal polyI:C treatment in mice significantly increased *TNF-α* mRNA levels in the hippocampus 2 h after final polyI:C treatment, compared with the saline-treated control (Table 3). Furthermore, *IFITM3* mRNA levels induced by type I interferon (Liu et al., 2002) were significantly increased, at least up to 24 h after polyI:C treatment. These findings suggested that repeated polyI:C treatment in neonatal

Table 4
Comparison of behavioral changes between prenatal and postnatal polyI:C injection models.

	Present study	Ozawa et al. (2006)	Meyer et al. (2005, 2008)	Smith et al. (2007)	Zuckerman et al. (2003) and Zuckerman and Weiner (2005)
Animal	ICR mouse	BALB/c mouse	C57BL6/J mouse	C57BL6/J mouse	Wistar rat
Time of polyI:C treatment	PD 2–6	GD12–GD17	GD9	GD12.5	GD15
Dose of polyI:C	5 mg/kg	5 mg/kg	5 mg/kg	20 mg/kg	4 mg/kg
Locomotor activity	=	=	=	↓	=
Methamphetamine-induced hyperactivity	=	↑	↑	n/a	↑
MK-801-induced hyperactivity	=	n/a	=	n/a	↑
Social interaction	↓	n/a	n/a	↓	n/a
Anxiety-like behavior in open-field	↑	↓	↑	↑	n/a
Object recognition memory	↓	↓	n/a	n/a	n/a
Prepulse inhibition	↓	↓	↓	↓	n/a
Latent inhibition	=	n/a	↓	↓	↓

PD: postnatal day, GD: gestation day, ↑: higher than vehicle-treated control, ↓: lower than vehicle-treated control, =: no difference, and n/a: not reported.

mice causes an inflammatory response in the hippocampus, while there are some differences in cytokine responses between gestational and neonatal immunochallenge induced by polyI:C.

It is proposed that hypofunction of the glutamatergic system plays a crucial role in the disease (Coyle and Tsai, 2004; Li et al., 2007). In the present study the animal model of early postnatal immune activation with polyI:C treatment showed an increased basal extracellular glutamate level in the hippocampus with an impairment of high K^+-induced glutamate release in the hippocampus in adulthood. These results suggest that activity-dependent changes in extracellular glutamate levels in the hippocampus are suppressed, leading to a lower signal/noise ratio in glutamatergic neurotransmission in polyI:C-treated mice. Such dysfunction of glutamatergic neurotransmission in the hippocampus may be associated with cognitive deficits in polyI:C-treated mice.

Real-time RT-PCR 2 h after the final treatment indicated that *PPP3CC*, *ATF4*, *KIF5B* and *IFITM3* mRNA levels in the hippocampus were significantly increased, but the changes were transient and disappeared 24 h later (Table 3). The only exception was the gene encoding *IFITM3* gene. Levels of *IFITM3* mRNA in the hippocampus were markedly increased at least from 2 to 24 h after the final polyI:C injection. Interestingly, it has been reported that mRNA levels of *IFITM3* are markedly increased in the brains of patients with schizophrenia (Arion et al., 2007), bipolar disorder (Iwamoto et al., 2004), and autism (Garbett et al., 2008), although the pathophysiological role of IFITM3 in mental disorders remains to be determined. Because the present study was conducted in ICR mice, an outbred stock, there is a considerable genetic heterogeneity (Chia et al., 2005). Accordingly, we repeated the experiment in C57BL/6 mice, and confirmed that neonatal polyI:C treatment significantly increased *IFITM3* mRNA levels in the hippocampus (data not shown). Thus, further studies to clarify the effect of *IFITM3* expression on brain development are warranted.

The neonatal immune activation model with polyI:C in mice exhibited emotional and cognitive impairments, but some behavioral changes, such as METH-induced hyperactivity and latent inhibition, were not evident in the neonatal polyI:C treatment model. Because schizophrenia is considered to develop as a result of the interactions of several genetic and environmental factors (Tsuang, 2000; Sawa et al., 2004), partial and subtle manifestation of schizophrenia-related behavioral changes in mice caused by a single environmental factor (e.g., viral infection during neurodevelopment) supports this hypothesis. Additional genetic factors and/or other environmental insults to prenatal viral infection may be required for full manifestation of the clinical symptoms and pathophysiology of schizophrenia in rodent models. Thus, the present model of neonatal immune activation has advantages for testing gene–environmental interactions for schizophrenia. Testing the effect of neonatal polyI:C treatment in mutant mice with susceptibility genes for schizophrenia would be intriguing for the development of an animal model of schizophrenia with gene–environmental interactions.

There is a disadvantage in the neonatal polyI:C treatment model: neonatal mice are directly exposed to immune activation with polyI:C, while the neuroimmunological factors involved in the human epidemiological association in schizophrenia may be due to complex mother-to-fetus immunological insults taking place in the maternal host, placenta and/or amnion following maternal virus infection. Accordingly, much attention should be paid when interpreting the behavioral and neurochemical abnormalities manifested in neonatal polyI:C treatment model, in relation to the pathogenesis/pathophysiology in schizophrenia.

Disclosure/conflict of interest

The authors declare that there are no conflicts of interest in the publication of the present work.

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Post-Training Dephosphorylation of eEF-2 Promotes Protein Synthesis for Memory Consolidation

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Abstract

Memory consolidation, which converts acquired information into long-term storage, is new protein synthesis-dependent. As protein synthesis is a dynamic process that is under the control of multiple translational mechanisms, however, it is still elusive how these mechanisms are recruited in response to learning for memory consolidation. Here we found that eukaryotic elongation factor-2 (eEF-2) was dramatically dephosphorylated within 0.5–2 hr in the hippocampus and amygdala of mice following training in a fear-conditioning test, whereas genome-wide microarrays did not reveal any significant change in the expression level of the mRNAs for translational machineries or their related molecules. Moreover, blockade of NMDA receptors with MK-801 immediately following the training significantly impeded both the post-training eEF-2 dephosphorylation and memory retention. Notably, with an elegant sophisticated transgenic strategy, we demonstrated that hippocampus-specific overexpression of eEF-2 kinase, a kinase that specifically phosphorylates and hence inactivates eEF-2, significantly inhibited protein synthesis in the hippocampus, and this effects was more robust during an "ongoing" protein synthesis process. As a result, late phase long-term potentiation (L-LTP) in the hippocampus and long-term hippocampus-dependent memory in the mice were significantly impaired, whereas short-term memory and long-term hippocampus-independent memory remained intact. These results reveal a novel translational underpinning for protein synthesis pertinent to memory consolidation in the mammalian brain.

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Introduction

The process of learning and memory may be divided into several sequential steps, including acquisition, consolidation, storage, and retrieval [1,2]. As the initial step, acquisition requires the brain to be at a higher arousal level in order to acquire new information as much as possible. However, only a very small portion of the acquired information may be further processed in the brain for long-term storage, a process that is called memory consolidation [3]. Consolidated memory traces are then transferred to certain brain regions such as the cortex for long-term storage. As consolidated information is retrievable over hours, days, months, and even up to the whole lifetime, this type of memory, called long-term memory, plays an essential role in human intelligent life. In contrast, information that does not undergo a consolidating process can only last for seconds or minutes. This type of memory is called short-term memory [4,5]. Without any doubt, to explore the molecular and neuronal mechanisms underlying memory consolidation is not only fundamental for our understanding of how acquired information is encoded in the brain but also insightful for disclosing how long-term memory formation could be impaired even though the acquisition is normal. This is of particular interest, as this kind of mnemonic dysfunction is often observed in many pathological conditions and clinical entities such as abnormal aging, mental retardation, and an early stage of neurodegenerative disease such as Alzheimer's disease [6–8].

A milestone over the past century for the studies of learning and memory is the demonstration that *de novo* new protein synthesis is required for memory consolidation [9,10], although recent evidence indicates that post-translational modifications of certain existing proteins may also be important for early consolidation [11]. This milestone has been established based essentially on the studies with the use of pharmacological/neurosurgical approaches. For example, post-training infusion of a protein synthesis inhibitor such as anisomycin into the hippocampus, amygdala, and motor cortex of the animals significantly impairs hippocampal, emotional, and motor memory, respectively [12–15]. An important advantage of

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Neonatal polyI:C treatment in mice results in schizophrenia-like behavioral and neurochemical abnormalities in adulthood

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ABSTRACT

It has been reported that viral infection in the first and second trimesters of pregnancy in humans increases the risk of subsequently developing schizophrenia. To develop a mouse model of immune activation during the early postnatal period, neonatal ICR mice were repeatedly injected with polyriboinosinic–polyribocytidilic acid (polyI:C; an inducer of strong innate immune responses) for 5 days (postnatal day 2–6) which may correspond, in terms of brain development, to the early second trimester in human. Cognitive and emotional behavior as well as the extracellular level of glutamate in the hippocampus were analyzed at the age of 10–12 weeks old. PolyI:C-treated mice showed anxiety-like behavior, impairment of object recognition memory and social behavior, and sensorimotor gating deficits, as compared to the saline-treated control group. Depolarization-evoked glutamate release in the hippocampus was impaired in polyI:C-treated mice compared to saline-treated control mice. Furthermore, to investigate the effect of neonatal immune activation on the expression levels of schizophrenia-related genes, we analyzed mRNA levels in the hippocampus 2 and 24 h after polyI:C treatment. No significant differences or only transient and marginal changes were observed between polyI:C-treated and saline-treated control mice in the expression levels of schizophrenia-related genes in the hippocampus.

1. Introduction

Schizophrenia is a chronic mental disorder characterized by psychosis (e.g., hallucinations and delusions), flattened emotions and impaired cognitive function, which affects about 1% of the general population. Although the disease etiology remains poorly understood, various hypotheses, including dopamine hyperfunction, glutamatergic hypofunction, GABAergic deficits, stress vulnerability and impaired neurodevelopment have been proposed as the etiology/pathophysiology of schizophrenia (Nawa and Takei, 2006; Tan et al., 2008). Among them, the neurodevelopmental hypothesis of schizophrenia supported by clinical, neuroimaging, neuropathologic, and genetic studies, is a more fundamental theory that does not conflict with other hypotheses (Sawa and Snyder, 2002; Ross et al., 2006).

The estimated heritability of schizophrenia is approximately 80% (Burmeister et al., 2008), and recent studies have identified possible candidate susceptibility genes for schizophrenia, such as *dysbindin*, *neuregulin1* and *disrupted-in-schizophrenia 1* (*DISC1*) (Harrison and Weinberger, 2005; Gogos and Gerber, 2006; Ross et al., 2006). Not only genetic factors, but also environmental factors are important in the etiology of mental disorders, and are believed to interact in most cases of schizophrenia (Caspi and

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the use of those approaches is the feasibility for both the temporal-specificity (within a special time window during the process of learning and memory) and spatial-specificity (targeting on a particular brain region), both of which allow a real-time/on-site coupling analysis of learning behavior in free-moving animals. However, as protein synthesis itself is a series of complicated biochemical reactions, it is still unclear how a neuronal process (learning) bridges to these biochemical reactions in the brain.

In mammalian cells, protein synthesis is mediated by interactions between a target mRNA and translational machineries including ribosomal proteins, eukaryote initiation factors (eIFs), and eukaryote elongation factors (eEFs). Upon mRNAs available, eIFs such as eIF4E recognize and bind to a target mRNA, and then eEFs such as eEF-2 mediate the polypeptide elongation [16,17]. Importantly, the activities of these translational machineries are regulated by many other molecules such as eEF-2 kinase (eEF-2K), eIF-binding proteins, etc. [16,17], all of which are called "translational machinery-related molecules" here. An important regulatory mechanism for most of these molecules is phosphorylation/dephosphorylation. Hyperphosphorylation of eIF4E-binding protein-1 by mammalian target of rapamycin (mTOR), for example, activates eIF4E, and consequently, promotes protein synthesis [18,19]. Phosphorylation of eEF-2 by eEF-2K inactivates eEF-2 and therefore, significantly inhibits protein synthesis [20], although the effect of eEF-2K might depend on certain condition in an *in vitro* system [21]. Originally known as Ca^{2+}-calmodulin-dependent protein kinase III [22], eEF-2K is present in all cells in the body [23]. It is therefore reasonably to speculate that an alteration in either the expression level or the phosphorylation state of these translational machineries or their related molecules including eEF-2K may significantly change the process of protein synthesis, which in turn may facilitate or impede a particular long-lasting biological process such as memory consolidation.

In this report, we provide compelling evidence at the molecular, pharmacological, genetic, and behavioral levels that post-training dephosphorylation of eEF-2 is a key translational mechanism for protein synthesis pertinent to memory consolidation in the brain.

Results

Post-training expression of mRNAs for translational machineries and their related molecules in brains of mice following training in a fear-conditioning test (FCT)

The FCT is the most commonly used behavioral paradigm for the studies of long-lasting fear memory in the rodent [24]. In order to determine whether training in the FCT altered the expression of translational machineries or/and their related molecules in the brain regions that are critically involved in consolidating fear memory, genome-wide cDNA microarrays were used to screen gene expression profiles in the hippocampus, amygdala, and cortex. A time-course of 30, 60, and 120 min after the training, together with a control group, was examined. As shown in Figure 1 and Table 1, some neuronal activity-related genes such as c-fos, BDNF, and Arc were up-regulated in the hippocampus of mice after the training, whereas the expression of mRNAs for ribosome, eIFs, eIF-binding proteins, eEFs, eIF kinase, eEF-2K, mTOR, p70S6 kinase, and p90 RSK1 kinase, etc. was not significantly changed, in comparison with those in control mice. Similar results were observed in the amygdala and cortex (data not shown), indicating that changes in the expression level of the mRNAs for the translational machineries and their related molecules are unlikely to be a mechanism that is pivotal for memory consolidation-associated protein synthesis.

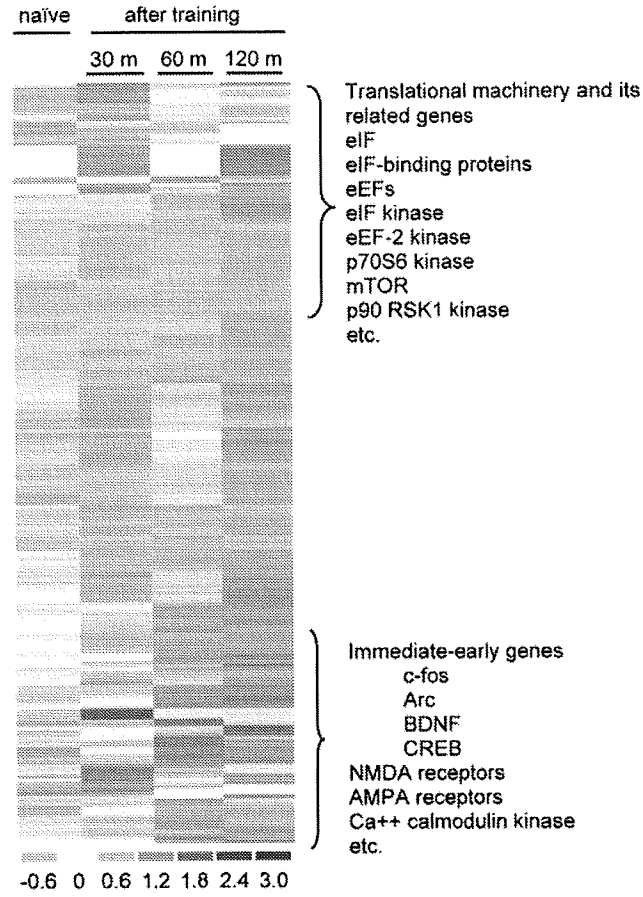

Figure 1. Gene expression profiles in the hippocampus of B6/CBA F1 mice following the training in FCT. A time-course of 30, 60, and 120 min (m) was examined. Over 100 probes that detected mRNAs that encode to transcriptional machineries or their related molecules and neuronal activity were used. No significant change in the expression level of translational machineries or their related molecules was found, whereas a number of neuronal activity-related genes were either up- or down-regulated, of which many immediate-early genes were up-regulated.
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Dephosphorylation of eEF-2 (dephospho-eEF-2) in both the hippocampus and amygdala was temporarily associated with post-training

We next focused on whether the phosphorylation of eEF-2 (phospho-eEF-2) in brains of mice altered after the training in FCT. A time-course of 30 min, 2 hr, and 4 hr was examined. In order to exclude any non-specific effect, four control conditions, naïve control (NC), shock control (SC), contextual control (CC), and tone control (TC), were examined. At 30 min, the level of phospho-eEF-2 in the hippocampus (Figure 2A), amygdala (Figure 2D), but not the cortex (data not shown), was dramatically decreased, compared to that in the same brain regions of control mice. After normalization to the NC level, about 25–35%, 24–38%, and 3–7% of eEF-2 was dephosphorylated in the hippocampus (Figure 2B), amygdala (Figure 2E), and cortex (data not shown), respectively, and an ANOVA revealed a significant difference in dephospho-eEF-2 between trained and control mice in either the hippocampus [$F(1,4) = 7.39$; $p<0.01$] or amygdala [$F(1,4) = 9.07$; $p<0.01$], but not in the cortex. Post-hoc tests revealed significant differences ($p<0.05$ or 0.01) between trained group and every control group, but not between any two control groups. For the total eEF-2 level (phospho-eEF-2 and dephospho-eEF-2), neither an

Table 1. Expression profiles of translational molecules and their related molecules in the hippocampus of B6/CBA F1 mice after training in a fear-conditioning test.

Gene Name	Profiles
eukaryotic translation initiation factor 3 (Eif3)	0.8
eukaryotic translation initiation factor 4E binding protein 2 (Eif4ebp2)	1.2
eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked (Eif2s3y)	1.1
eukaryotic translation initiation factor 4E binding protein 1 (Eif4ebp1)	0.9
eukaryotic translation initiation factor 2, subunit 2 (beta, 38kDa)	1.0
Similar to eukaryotic translation initiation factor 3, subunit 4 (delta, 44kD)	1.0
eukaryotic translation initiation factor 2A (Eif2a)	1.5
eukaryotic translation initiation factor 2 alpha kinase 4 (Eif2ak4)	1.2
eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked (Eif2s3x)	1.5
heme-regulated eIF2 alpha kinase (Hri)	1.1
eukaryotic translation initiation factor 2 alpha kinase 1	1.0
eukaryotic translation initiation factor 2 alpha kinase 2	1.8
eukaryotic translation initiation factor 4E	1.0
eukaryotic translation initiation factor 1A	1.1
eukaryotic translation initiation factor 3, subunit 5 (epsilon)	1.0
eukaryotic translation initiation factor 4A1	1.1
Highly similar to e2be rat translation initiation factor eIF-2B epsilon subunit	1.0
eukaryotic translation initiation factor 3, subunit 3 (gamma, 40kD)	0.4
eukaryotic translation initiation factor 4E binding protein 1	1.4
eukaryotic translation initiation factor 4A1	1.0
eukaryotic translation initiation factor 4E binding protein 2	0.9
eukaryotic translation initiation factor 5A	1.0
eukaryotic translation initiation factor 4A2	1.0
eukaryotic translation initiation factor 3, subunit 2 (beta, 36kD) (Eif3s2)	1.1
eukaryotic translation initiation factor 3	1.1
eukaryotic translation initiation factor 2, subunit 2 (beta, 38kDa) (Eif2s2)	1.1
eukaryotic translation initiation factor 2 alpha kinase 3 (Eif2ak3)	1.0
eukaryotic translation initiation factor 2B (Eif2b)	1.2
eukaryotic translation initiation factor 4E	1.1
eukaryotic translation initiation factor 4A2	1.0
eukaryotic translation initiation factor 5A	1.0
Mouse RNA-dependent EIF-2 alpha kinase	1.8
eukaryotic translation initiation factor 2A	1.2
eukaryotic translation initiation factor 2 alpha kinase 1	1.3
eukaryotic translation initiation factor 4E binding protein 2	1.1
eukaryotic translation initiation factor 3, subunit 8 (110 kDa)	0.9
eukaryotic translation initiation factor 2, subunit 2 (beta, 38kDa)	1.0
Highly similar to S72266 translation initiation factor eIF2B gamma chain	0.9
eukaryotic translation initiation factor 5A2	1.3
eukaryotic translation initiation factor 3, subunit 8 (110 kDa) (Eif3s8)	1.1
eukaryotic translation initiation factor 3, subunit 8 (110 kDa)	0.9
eukaryotic translation initiation factor 4, gamma 2 (Eif4g2)	1.1
eukaryotic translation initiation factor 3, subunit 3 (gamma, 40kD) (Eif3s3)	1.0
eukaryotic translation initiation factor 3, subunit 7 (zeta, 6667 kDa) (Eif3s7)	0.8
eukaryotic translation initiation factor 3, subunit 2 (beta, 36kD) (Eif3s2)	0.9
eukaryotic elongation factor, selenocysteine-tRNA-specific (Eefsec)	1.3
eukaryotic translation elongation factor 1 alpha 2 (Eef1a2)	0.9
eukaryotic translation elongation factor 1 alpha 1	0.9
eukaryotic translation elongation factor 2	1.0

Table 1. Cont.

Gene Name	Profiles
eukaryotic translation elongation factor 1 beta 2	1.0
eukaryotic translation elongation factor 1 beta 2 (Eef1b2)	1.0
eukaryotic elongation factor-2 kinase	1.2
eukaryotic translation elongation factor 1 epsilon 1 (Eef1e1)	1.1
eukaryotic translation elongation factor 1 delta	1.0

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observable difference (Figure 2A and D) nor a statistical significance (Figure 2C and F) was noted in the hippocampus (Figure 2A and C), amygdala (Figure 2D and F), or cortex (data not shown) between trained and control mice. Moreover, at 2 hr after the training, a very similar pattern of dephopho-eEF-2 and the total eEF-2 level to those observed at 30 min after the training was found in both the hippocampus and amygdala, together with no any significant change in both of the dephopho-eEF-2 and total eEF-2 level in the cortex (data not shown). Four hours after the training, the dephospho-eEF-2 in the hippocampus (Figure 2G and H) and amygdala (Figure 2G and I) returned to the pre-training level, indicating that there was a time window for the dephosphorylation. Similarly, the phospho-eEF-2 in the cortex and the total eEF-2 level in all these three brain regions were not significantly different between trained and control mice. All these results indicated that the dephospho-eEF-2, but not the change in its expression level, in both the hippocampus and amygdala was temporally but significantly associated with post-training while this change was not noted in the cortex.

MK-801 concurrently blocked memory consolidation and post-training dephospho-eEF-2

Post-training dephospho-eEF-2 provided a clue for us to explore whether this change served as a working mechanism for memory consolidation. We first addressed whether there was a functional link. Based on the essential role of the NMDA receptor in memory consolidation [25,26], we examined whether antagonism of NMDA receptors could block both memory consolidation and dephospho-eEF-2. Mice were treated with MK-801 (0.2 mg/kg; i.p.) immediately following the training, and memory retention and phospho-eEF-2 were examined 2 hr thereafter. To exclude an acute effect of MK-801, another group of mice was examined for their memory retention 24 hr after the treatment. As expected, mice treated with MK-801 were significantly impaired in both contextual and cued conditionings at either 2 hr (Figure 3A and B) or 24 hr (data not shown), compared to those in mice treated with vehicle ($p<0.01$; Student's t test). Since the treatment (i.p.) lacked a brain region-specificity, the overall mnemonic function was affected. Based on our previous findings [25] and others [27,28], together with the effect observed at 24 hr here, this impairment should attribute to a deficit in memory consolidation. Very interestingly, the same MK-801-treatment prevented the post-training dephospho-eEF-2 in the hippocampus (Figure 3C) and amygdala (Figure 3E) at 2 hr after the treatment. A statistical significance was observed in the hippocampus (Figure 3D; $p<0.01$; Student's t test) and amygdala (Figure 3E; $p<0.01$; Student's t test) between MK-801-treated trained mice and vehicle-treated trained mice, as well as between trained mice and naïve mice ($p<0.01$; Student's t test). This concurrent effect on memory consolidation and post-training dephospho-eEF-2 strongly suggested a functional link between these two events, because the blockade of NMDA receptors was able to block both of them.

Generation of hippocampus-specific eEF-2K transgenic (hip-eEF-2K-tg) mice

The functional link between the dephospho-eEF-2 and memory consolidation conferred an opportunity to further study whether a blockade of the dephospho-eEF-2 following training impaired memory consolidation via blocking protein synthesis. Based on the findings that (1) eEF-2K is the most important kinase that phosphorylates and inactivates eEF-2, (2) the only identified substrate for eEF-2K is eEF-2 [23,26], and (3) training in the FCT in the mouse creates two types of conditionings that are respectively hippocampus-dependent and -independent, to over-express eEF-2K in the hippocampus only would provide an ideal system to specifically analyze whether the post-training dephospho-eEF-2 plays a role in memory consolidation. Accordingly, a Cre/loxP recombination system and a transcriptional silencing strategy were used to generate hip-eEF-2K-tg mice. Two independent transgenic mouse strains, eEF-2K transgenic mice and Cre transgenic mice, were needed (Figure S1). The eEF-2K transgenic mice were featured by Cre recombination-dependent deletion of a transcriptional stop signal that located upstream of the eEF-2K transgene so that the transgene would only express in the cells or brain region where Cre expressed. Fortunately, as the Cre expression in our Cre transgenic mice was limited to neurons in most parts of the hippocampus, Cre/eEF-2K double transgenic mice exhibited hippocampus-specific eEF-2K overexpression, which was evidenced by *in situ* hybridization (Figure 4A to D). The highest level of the transgene mRNA was observed in the CA1/CA3 regions, a lower level in the dentate gyrus, and little expression in the CA2 region (Figure 4D). Real-time RT-PCR showed a 10-fold higher level of the total eEF-2K mRNAs (endogenous and transgenic eEF-2K) in the hippocampus of hip-eEF-2K-tg mice than in wild-type mice (data not shown), whereas Western blot analysis showed a 5.5-fold higher level of eEF-2K protein in the hippocampus of hip-eEF-2K-tg mice than in wild-type mice (Figure 4E, upper panel and F). Moreover, a significantly higher level (about 2.5-fold) of phospho-eEF-2 was observed in the hippocampus of hip-eEF-2K-tg mice, compared to that in wild-type mice (Figure 4E middle panel and Figure 4G). A tendency of decrease in the total eEF-2 expression was observed in hip-eEF-2K-tg mice (Figure 4E low panel) but was not statistically significant. In the cortex and amygdala, no observable difference in the expression of eEF-2K, phospho-eEF-2, or the total eEF-2 was found between wild-type and hip-eEF-2K-tg mice (data not shown). These results indicated that we successfully generated hip-eEF-2K-tg mice and the eEF-2K transgene was functional.

Overall characterization of hip-eEF-2K-tg mice

To exclude a possibility that a random insertion of a transgene into the mouse genome might produce some unexpected effects, the general conditions of the transgenic mice were carefully

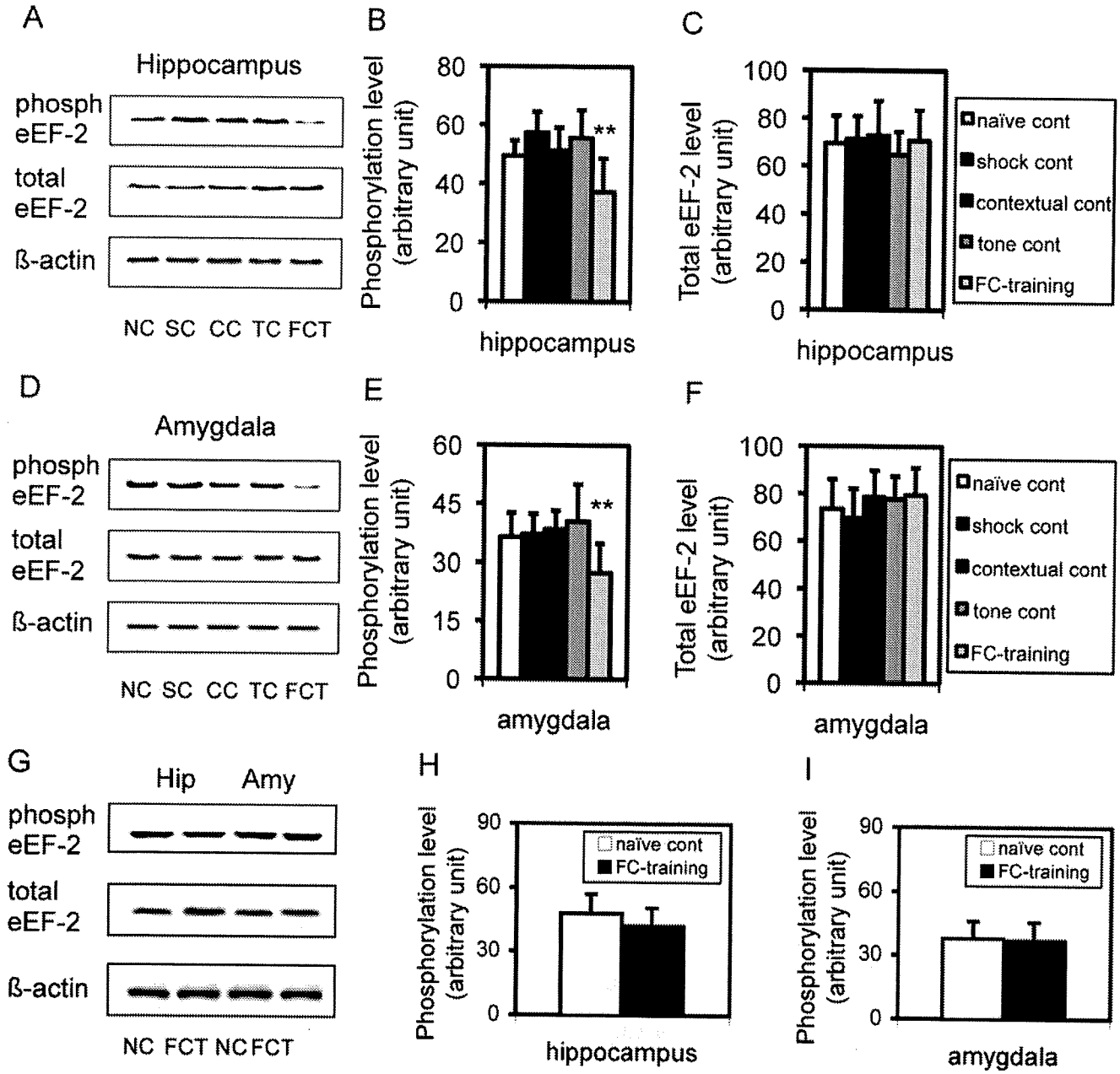

Figure 2. Dephospho-eEF-2 in both the hippocampus and amygdala is temporally associated with post-training. A. Representative Western blots showing the expression level of phospho-eEF-2 (phosph eEF-2; upper panel), total eEF-2 (middle panel), and β-actin (low panel) in hippocampi from mice that were sacrificed 30 min after the training. NC: naïve control; SC: shock control; CC: contextual control; TC: tone control; FCT: fear-conditioning training. **B.** Quantitative analysis of phospho-eEF-2 in hippocampi from mice that were sacrificed 30 min after the training (n = 5), in comparison to NC (n = 6), SC (n = 5), CC (n = 5), and TC (n = 5). **C.** Quantitative analysis of the total eEF-2 level in hippocampi of the same mice as described in B. **D.** Representative Western blots showing the expression level of phospho-eEF-2 (phosph eEF-2; upper panel), total eEF-2 (middle panel), and β-actin (low panel) in amygdalae from the same mice as described in B. **E.** Quantitative analysis of phospho-eEF-2 in amygdalae from the same mice as described in B. **F.** Quantitative analysis of the total eEF-2 level in amygdalae of the same mice as described in B. **G.** Representative Western blots showing the expression level of phospho-eEF-2 (phosph eEF-2; upper panel), total eEF-2 (middle panel), and β-actin (low panel) in both hippocampi (Hip) and amygdalae (Amy) from mice that were sacrificed 4 hr after the training. (H). Quantitative analysis of phospho-eEF-2 in hippocampi from NC (n = 6) and trained mice (n = 5) that were sacrificed 4 hr after the training in FCT. (H). Quantitative analysis of phospho-eEF-2 in amygdalae from the same mice as described in H. **, $p<0.01$, one-way ANOVA.
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examined. Hip-eEF-2K-tg mice showed normal viability without observable abnormality in growth, body size, and mating, eating, and general behaviors, compared to those in their wild-type littermates (data not shown). Nissl staining and Golgi-impregnated staining did not reveal an observable difference between wild-type (Figure 4H) and hip-eEF-2K-tg mice (Figure 4I). Open-field behaviors indexed by movement time, total distance traveled, and rearing numbers were indistinguishable between these mice (Figure S2A-C). These results indicated that overall hip-eEF-2K-tg mice were similar to their wild-type littermates.

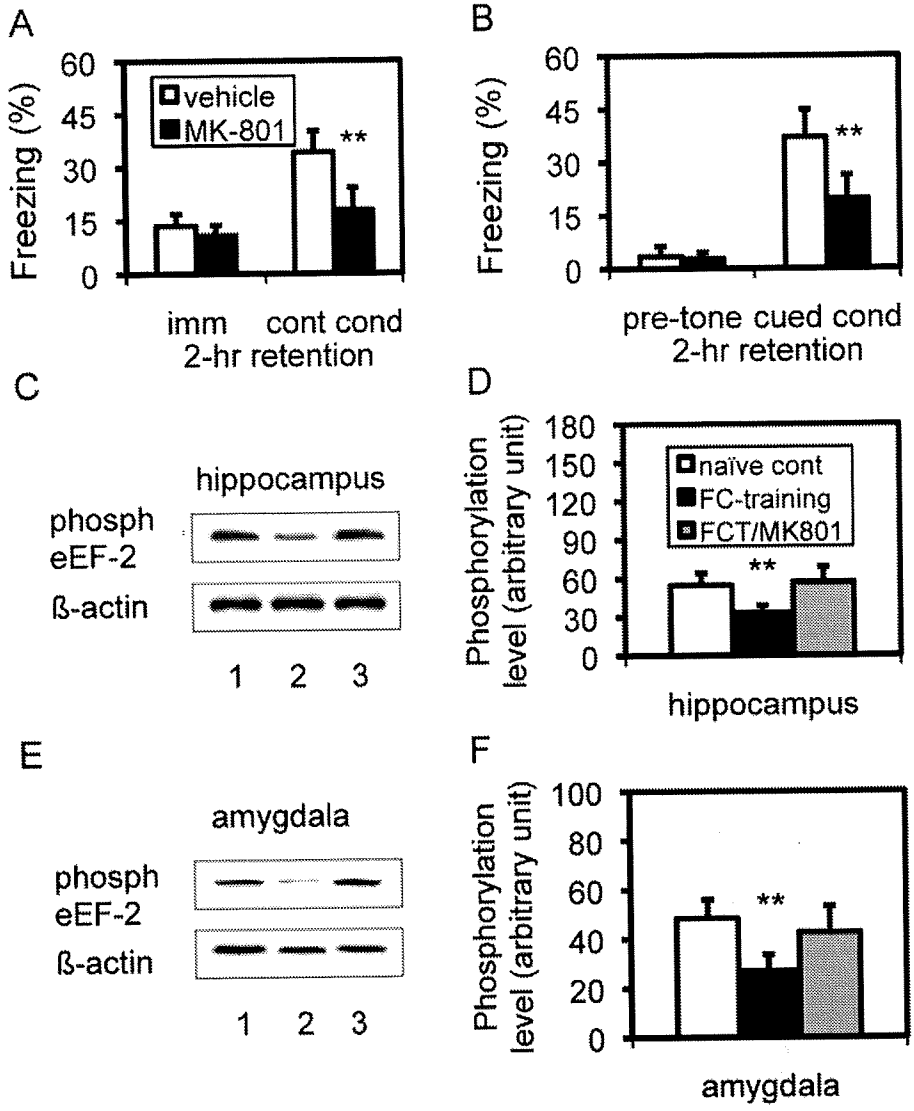

Figure 3. Effect of MK-801 on post-training dephospho-eEF-2 and memory retention. A. Contextual conditioning 2 hr after training/MK-801 treatment. **, $p<0.01$, Student's *t* test. Imm: immediate freezing; cont cond: contextual conditioning. **B.** Cued conditioning memory in the same mice. **, $p<0.01$, Student's *t* test. cued cond: cued conditioning. **C.** Expression level of phospho-eEF-2 (phosph) in hippocampi from mice 2 hr after training/MK-801 treatment. 1: naïve control; 2: mice with training/vehicle; 3: mice with training/MK-801. **D.** Quantitative analysis of the expression of phospho-eEF-2 in hippocampi from mice 2 hr after the training. **, $p<0.01$; Student's *t* test, compared between trained group-treated with vehicle and either naïve group or trained group-treated with MK-801. **E.** The expression level of phospho-eEF-2 (phosph) in amygdalae from mice 2 hr after the training. The same mice, as described above, were used. **F.** Quantitative analysis of the expression of phospho-eEF-2 in amygdalae of mice 2 hr after training. **, $p<0.01$; Student's *t* test, compared between trained group-treated with vehicle and either naïve group or trained group-treated with MK-801.
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Hippocampus-specific overexpression of the eEF-2K transgene specifically inhibited protein synthesis in the hippocampus, but not any other brain regions

We then asked whether the increased phospho-eEF-2 affected protein synthesis. First, we used an *in vivo* [35S]-methionine labeling system [29] to examine the rate of [35S]-methionine incorporation into new proteins in the brain. As shown in Figure 5A, the [35S] incorporation in hippocampi from hip-eEF-2K-tg mice was slightly, but significantly, lower than in wild-type mice ($p<0.05$; Student's *t* test); whereas in either cortices (data not shown) or amygdalae (Figure 5A), no significant difference was found. In should be noted that as the assay was conducted with the lysates from the whole hippocampus that contained cells that expressed very little of the transgene (CA2 neurons) or that did not express the transgene at all (all glial cells), the results showed in Figure 5A did not represent the overall protein synthesis inhibition rate in all hippocampal neurons. This was also evidenced by autoradiography, which showed the lowest density of [35S]-labeling in the CA1/CA3 regions, a fairly low level in the dentate gyrus, and almost no change in the CA2 region of hip-eEF-2K-tg mice (Figure 5E and F), compared to those in wild-type mice (Figure 5B and C). Similarly, no observable changes could be detected in any other brain regions including the amygdala (Figure 5D and G) and cortex (Fig. 5B and E), confirming the hippocampus-specific effect. The pattern of the protein synthesis inhibition in different hippocampal sub-regions was almost the same as that in the transgene mRNA expression (Figure 4D). It

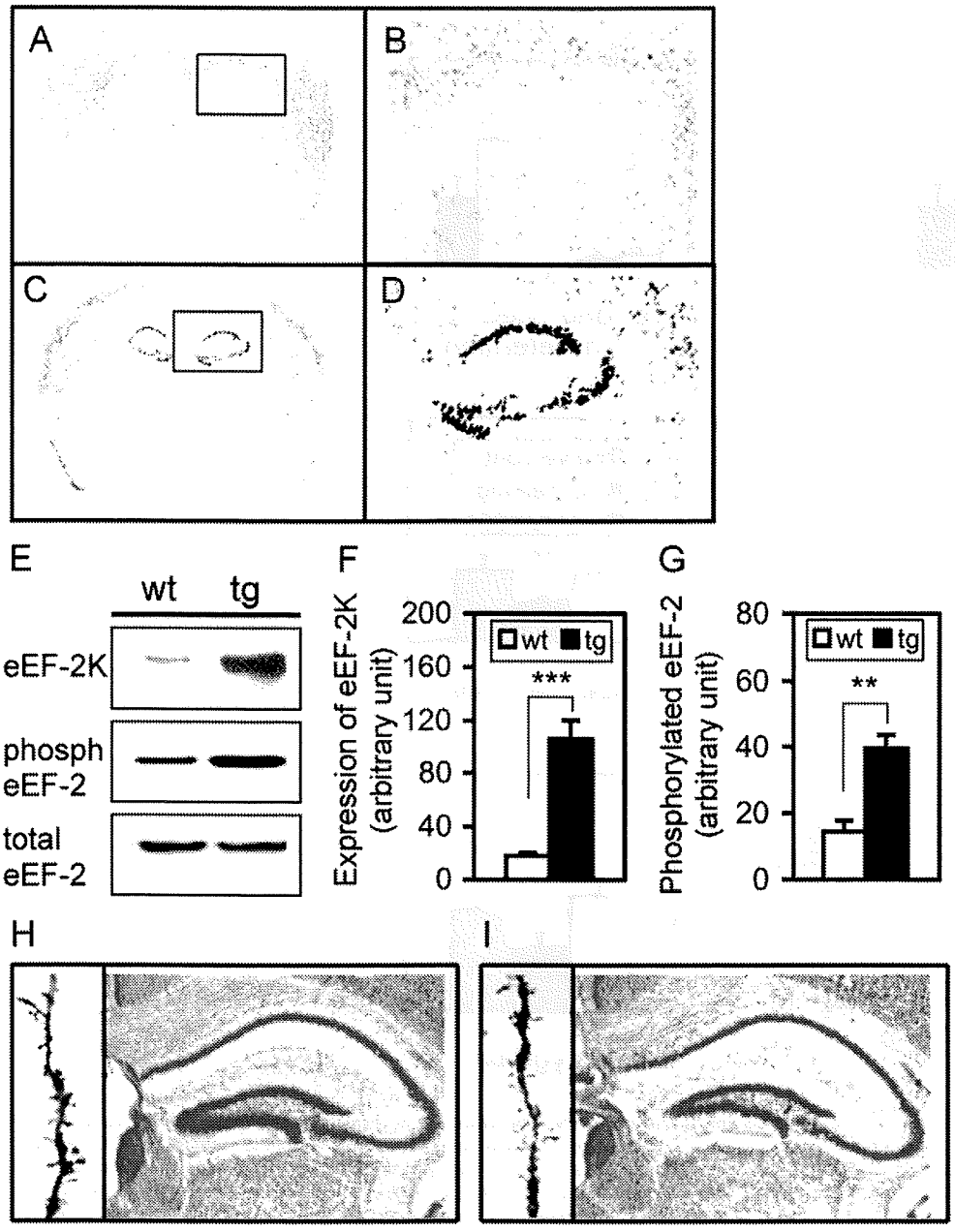

Figure 4. Generation of hip-eEF-2K-tg mice. A-D *in situ* hybridization showing the expression of the eEF-2K transgene in wild-type (A) and hip-eEF-2K-tg mice (C) or a higher magnification in wild-type (B) and hip-eEF-2K-tg mice (D). Exclusive expression of the transgene mRNA was found in the CA1, CA3, and dentate gyrus, but not the CA2 region. **E.** Representative Western blots showing the expression of the total eEF-2K (including the endogenous and transgenic eEF-2K; upper panel), phospho-eEF-2 (phosph) (middle panel), and the total eEF-2 (including phospho- and dephospho-eEF-2; low panel) in the hippocampi from wild-type (wt) and hip-eEF-2K-tg (tg) mice. **F.** Quantitative analysis of the expression of eEF-2K in wt (n = 6) and tg mice (n = 5). ***, $p<0.001$, Student's *t* test. **G.** Quantitative analysis of the expression of phospho-eEF-2 in wt (n = 5) and tg mice (n = 5). **, $p<0.01$, Student's *t* test. **H** and **I.** Representative microphotography of Nissl staining and Golgi staining in wild-type (h) and hip-eEF-2K-tg mice (i).
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should be noted that even in the CA1/CA3 regions where the transgene expression was at the highest level, the [35S]-labeling signal was still observable, indicating that protein synthesis was not completely diminished by the transgene.

We further employed a dynamic modeling system to evaluate the transgene effect. It has been well known that the expression of certain immediate-early genes such as Arc and c-fos can be quickly triggered by neuronal activities [30,31]. To compare the difference between the mRNA transcription and protein translation in the same brain region of the same animal or between different animals provided a valuable means to analyze how the protein synthesis was specifically affected by the transgene. Accordingly, mice were either treated with vehicle (basal level, BL) or a single dose of kainic acid (KA; 20 mg/kg; i.p.), and then brains were collected with a time-course from 5 min to 3 hr. A robust induction of both Arc and c-fos mRNAs was observed in the hippocampus, with a peak at 60 min for Arc mRNA in either wild-type (Figure 5H) or hip-eEF-2K-tg mice (Figure 5I) and a peak at 30 min for c-fos

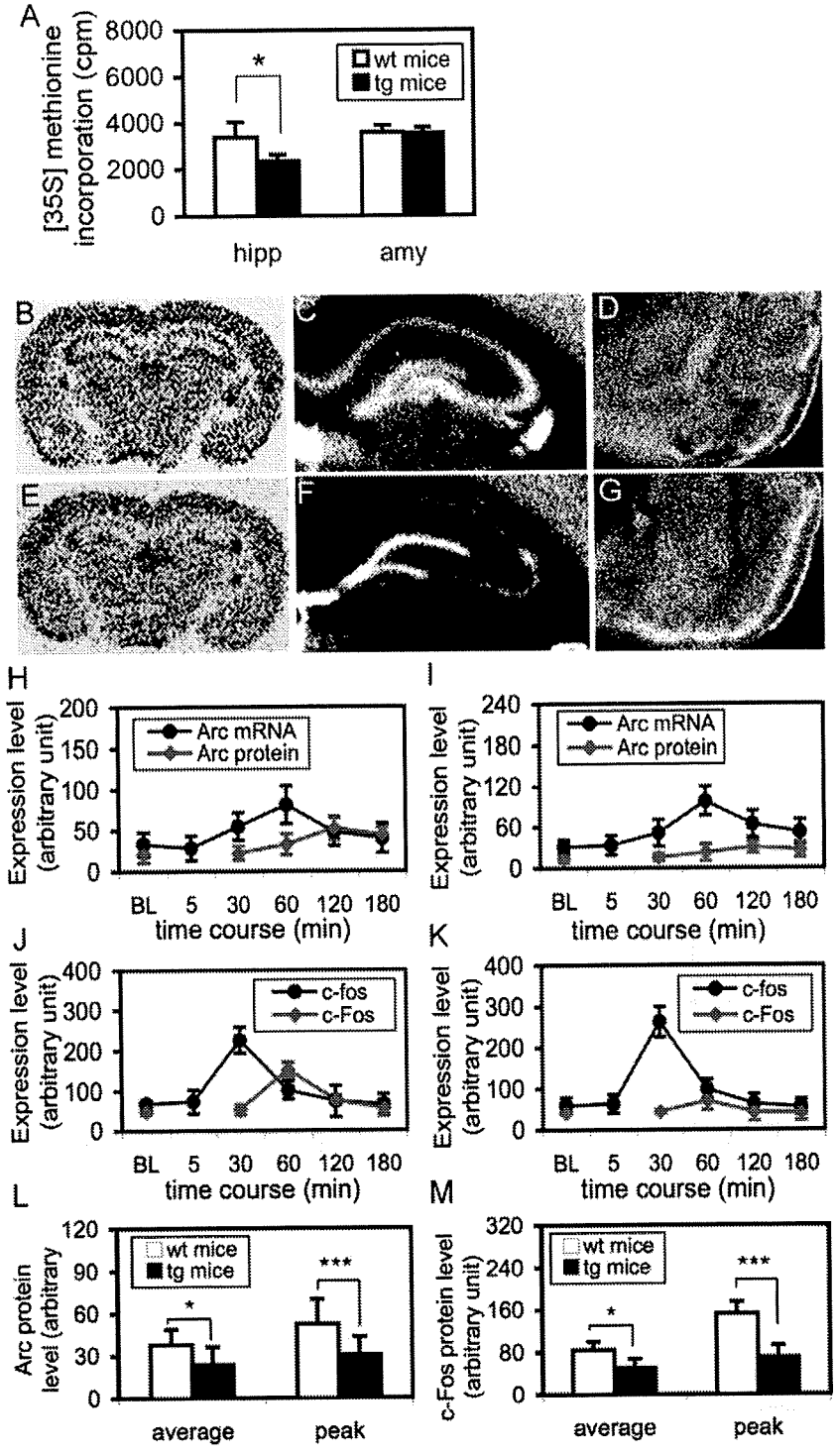

Figure 5. Protein synthesis inhibition in the hippocampus, but not amygdala, of hip-eEF-2K-tg mice. A. Quantitative analysis of [^{35}S]-methionine incorporation into proteins in the hippocampi (hipp) and amygdalae (amy) from wild-type (wt; n = 6) and hip-eEF-2K-tg (tg) mice (n = 7). *, $p<0.05$, Student's *t* test. **B** and **E**. Representative autoradiography microphotographs showing protein synthesis inhibition in coronal brain sections from wild-type (B) and hip-eEF-2K-tg mice (E). **C** and **F**. A higher magnification of microphotographs showing protein synthesis inhibition in the hippocampus of hip-eEF-2K-tg mice (F), compared to wild-type mice (C). **D** and **G**. A higher magnification of microphotographs showing no observable protein synthesis inhibition in the amygdala of hip-eEF-2K-tg mice (G) compared to wild-type mice (D). **H** and **I**. Expression of Arc mRNA (black line) and Arc protein (red line) in the hippocampi from wild-type (H; n = 5 in each group) and hip-eEF-2K-tg mice (I; n = 5 in each group). BL: basal line from mice treated with vehicle. **J** and **K**. Expression of c-fos mRNA (black line) and c-Fos protein (red line) in the hippocampi from wild-type (J; n = 4 in each group) and hip-eEF-2K-tg mice (K; n = 4 in each group). BL: basal line from mice treated with vehicle. **L** and **M**. Quantitative analysis of the expression of Arc protein (L) and c-Fos protein (M) in hippocampi from mice after KA injection at the average level and peak level. *, $p>0.05$, Student's *t* test, ***, $p<0.001$, *post hoc* test, compared between wild-type (wt) and hip-eEF-2K-tg (tg) mice.
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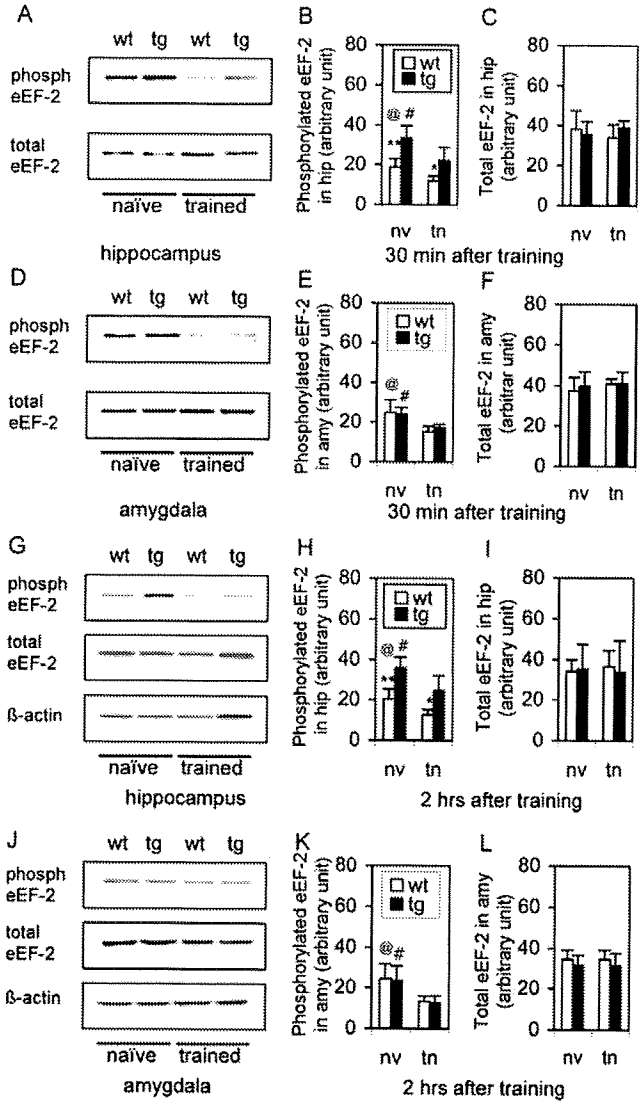

Figure 6. Overexpression of eEF-2K in the hippocampus prevented post-training dephospho-eEF-2. A-F. Expression of phospho-e-EF-2 in mice 30 min after the training. **A.** Representative Western blots showing the expression level of phospho-eEF-2 (phosph; upper panel) and total eEF-2 (low panel) in the hippocampi from wild-type (wt) and hip-eEF-2K-tg (tg) mice 30 min after training (trained) or without training (naïve). **B.** Quantitative analysis of the expression level of phospho-eEF-2 in hippocampi 30 min after the training. **, $p<0.01$, naïve wt mice vs. naïve tg mice; @, $p<0.05$, naïve wt mice vs. trained wt mice. #, $p<0.05$, naïve tg mice vs. trained tg mice; *, $p<0.05$, trained wt mice vs. trained tg mice. nv: naïve; tn: trained. **C.** Quantitative analysis of the expression level of the total eEF-2 in hippocampi 30 min after the training. **D.** Representative Western blots showing the expression level of phospho-eEF-2 (upper panel) and total eEF-2 (low panel) in the amygdalae of wt and tg mice 30 min after the training and without training. **E.** Quantitative analysis of the expression level of phospho-eEF-2 in amygdalae 30 min after the training. @, $p<0.01$, naïve wt mice vs. trained wt mice. #, $p<0.05$, naïve tg mice vs. trained tg mice. **F.** Quantitative analysis of the expression level of the total eEF-2 in amygdalae 30 min after the training. **G-I.** Expression of phospho-e-EF-2 in tg mice 2 hr after the training. **G.** Representative Western blots showing the expression level of phospho-eEF-2 (upper panel), total eEF-2 (middle panel), and β-actin (low panel) in hippocampi 2 hr after the training and without training. **H.** Quantitative analysis of the expression level of phospho-eEF-2 in hippocampi 2 hr after the training. **, $p<0.01$, naïve wt mice vs. naïve tg mice; @, $p<0.05$, naïve wt mice vs. trained wt mice. #, $p<0.05$, naïve tg mice vs. trained tg mice; *, $p<0.05$, trained wt mice vs. trained tg mice. **I.** Quantitative analysis of the expression level of the total eEF-2 in hippocampi of mice 2 hr after the training. **J.** Representative Western blots showing the expression level of phospho-eEF-2 (upper panel), total eEF-2 (middle panel), and β-actin (low panel) in amygdalae 2 hr after the training and without training. **K.** Quantitative analysis of the expression level of phospho-eEF-2 in amygdalae of mice 2 hr after the training. @, $p<0.01$, naïve wt mice vs. trained wt mice. #, $p<0.05$, naïve tg mice vs. trained tg mice. **L.** Quantitative analysis of the expression level of the total eEF-2 in amygdalae of mice 2 hr after the training. Sample size in each group was 4–5 mice, with at least 2 measures in each animal. Student's *t* test was used for all statistical analyses.

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mRNA in either wild-type (Figure 5J) or hip-eEF-2K-tg mice (Figure 5K). For a within-genotype analysis between vehicle- and KA-treated mice, we found a highly significant difference in Arc mRNA expression in either wild-type [$F(5,25) = 6.24$, $p<0.001$] or hip-eEF-2K-tg mice [$F(5,25) = 10.34$, $p<0.001$], and in c-fos mRNA expression in either wild-type [$F(5,20) = 29.34$, $p<0.001$] or hip-eEF-2K-tg mice [$F(5,20) = 31.29$, $p<0.001$]. A between-genotype analysis with a repeated ANOVA did not reveal a significant difference in the expression of either mRNA in these mice, indicating that the transgene did not affect the transcriptions of the mRNAs that were associated with neuronal activity. At the protein level, a delayed but similar expression pattern for both Arc and c-Fos was observed (red line; Figure 5H-K). In KA-treated wild-type mice, an one-way ANOVA confirmed the increase in both Arc expression [$F(4,20) = 8.01$, $p<0.001$] and c-Fos expression [$F(4,15) = 37.88$, $p<0.001$]. In KA-treated hip-eEF-2K-tg mice, the increase in the expression of either Arc [$F(4,20) = 3.27$, $p<0.05$] or c-Fos [$F(4,15) = 4.65$, $p<0.05$] was at a less significant level. Moreover, a repeated ANOVA revealed a highly significant difference in the expression of Arc [$F(4,40) = 9.69$, $p<0.001$] or c-Fos [$F(4,30) = 39.79$, $p<0.001$] between wild-type and hip-eEF-2K-tg mice, confirming the effect of the transgene on protein synthesis inhibition. The p values at the average level from four time-points of the time-course ($p<0.05$) and at the peak level ($p<0.001$) were different in either Arc (Figure 5L) or c-Fos expression (Figure 5M), indicating that the effect of the transgene was more robust during an "on-going" protein synthesis process.

Expression of the eEF-2K transgene prevented post-training dephospho-eEF-2 in the hippocampus

Before the training in the FCT, the phospho-eEF-2 level in the hippocampus (Figure 6A), but not the amygdala (Figure 6D), of hip-eEF-2K-tg mice was higher than that in wild-type mice. Thirty minutes after the training, the phospho-eEF-2 level significantly decreased ($p<0.05$; Student's *t* test) in both the hippocampus (Figure 6A and B) and amygdala (Figure 6D and E), without a significant change in the total eEF-2 level in either group (Figure 6C and F). Interestingly, a significant difference in the phospho-eEF-2 level was still observed in the hippocampus (Figure 6B and H; $p<0.05$; Student's *t* test), but not the amygdala (Figure 6E and K), between trained wild-type and trained hip-eEF-2K-tg mice, indicating that the effect was due to the transgene expression. Similar changes were observed in mice sacrificed at 2 hr after the training (Figure 6G-L). These results indicate that the transgene was able to largely, but not completely, prevent post-training dephospho-eEF-2 in the hippocampus, but not amygdala.

Long-term hippocampus-dependent memory was specifically impaired in hip-eEF-2K-tg mice

The results above validated an ideal system to test whether the post-training dephospho-eEF-2 was functionally associated with memory consolidation. In order to exclude a non-specific effect of