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Distinct Influences of Neonatal Epidermal Growth Factor Challenge on Adult Neurobehavioral Traits in Four Mouse Strains

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Epidermal growth factor (EGF) receptor (ErbB1) signals regulate dopaminergic development and function and are implicated in schizophrenia. We evaluated genetic effects on neurobehavioral changes induced by neonatal EGF administration, using four mouse strains. Subcutaneous EGF administration increased phosphorylation of brain ErbB1 in all strains, although DBA/2 and C57BL/6 mice had lower basal phosphorylation. Neonatal EGF treatment differentially influenced physical and behavioral/cognitive development, depending on mouse strain. Prepulse inhibition was decreased in DBA/2 and C57BL/6 mice but not C3H/He and ddY mice. Locomotor activity was accelerated in DBA/2 mice, but reduced in ddY mice. EGF treatment enhanced fear-learning performance with a tone cue in DBA/2 mice, but decreased performance with tone and context cues in C3H/He and ddY mice, respectively. The strain-dependent behavioral sensitivity was correlated with basal ErbB1 phosphorylation. Genetic components regulating brain ErbB1 signaling strongly influence the direction and strength of behavioral responses stemming from the neonatal neurotrophic perturbation.

KEY WORDS: Animal behavior; cytokine; environment; genetic influence; neurotrophic factor; body weight; schizophrenia.

INTRODUCTION

Epidermal growth factor (EGF) and EGF-related peptide growth factors, such as transforming growth factor alpha (TGFα), heparin-binding EGF-like factor (HB-EGF), amphiregulin, betacellulin, and epiregulin, are distributed in various brain regions and all bind to EGF receptors (ErbB1) (Gomez-Pinilla *et al*., 1988; Kaser *et al*., 1992; Kornblum *et al*., 1997; Nakagawa *et al*., 1998). These ErbB1 ligands enhance neuronal survival and promote differentiation, particularly that of midbrain dopaminergic neurons (Alexi and Hefti, 1993; Casper *et al*., 1991; Ferrari *et al*., 1991; Hanke *et al*., 2004). ErbB1 is expressed in many types of brain neurons, but is especially enriched in midbrain dopaminergic neurons (Adamson and Meek, 1984; Gomes-Pinilla *et al*., 1988; Kaser *et al*., 1992; Seroogy *et al*., 1994). Consistent with ErbB1 distribution, *in vivo* experiments demonstrate neurotrophic activity of ErbB1 receptor ligands in midbrain dopaminergic neurons. Intracerebroventricular infusion of EGF increases the survival and function of dopaminergic neurons in animal models of Parkinson's disease (Hadjiconstantinou *et al*., 1991; Iwakura *et al*., 2005; Ventrella *et al*., 1993). Gene-targeting and transgenic studies of ErbB1 receptor and its ligands, confirms their biological significance in prenatal and postnatal

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development of neocortical structures as well as in dopamine physiology (Blum, 1998; Hilakivi-Clarke *et al*., 1995; Sibilia *et al*., 1998).

EGF was first described as Tooth-Lid Factor because it enhances eyelid opening and tooth eruption in newborn mice. Using this activity as an assay, Cohen (1962) purified this factor and nerve growth factor to homogeneity from mouse salivary gland extracts. EGF is alternatively called urogastrone because receptor-saturating concentrations of EGF are often present in urine, blood, and amniotic fluid (Gregory and Preston, 1977). EGF concentrations in blood and amniotic fluid vary among individuals (Peracchi *et al*., 2001; Varner *et al*., 1996). Although EGF efficiently penetrates the immature blood–brain barrier during early development (Futamura *et al*., 2003; Pan and Kastin, 1999), the effects of peripheral EGF and other ErbB1 ligands on brain development and behaviors remained unknown for many years. We subsequently found that subcutaneously administered EGF acts on neonatal brain neurons to perturb dopaminergic development and alter animal behaviors, such as sensory motor gating, social interaction, exploratory motor activity and amphetamine sensitivity (Futamura *et al*., 2003; Mizuno *et al*., 2004). These findings indicate that severe abnormalities in EGF/ErbB1 signaling are induced in some human fetuses or neonates and might influence their risk of schizophrenia.

Genetic background and rearing environment mutually interact with each other and influence later behavioral traits such as sensorimotor gating and learning (Gendreau *et al*., 1997; Tong *et al*., 2001; Waddell *et al*., 2004). Neurotrophic molecules including ErbB1 ligands might mediate various environmental signals and insults to the brain. For example, the production of ErbB1 ligands, EGF and HB-EGF, is regulated by neuronal activity, inflammation, and brain injury (Isono *et al*., 2003; Kawahara *et al*., 1999; Opanashuk *et al*., 1999; Schaudies and Johnson, 1993; Tanaka *et al*., 1999). Maternal separation also influences production of TGFα in the neonatal brain (Romeo *et al*., 2004). Thus, the injury-, stress- or inflammation-triggered abnormal expression of ErbB1 ligands impairs normal brain development, potentially leading to dopamine-related cognitive/behavioral abnormalities such as schizophrenia (Isono *et al*., 2003; Opanashuk *et al*., 1999; Tanaka *et al*., 1999). Consistent with this hypothesis, abnormal ErbB signals are implicated in schizophrenia (Moldin and Gottesman, 1997; Nawa et al., 2000; Petronis, 2004; Stefanson *et al*., 2002). A single nucleotide polymorphism in the EGF gene that positively regulates its transcription is associated with the risk of schizophrenia in Finnish men (Anttila *et al*., 2004). Abnormal expression of EGF and ErbB1 receptors is found in the forebrain regions of schizophrenic patients (Futamura *et al*., 2002).

Although abnormal EGF/ErbB1 signaling might be associated with the risk of schizophrenia or contribute to its pathology, it remains to be determined how genetic components can be incorporated into this hypothesis. In the present investigation, we used EGF-treated mice as a schizophrenia model and evaluated the significance of genotype in determining behavioral responses to EGF at the adult stage. We peripherally administered EGF to four different mouse strains as newborns and compared the strength and the direction of EGF-behavioral alterations in each mouse strain. Mice were subjected mainly to the behavioral tests that are often used to evaluate schizophrenia models (Gendreau *et al*., 1997; Swerdlow and Geyer, 1998). In addition, strain difference in basal and EGF-triggered ErbB1 activation was correlated with strain-dependent behavioral changes.

MATERIALS AND METHODS

Animal Protocols

Four strains of neonatal mice, DBA/2 (DBA/2 Cr Slc; 4–6 per litter, 12 l total; SLC, Shizuoka, Japan), ddY (8–15 per itter, 6 l total; SLC), C57BL/6 (C57BL/6 Crj; 7–9 per litter, 7 l total; Nihon Charles River, Yokohama, Japan) and C3H/He (C3H/He Cr Slc; 4–6 per litter, 11 l total; SLC), were purchased together with their dams. The number of pups per dam was different among strains and not artificially adjusted to other mouse strains. Recombinant human EGF (0.875 μg/g; Higeta Syoyu, Chiba, Japan) was administered subcutaneously to half of individual litters of each mouse strain daily during postnatal days (PND) 2–10. The amount of EGF injected was the minimum dose that reproducibly induces behavioral impairments in rats (Futamuta *et al*., 2003). Control littermates of the other half received a saline injection. After PND 20, mice were separated according to gender and raised separately (3–5 mice per cage; 20L × 30W × 12H cm). All mice treated with EGF or saline as neonates were housed on a 12-h light-dark cycle with free access to food and water. Physical indices such as body weight, eyelid opening and tooth eruption were monitored daily (18:00–20:00 hour). All animal experiments were

authorized by the Animal Use and Care Committee of Niigata University and performed during the night cycle (20:00–2:00 hour). Animals (EGF-treated or saline-treated mice, n = 12–17 per group for each strain) were assigned to two behavioral test batteries to minimize the effects of the preceding behavioral test; (a) locomotor test followed by prepulse inhibition (PPI) or (b) startle response followed by the contextual conditioning task. Mice were subjected to behavioral tests during PND 56–70 with 3–7-day interval between tests. Both sexes were equally represented. Mice were habituated to experimental rooms, at least, 2–3 hours before testing. After completion of the behavioral testing, mice were weighed and dissected, and the wet tissue weight of brain, spleen and adrenal gland was measured.

Immunoblot Analysis

Using an independent set of animals, EGF (0.875 μg/g) or saline was injected subcutaneously into mouse pups (PND2) to examine the acute effect on ErbB1 activation in the brain. At this dose, the level of circulating EGF in the blood is greater than 10 ng/ml (ErbB1 receptor-saturating concentrations) 0.5–2 hour after administration in neonatal rats (data not shown; Futamura *et al*., 2003). Thirty minutes after acute EGF injection, brains were removed and homogenized by sonication with 2× sample loading buffer. Protein (50 μg/lane) was subjected to an 8–12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was probed with antibodies directed against phosphorylated ErbB1 (1:1000; Cell Signaling, Beverly, MA) or against ErbB1 (1:1000, Santa Cruz Biotech, Santa Cruz, CA), and subjected to chemiluminescence reaction (Amersham-Pharmacia, Tokyo, Japan).

Analysis of Locomotor Activity

Exploratory motor activity was measured in a novel environment with the following test equipment under dim light (27L × 27W × 20H cm, MED Associates, St. Albans, VA). Mice were placed in an automated activity monitor equipped with infrared photosensors at 1.62-cm intervals. Horizontal activity for every 5 minute was measured as beam crossings during the initial 60 minute and analyzed by using fully automated tracking system (Activity Monitor, Med Associates).

Measurement of Acoustic Startle Response and PPI

The mice were placed in a plastic cylinder and fixed in an automated startle chamber (SR-Lab Systems, San Diego, CA). After a 5-minute acclimation period with 70-dB-background noise (white noise), an 80, 90, 100, 110 or 120-dB white noise stimulus (40 millisecond duration) was given eight times to each mouse in the same pseudorandom order at 15 second-intervals. Analysis for startle amplitudes was based on the mean of the seven trials (ignoring the first trial) for each trial type.

Using a different set of mice, PPI responses were measured with 120-dB acoustic stimuli combined with four different prepulse intensities. Each mouse was placed in the startle chamber (SR-Lab) and initially acclimatized for 5 minute with background noise alone (70-dB white noise). The mouse was then subjected to 48 startle trials, each trial consisting of one of six conditions: (i) a 40-millisecond 120-dB noise burst presented alone (S), (ii–v) a 40-millisecond 120-dB noise burst following prepulses by 100 millisecond (20-millisecond noise burst) that were 3, 6, 9, or 12 dB above background noise (i.e., 73-, 76-, 79- or 82-dB prepulse, respectively), or (vi) no stimulus (background noise alone), which was used to measure baseline movement in the chamber. These six trial types (i–vi) were each repeated eight times in a pseudorandom order to give 48 trials. The inter-trial interval was 15 seconds. Each trial type was presented once within a block of six trials and the order of 48 trial presentations was fixed for all mice. Analysis was based on the mean of the seven trials for each trial type. The percentage PPI of a startle response 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).

Contextual Conditioning

The test paradigm of contextual conditioning was based on a work by Frankland *et al*. (2004). Mice were placed in a shock chamber with a grid floor (10L × 10W × 10H cm box; Obaraika Ltd. Tokyo, Japan) for 2 minute to monitor baseline movement/freezing and then exposed to 0.8-mA electric shocks (2-second duration, six times for DBA/2, ddY, and C3H/He, and three times for C57BL/6) all together with 30-second tone cues (60 dB, 10 kHz). Preliminary tests revealed that DBA/2, C3H/He, and ddY mice were

relatively insensitive to three electric shocks and failed to produce more than 50% freezing rates compared with C57BL/6 mice (data not shown). The number of shocks was therefore increased for the DBA/2, C3H/He and ddY mice. One day after conditioning, mice were returned to the chamber. The time spent freezing, (no movements except those necessary for respiration) was recorded and scored at 30-second intervals for 3 minute. After 3 hour, mice were moved to a different chamber with a flat floor (10L× 10W × 10H cm box) and the time spent freezing was recorded and scored for 3 minute before and after the tone cue. Freezing behavior was automatically monitored by a video camera during all sessions and analyzed by imaging software (Obaraika Ltd.).

Statistical Analysis

Behavioral scores were initially analyzed using four-way analysis of variance (ANOVA) with genotypes (four strains), gender (two levels), and treatment (two levels) being between-subject factors and with time (6 or 10 time points) or prepulse (four levels) being within-subject factors. As the initial ANOVAs yielded no significant outcomes involving the gender variable, the data of the two genders were combined for the final analyses. When there were significant interactions between genotype and treatment for locomotor activity, and startle response, these behavioral scores were analyzed separately in each strain to evaluate treatment effects or at each treatment level (EGF or saline) to examine the genotype effects. A Lavene test revealed significant differences in variance among strains or among groups in almost all behavioral, physical and biochemical data (data not shown). Accordingly a non-parametric analysis using a Student–Newman–Keuls test was performed for *post hoc* multiple comparisons to avoid type-2 errors. Only to evaluate EGF effects at individual data points during repeated measurements or in biochemical examination, a planned Student's *t*-test with Bonferroni's alpha compensation was used alternatively. These statistical analyses were performed using SPSS 11.0 for Windows (n values all represent the number of animals used).

RESULTS

Strain Differences in ErbB1 Activation Triggered by Peripheral Injection of EGF

We have previously reported that peripheral administration of EGF activates ErbB1 in the neonatal brain through the immature blood–brain barrier (Futamura *et al.*, 2003; Kleshcheva, 1988). In the present study, we examined whether there are strain differences in ErbB1 activation by monitoring ErbB1 expression and phosphorylation in the brain (Fig. 1a). In control mice, there was no significant difference in total ErbB1 levels among mouse strains. A single subcutaneous injection of EGF increased phosphorylation of ErbB1 in all four-mouse strains ($F[1,24] = 120$, $p < 0.001$) with significant strain differences in ErbB1 phosphorylation levels ($F[3,24] = 5.65$, $p = 0.004$) (Fig. 1b). There was a marginally non-significant interaction between treatment and strain ($F[3,24] = 2.74$, $p = 0.065$). A *post hoc* test indicated that basal ErbB1 phosphorylation levels in C3H/He and ddY mice were higher than those

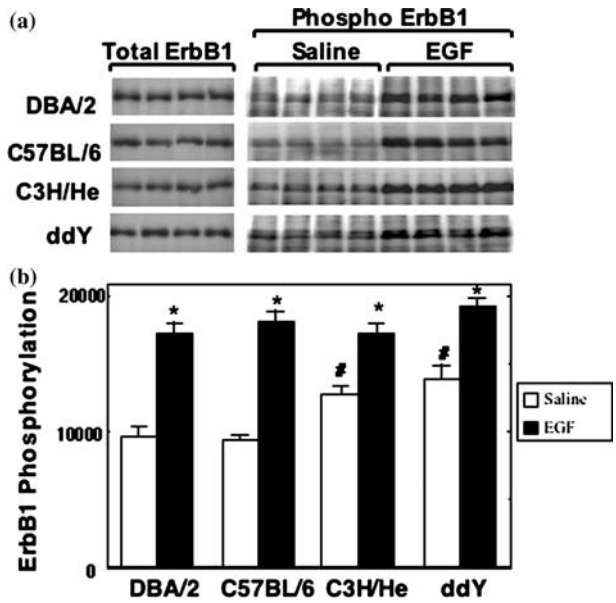

Fig. 1. Brain ErbB1 phosphorylation induced by subcutaneously administered EGF in mouse pups. Relative magnitudes of ErbB1 phosphorylation in the brain were measured after EGF or saline administration at postnatal day (PND) 2. Expression of ErbB1 levels was monitored in parallel. EGF solution (0.875 μg/g) or saline was subcutaneously injected into neonatal DBA/2, C57BL/6, C3H/He, and ddY mice ($n = 4$ each). (a) Thirty minutes after injection, the whole brain was dissected and protein extracts were subjected to immunoblotting for the anti-ErbB1 antibody or anti-phospho ErbB1 antibody. (b) The ordinate represents optical density of immunoreactive bands for phospholylated ErbB1 (arbitrary units, mean ± SEM). $^{*}p < 0.05$, compared with saline-injected control littermates for each strain and $^{\#}p < 0.05$, compared with saline-treated DBA/2 or C57BL/6 mice by Student's *t*-test with Bonferroni's alpha compensation. Note; There was no difference in ErbB1 levels between saline-treated and EGF-treated mice in all strains (data not shown).

Table I. EGF Effects on Physical Development of Four Mouse Strains

		Control			EGF			
	Strain	Mean	s.e.	Rank	Mean	s.e	Rank	% Change
Eyelid open (PND)	DBA/2	13.1	0.2	e	9.5	0.2	ab	−27*
	C57BL/6	14.3	0.3	f	9.1	0.09	a	−36*
	C3H/He	12.3	0.3	d	9.5	0.2	ab	−23*
	ddY	14.5	0.2	f	10.1	0.1	c	−30*
Tooth erupt (PND)	DBA/2	10.0	0.2	d	8.3	0.3	a	−17*
	C57BL/6	11.1	0.2	e	9.0	0.2	ab	−19*
	C3H/He	10.2	0.2	d	8.8	0.3	ab	−13*
	ddY	9.4	0.3	cd	8.2	0.1	a	−13*
Body weight PND12 (g)	DBA/2	4.8	0.2	ab	4.0	0.2	a	−16
	C57BL/6	4.7	0.3	ab	4.4	0.3	a	−7
	C3H/He	5.9	0.3	c	5.7	0.1	c	−4
	ddY	5.9	0.09	c	5.3	0.2	bc	−9

*Marks significant differences between EGF-treated mice and saline-treated controls by Student–Newman–Keuls rank test ($n = 11$–19 per group) as a Levene test revealed significant differences in variance in all categories ($p = 0.002$–0.024). The physical indices were indistinguishable between males and females at the early juvenile stage.

in the other strains, but the strain difference was not apparent following EGF treatment.

Effects of Neonatal EGF Treatment on Physical Development of Mice

Repeated administration of EGF to mouse newborn pups accelerates eyelid opening and tooth eruption (Cohen *et al.*, 1962). To compare the peripheral effects of EGF in the four mouse strains, we administered EGF or saline subcutaneously into mouse pup (PND 2) daily until PND 10 and monitored these physical indices at the end of the EGF administration (Table I). Two-way ANOVA with strain (4) × treatment (2) indicated that EGF treatment stimulated eyelid opening ($F[1,102] = 837$, $p < 0.001$) and tooth eruption ($F[1,107] = 109$, $p < 0.001$). There was a strain × treatment interaction in eyelid opening ($F[3,102] = 13.2$, $p < 0.001$), but not in tooth eruption. Although *post hoc* comparisons revealed significant differences in EGF-triggered eyelid opening among strains, the basal speed of lid opening in control mice (C3H/He > DBA/2 > ddY = C57BL/6) was negatively correlated with the magnitude of EGF effects on eyelid opening (i.e. accelerated days; C57BL/6 > ddY > DBA/2 > C3H/He). Administration of EGF had similar effects on tooth eruption in all strains, although there was a basal strain difference in tooth eruption of saline-treated mice.

We also monitored the immediate influence of repeated EGF administration on body weight (Table I). We compared mean body weight on PND12, 2 day after completion of EGF treatment. Two-way ANOVA indicated that EGF treatment affected body weight ($F[1,93] = 9.36$, $p = 0.003$ for treatment). There was no strain × treatment interaction. *Post hoc* comparisons of body weights among saline- and EGF-treated mice failed to detect a statistical difference in the effect of EGF in each strain at this developmental stage. The immediate effect contrasted with the delayed effects of the treatment on body weight at the adult stage (see below).

Influences of Neonatal EGF Treatment on Exploratory Motor Behavior in Adult Mice

The effect of EGF on locomotor activity was assessed in a novel environment at the adult stage (PND56-62), more than 6 weeks following the final EGF injection. A multiple ANOVA with factors of strain (4) × gender (2) × treatment (2) × test duration (12, repeated) revealed a significant main effect of strain ($F[3,101] = 16.0$, $p < 0.001$), but there was significant interactions between strain and treatment ($F[3,101] = 2.76$, $p = 0.046$) and between gender and strain ($F[3,101] = 3.21$, $p = 0.026$). Test duration had a significant overall effect ($F[11,91] = 44.6$, $p < 0.001$) and an interaction with strain ($F[33,279] = 3.40$,

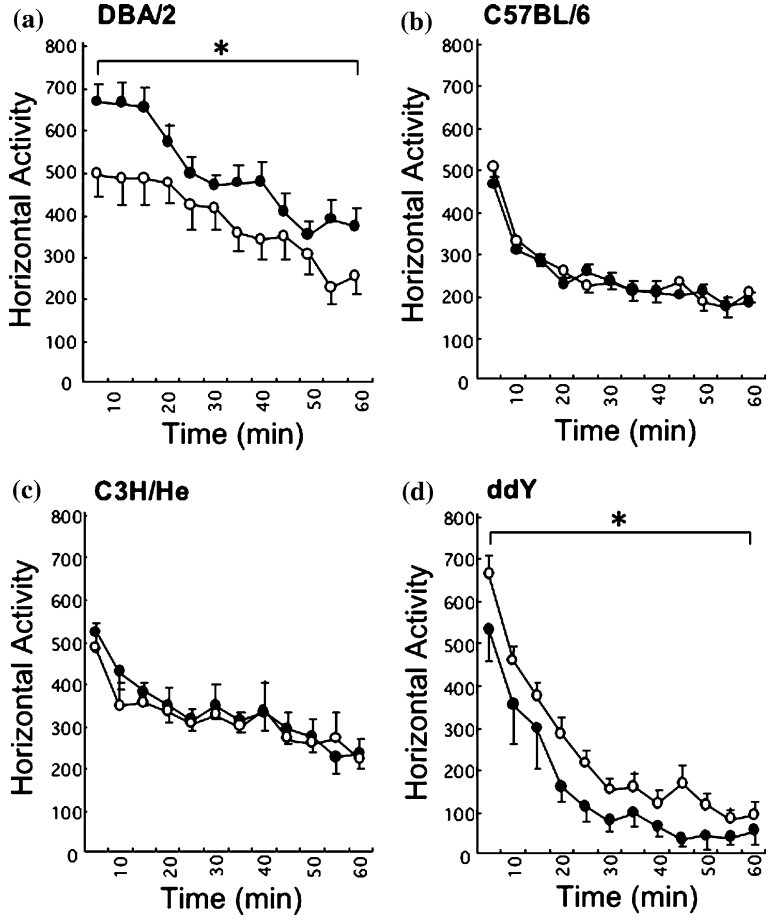

Fig. 2. Exploratory locomotor activity of four mouse strains. Neonatal mice (DBA/2, C57BL/6, C3H/He, and ddY strains) were treated daily with EGF (0.875 μg/g; closed circle) or saline (25 μl/g; open circle) ($n = 13$–17 each group) from PND 2 to PND 10. Horizontal (the number of 1.7 cm-line crossing) activity in a novel open field was measured every 5 minute in adult mice (PND56-62) and was plotted on the ordinate. Asterisks indicate significant relative differences in ANOVA (*$p < 0.05$). Note: The changes in locomotor activity were indistinguishable between males and females.

$p < 0.001$) as well. Because of the significant interaction between treatment and strain and no interaction between gender and treatment, we pooled male and female data to analyze the main effect of EGF on locomotor activity separately in each strain by using one-way repeated ANOVA (Fig. 2). Neonatal EGF treatment significantly increased exploratory locomotor activity in the DBA/2 mice, compared to their saline-treated control littermates ($F[1,24] = 4.95$, $p = 0.036$). In contrast, EGF treatment suppressed locomotor activity in ddY mice ($F[1,25] = 5.89$, $p = 0.023$). There was no significant effect of EGF in C57BL/6 and C3H/He mice. Thus, neonatal EGF treatment can either increase or decrease exploratory locomotor activity, depending upon mouse genetic background.

Influence of Neonatal EGF Treatment on Acoustic Startle Response

The effect of EGF on startle response was tested with five intensities of white noise in four mouse strains at the adult stage (Fig. 3). A multiple ANOVA with factors of strain (4) × gender (2) × treatment (2) × sound intensity (5, repeated) revealed significant main effects of EGF ($F[1,91] = 10.7$, $p = 0.002$) and strain ($F[3,91] = 65.6$, $p < 0.001$), but not gender ($F[1,91] = 0.43$, $p = 0.51$). There were interactions between sound intensity and strain ($F[12,270] = 8.54$, $p > 0.001$) and between sound intensity and treatment ($F[4,88] = 5.87$, $p < 0.001$). Because of significant sound intensity × treatment interaction and no gender × treatment interaction, EGF effects were

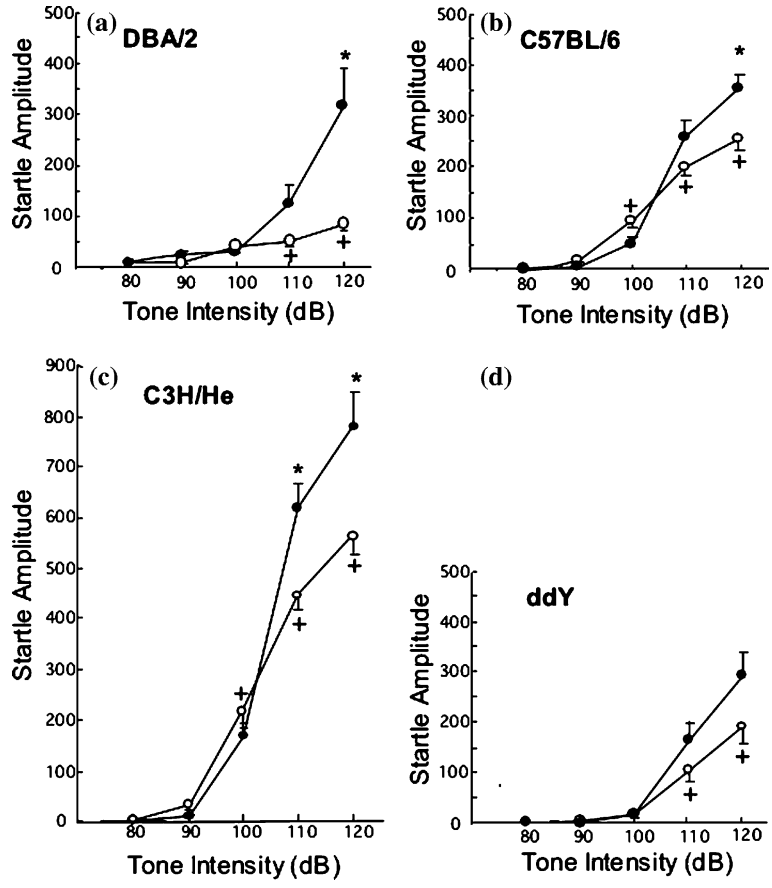

Fig. 3. Comparison of acoustic startle reactions among mouse strains. Neonatal mice (DBA/2, C57BL/6, C3H/He, and ddY strains) were given EGF (closed circle) or saline (open circle) ($n = 10$–17 for each group) during the early postnatal period. At the adult stage (PND56–62), relative amplitudes of the startle response to 80-, 90-, 100-, 110- and 120-dB white noise were measured. Data represent mean ± SEM (arbitrary units). $^{*}p < 0.05$, a single startle level compared with saline-injected control littermates by Student's *t*-test with Bonferroni's alpha compensation. A Student–Newman–Keuls test was applied to detect minimum sensitivity of startle in each strain, comparing with the background startle at 80-dB noise ($^{+}p < 0.05$), or to compare basal startle amplitudes at 120-dB among strains; C3H/He > C57BL/6 = ddY > DBA/2. Note: The changes in startle amplitude were indistinguishable between males and females. A Levene test revealed significant differences in variance at 120-dB level among groups ($F[7,99] = 3.65$, $p = 0.002$).

examined in each strain at 120-dB pulses, pooling male and female data. Startle amplitudes were significantly elevated in DBA/2, C3H/He and C57BL/6 mice but not ddY mice. A Student–Newman–Keuls test revealed significant stain difference in basal sensitivity of startle responses: A minimum response was detected with the 100-dB pulse in C3H/He and C57BL/6 mice while that in DBA/2 and ddY mice was with the 110-dB pulse. These results indicated that the magnitudes or threshold levels of sound startle were not associated with EGF responsiveness of individual mouse strains. Thus, EGF treatment differentially affected startle responses, depending on mouse strain, but irrespective of basal startle sensitivity or amplitudes.

Influence of EGF on PPI in Four Mouse Strains

We measured PPI in all mouse strains at the adult stage (Fig. 4). A multiple ANOVA with factors of strain (4) × gender (2) × treatment (2) × prepulse intensity (4, repeated) yielded significant main effects for treatment ($F[1,97] = 12.5$, $p < 0.001$), strain ($F[3,97] = 12.0$, $p < 0.001$), prepulse intensity ($F[3,97] = 69.8$, $p < 0.001$) but not for gender. There was a marginally non-significant interaction between strain and treatment ($F[3,97] = 2.20$, $p = 0.093$). There was no other significant two-way, three-way, or four-way interaction. Accordingly, PPI scores of males and females were combined and overall PPI levels were compared in each strain and at each treatment

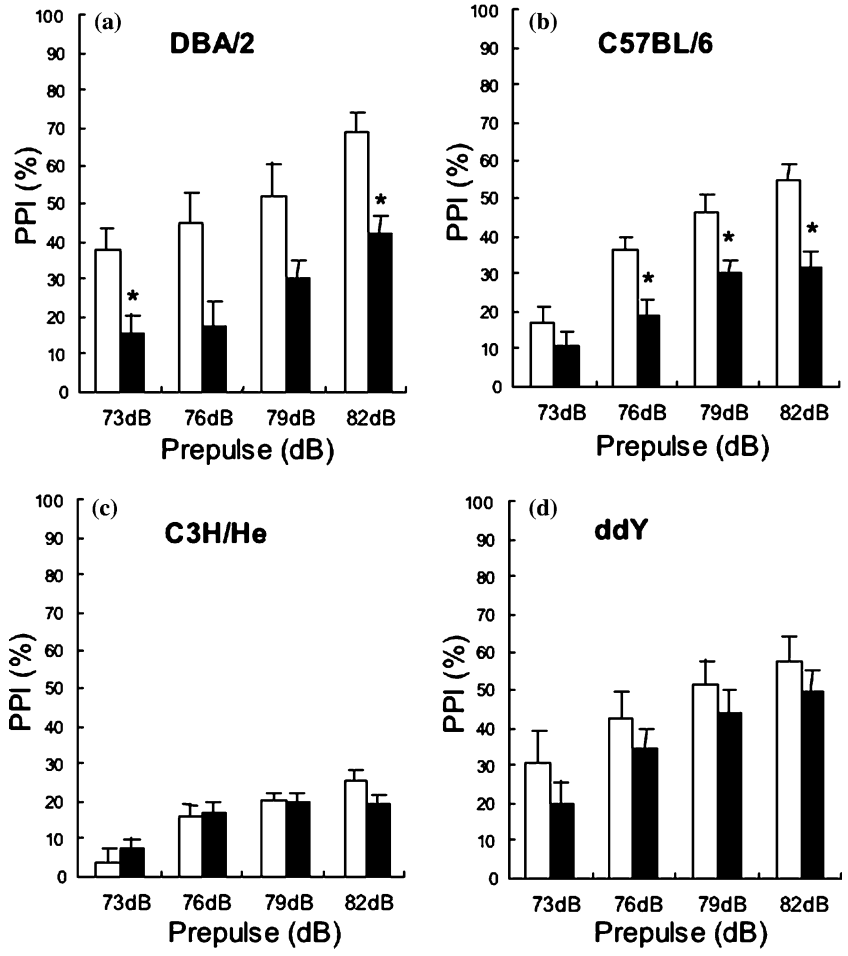

Fig. 4. Strain-specific effect of neonatal EGF administration on PPI. PPI was measured with 73-, 76-, 79- and 82-dB prepulse in mice treated neonatally with EGF (closed bar) and control (open bar) littermates of four mouse strains (DBA/2, C57BL/6, C3H/He, and ddY, $n = 12$–17 for each group). Data represent mean ± SEM (% inhibition of main pulse responses). *$p < 0.05$, a single PPI level compared with saline-injected control littermates by planned Student's *t*-test with Bonferroni's alpha compensation. A Student–Newman–Keuls test was applied to compare basal PPI levels among strains; ddY = DBA/2 = C57BL/6 > C3H/He Note: The changes in PPI were indistinguishable between males and females. A Levene test revealed significant differences in variance at all prepulse levels ($F[7,105] = 3.38$–4.53, $p < 0.003$).

level. The neonatal EGF treatment significantly decreased PPI levels in DBA/2 ($F[1,24] = 12.4$, $p = 0.002$) and C57BL/6 mice ($F[1,24] = 9.80$, $p = 0.005$), but not in C3H/He or ddY mice. Comparisons of baseline PPI among strains revealed that only C3H/He mice displayed the lower PPI (see Fig. 4 legend). Although both control ddY and DBA/2 had the similar basal PPI level, DBA/2 mice reacted with EGF to reduce PPI levels and ddY mice did not respond to EGF, retaining the higher basal PPI level. Strength of the EGF effects on PPI differed among mouse stains and appeared not to correlate with baseline PPI levels.

Effect of EGF on Contextual and Auditory Conditioning in Four Strains of Mice

The effect of EGF on learning was examined at the adult stage by measuring freezing behavior after training, in which an electric shock was coupled with a context plus a tone. Because of the difference in shock sensitivity strains, a factorial ANOVA was not applied to detect strain differences and a strain-treatment interaction. Freezing levels were compared separately in each strain as well as in each testing session with ANOVA [treatment × gender × test duration (repeated, 6 points over 3 minute)]. In DBA/2 mice, ANOVA

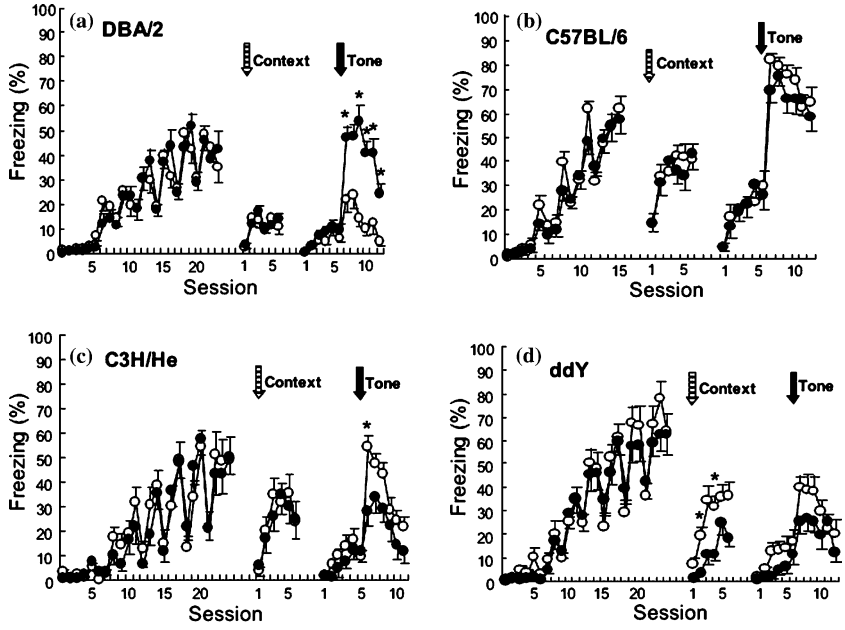

Fig. 5. Tone-dependent and context-dependent learning in EGF-treated and untreated mice. EGF- and saline-treated mice of each strain (DBA/2, C57BL/6, C3H/He, and ddY; $n = 12$–16 each) were subjected to shock-paired contextual conditioning with a tone cue at PND 63–70 and, after 1 day, their learning performance was measured with the contextual cue and the tone cue. Mean percentage (±SEM) of freezing time of saline-treated (open circle) and EGF-treated mice (closed circle) was scored every 30 seconds during conditioning as well as during testing periods (3 minute). Striped arrows and closed arrows indicate the timing of the context cue given and that of the tone cue given during test sessions, respectively. Note. The number of electric shocks was reduced in C57BL/6 mice to adjust their freezing levels to those of the other mouse strains (see Materials and methods).

detected a significant EGF effect on freezing time coupled with the tone cue ($F[1,27] = 18.5$, $p < 0.001$), but not with context. In contrast, the effect of EGF was reversed in C3H/He mice (Fig. 5). Neonatal EGF treatment impaired tone-dependent learning ($F[1,26] = 5.02$, $p = 0.034$), but not context-dependent learning in C3H/He mice. EGF treatment in C57BL/6 mice did not alter learning performance in either paradigm. Neonatally EGF-treated ddY mice exhibited lower learning ability in the context-dependent test ($F[1,21] = 11.0$, $p = 0.003$) but not in the tone-dependent test. In any of mouse strains, there were no significant differences in freezing rate during conditioning between EGF- and saline-treated animals, suggesting no influence of neonatal EGF treatment on shock sensitivity. There was no gender effect or no gender × treatment interaction in the above learning performance of any strain, either. Thus, shock-triggered learning ability in EGF-treated mice was differentially altered, depending upon mouse strain and learning paradigm.

Impact of EGF on other Physical Indices at the Adult Stage

We also monitored EGF effects on body weight and tissue weight of brain, spleen and adrenal gland after completion of the behavioral testing. A multiple ANOVA of body weight with main factors of strain (4) × gender (2) × treatment (2) revealed that EGF treatment ($F[1,115] = 7.07$, $p = 0.009$), mouse strain ($F[3,115] = 97.9$, $p < 0.001$), and gender ($F[1,115] = 13.4$, $p < 0.001$) had significant effects on body size without any interaction between main factors or among the factors. EGF effects on body weight were further analyzed in each gender by Student–Newman–Keuls test (Table II). In male adult mice, there was a significant increase in body weight detected in ddY mice. Female C57BL/6 and C3H/He mice exhibited increasing trends in averaged body weight, which failed to reach a significance range. The overall increasing trend in body weight of the adult EGF-treated mice is contrasted with the overall decreasing trend during postnatal EGF treatment as shown in Table I.

Table II. Delayed Effects of Neonatal EGF Treatment on Body Weight at the Adult Stage

		Control			EGF			
	Strain	Mean	s.e.	Rank	Mean	s.e.	Rank	% Change
Male body weight (g)	DBA/2	21.1	0.4	a	22.6	0.7	a	+7.1
	C57BL/6	23.7	0.2	a	24.5	0.4	a	+3.3
	C3H/He	23.2	0.7	a	22.8	0.7	a	−1.8
	ddY	33.8	1.6	b	38.1	1.4	c	+12.7*
Female body weight (g)	DBA/2	19.3	1.0	ab	20.2	0.8	ab	+4.6
	C57BL/6	21.9	0.8	ab	25.4	2.2	b	+16.0
	C3H/He	16.2	1.0	ab	19.4	1.4	ab	+19.8
	ddY	31.4	2.0	c	33.5	2.2	c	+6.7

Adult mice, which were treated with EGF or saline as neonates, were weighed on PND 70–84 (n = 5–13 per group for males and n = 4–11 per group for females). *Marks a significant difference between EGF-treated mice and saline-treated controls by Student–Newman–Keuls test. A Levene test revealed significant differences in variance among groups ($p < 0.001$).

ANOVA revealed that EGF treatment (F [1,131] = 19.8, $p < 0.001$) and mouse strain (F [3,131] = 72.1, $p < 0.001$) had significant effects on brain size without any interaction between main factors or among the factors (Table III). There was a significant decrease in brain size of the ddY mice, but not in that of the other strains. It is noteworthy that the EGF-treated ddY mice were smaller in brain size but larger in body size at the adult stage. There was no significant effect of EGF treatment on tissue size of spleen or adrenal gland and no factorial interaction between EGF treatment and strain or between treatment and gender, although there were strain and gender differences in their sizes (data not shown).

DISCUSSION

Our previous study demonstrated that peripherally administered EGF in neonatal Sprague–Dawley rats efficiently penetrates the brain–blood barrier and acts on immature neurons in the developing brain (Futamura *et al.*, 2003). The immediate effects of EGF injections include the upregulation of tyrosine hydroxylase and dopamine turnover in the striatum. There are, however, no detectable alterations in cell proliferation and brain structures. Although these neurochemical influences disappear until adulthood, neonatal EGF treatment conversely induces several abnormalities in various behavioral

Table III. Influence of Neonatal EGF Treatment on Tissue Sizes at the Adult Stage

		Control			EGF			
	Strain	Mean	s.e.	Rank	Mean	s.e.	Rank	% Change
Brain weight (mg)	DBA/2	370	5.2	a	354	3.6	a	−4.3
	C57BL/6	427	2.8	bc	411	8.2	b	−3.7
	C3H/He	431	7.5	bc	410	4.8	b	−4.9
	ddY	461	8.5	d	436	5.1	c	−5.4*
Spleen size (mg)	DBA/2	95	7.9	a	102	6.3	a	+7.3
	C57BL/6	88	8.7	a	84	5.3	a	−4.5
	C3H/He	72	2.7	a	71	3.7	a	−1.4
	ddY	148	17	b	147	17	b	−0.7
Adrenal gland size (mg)	DBA/2	1.8	0.1	a	1.8	0.2	a	0
	C57BL/6	1.9	0.1	a	1.8	0.1	a	−5.2
	C3H/He	1.7	0.1	a	1.6	0.1	a	−5.9
	ddY	3.0	0.5	b	3.2	0.5	b	+6.6

Adult mice, which were treated with EGF or saline as neonates were weighed and dissected on PND 70–84 (n = 15–22 per group). Wet tissue weight of brain, spleen and adrenal gland was measured. *Marks a significant difference between EGF-treated mice and saline-treated controls by Student–Newman–Keuls test. A Levene test revealed significant differences in variance in spleen ($p = 0.013$) and adrenal sizes ($p < 0.001$).

and pharmacological tests for locomotion, PPI, antipsychotic sensitivity and methamphetamine sensitization (Futamuta *et al*., 2003; Mizuno *et al*., 2004), which involve the dopaminergic system (Gendreau *et al*., 1997; Swerdlow and Geyer, 1998; Zocchi *et al*., 1998). Thus, this animal appears to be useful as an animal model for schizophrenia (Nawa and Futamura, 2003). The present study demonstrates that neonatal EGF treatment produces similar behavioral alterations in mice, but EGF influences on individual behaviors varied considerably depending on mouse strain (Table IV).

Irregular Behavioral Responses of Mouse Strains and Brain ErbB1 Signaling

Neonatal EGF treatment had opposite effects on exploratory locomotor activity in DBA/2 and ddY mice (Table IV). Similar strain differences following neonatal EGF treatment were observed in the PPI test and learning tasks. EGF treatment enhanced auditory learning performance in DBA/2 mice, whereas it depressed contextual and auditory learning in ddY and CH3/He mice, respectively. Neonatal EGF treatment reduced PPI levels in DBA/2 and C57BL/6, but not in C3H/He and ddY mice. Based on the behavioral influence of EGF, we classified the mouse strains into two categories: PPI responders and PPI non-responders. The PPI responders include DBA/2 and C57BL/6 mice, which show a decrease in PPI and/or improved learning following neonatal EGF challenge. The PPI non-responders include C3H/He and ddY mice, which exhibit decreased learning and no significant response to EGF in PPI. These behavioral variations among mouse strains are not correlated with physical responses to EGF. The present findings highlight the biological significance of genetic background in determining the direction and strength of EGF influences on neurobehavioral traits. Endogenous expression of ErbB1 receptor ligands (EGF, TGFα, HB-EGF, amphiregulin etc.) is induced by neuronal activity, inflammation, and injury (Isono *et al*., 2003; Opanashuk *et al*., 1999; Tanaka *et al*., 1999). Rearing conditions also affect the production of TGFα in the neonatal brain (Romeo *et al*., 2004). Therefore, genetic background of individual animals might influence physiologic and pathologic consequences of brain ErbB1 activation during brain development.

Strain-specific Behavioral Responses and Peripheral Reactions to EGF

In our experiments, neonatal EGF treatment facilitated eyelid opening and tooth eruption, confirming the biological activity, as described previously (Cohen, 1962). There was no correlation, however, between the acceleration of these physical indices and the adult behavioral changes induced by EGF. Both PPI responders (DBA/2 and C57BL/6 mice) and PPI non-responders (C3H/He and ddY mice) had the same degree of accelerated eyelid opening and tooth eruption. The strength of EGF effects on body weight or brain weight at the adult stage was most marked in ddY mice and, therefore, was not consistent with that on behavioral traits, either. These results suggest that strain-dependent behavioral responses to EGF are independent of the peripheral actions of EGF. In contrast, the behavioral responses appeared to be correlated with basal phosphorylation levels of brain ErbB1 in individual mouse strains. Although there was no significant difference in total ErbB1 levels, basal phosphorylation levels were lower in PPI responders and higher in PPI non-responders. After treatment with receptor-saturating EGF, however, ErbB1 phosphorylation was approximately equivalent in all four strains. Therefore, the strain difference in basal ErbB1 phosphorylation might reflect

Table IV. Summary of Behavioral Effects of Neonatal EGF Treatment in Four Mouse Strains

				Context and auditory fear learning	
Mouse strains	Exploratory locomotion	Startle response	PPI	Context	Tone cue
DBA/2	UP	UP	Down	=	UP
C57BL/6	=	UP	Down	=	=
C3H/He	=	UP	=	=	Down
ddY	Down	=	=	Down	=

= not statistically significant. All behavioral data were obtained at the adult stage (PND56-75).

variations in endogenous EGF levels among mouse strains. In agreement, C57BL/6 mice are reported to produce the lowest level of EGF in the salivary gland among many mouse strains (Tom-Moy and Barka, 1981). EGF/TGFα/ErbB1 signals have crucial roles in the development of dopaminergic neurons and neural stem cell proliferation (Alexi and Hefti, 1993; Blum, 1998; Casper *et al.*, 1991; Ferrari *et al.*, 1991; Hanke *et al.*, 2004; Sibilia *et al.*, 1998). There are genetic differences among mouse strains in basal tyrosine hydroxylase activity and the number of dopamine neurons in the midbrain area (Baker *et al.*, 1980). Thus, the strength of the ErbB1 phosphorylation before or after neonatal EGF treatment influences ErbB1-dependent dopaminergic differentiation or maturation, leading to strain-specific behavioral alterations.

Interaction between EGF-triggered Behavioral Changes and Basal Traits

Many behavioral traits, such as sensorimotor gating, cognitive performance, and learning ability, are under apparent genetic control (Crawley *et al.*, 1997; Marks *et al.*, 1989; Nguyen *et al.*, 2000; Wehner *et al.*, 1997). Inbred mouse strains differ in basal acoustic startle response and in PPI levels; Among the various mouse strains, DBA/2 mice are relatively insensitive in sound-startle reactions and C3H/He mice have a lower PPI level, which is consistent with the present results (Bullock *et al.*, 1997; Crawley *et al.*, 1997; Dulawa and Geyer, 1996; Marks *et al.*, 1989). The baseline startle amplitudes of PPI non-responder mice, ddY, were similar to those of the PPI responder mice, C57BL/6. Control ddY, C57BL/6, and DBA/2 mice all had similar basal PPI levels, but only ddY mice failed to alter PPI levels in response to neonatal EGF challenge. Thus, there was no correlation between baseline startle amplitudes and EGF effects on PPI or between baseline PPI levels and the EGF effects.

Neonatal EGF treatment in DBA/2 mice improved performance in learning with an auditory cue. In contrast, EGF treatment led to the opposite behavioral changes in learning of other strains: PPI non-responders of C3H/He and ddY mice displayed reduced performance of learning with tone and visual cues in response to EGF, respectively. Although higher auditory sensitivity of EGF-treated DBA/2 mice contributed to their better tone-dependent learning, (Nguyen *et al.*, 2000; Wehner *et al.*, 1997), it is difficult to correlate the strength and direction of the EGF-triggered changes in learning of C3H/He and ddY mice with higher or lower sensory sensitivity of these animals. Learning involves multiple brain processes including primary sensation, sensory modality, and integration, which are supported by different brain regions. For example, visual fear learning is known to depend hippocampal mechanism while auditory fear learning involves amygdala processing (Anagnostaras *et al.*, 2000; Phillips and LeDoux. 1992; Sacchetti *et al.*, 1999). Thus, one of the potential explanations is that the strain-dependent or area-specific sensitivity to EGF might differ among strains and contribute to the above strain-difference in EGF responses in learning. The fact that the cell proliferation activity of EGF is significantly different among mouse strains supports this hypothesis (Parzefall *et al.*, 2002). Alternatively, it is possible that EGF permeability into these brain regions might differ considerably among mouse strains, although this assumption also remains to be tested.

Implication of Genetic Background in Schizophrenia

Abnormal brain development as well as dopaminergic dysfunction is implicated in the etiology or pathology of schizophrenia (Murray and Lewis, 1987; Weinberger, 1987). Among many neurodevelopmental regulators, ErbB1 ligands (EGF, HB-EGF, TGFα, etc.) have strong trophic activity on midbrain dopaminergic neurons (Hadjiconstantinou *et al.*, 1991; Iwakura *et al.*, 2005; Seroogy *et al.*, 1994; Ventrella *et al.*, 1993). Our previous postmortem study on schizophrenic patients indicates that EGF content is specifically reduced and ErbB1 receptor levels are conversely upregulated in the striatum (Futamura *et al.*, 2002). Based on the above hypothesis, as well as the fact that schizophrenic patients carry abnormal EGF/ErbB1 signals (Futamura *et al.*, 2002), the present animal model was initially established with EGF (Futamura *et al.*, 2003). This animal has several characteristics that fit a schizophrenia model; (1) Behavioral abnormalities, such as reduced sensorimotor gating and accelerated rearing behaviors, develop at post-pubertal stages and persist for life. (2) Most of these abnormalities can be ameliorated by typical or atypical antipsychotic drugs. (3) There is a reduction in brain weight with no apparent sign of gliosis or neurodegeneration. Accordingly, the EGF-treated rat presumably serves as a good model for schizophrenia. EGF-treated mice exhibited or did not exhibit impairments in sensorimotor gating and context learning, depending on mouse genetic

backgrounds. Although patients with schizophrenia display similar sets of cognitive and learning impairments such as sensorimotor gating and context learning, there are large inter-individual differences in patients' cognitive/learning performance (Braff *et al*., 1992; Hsieh *et al*., 2004; Kumari *et al*., 2004; Sullivan *et al*., 1997). Thus, the present mouse study indicates that the inter-individual variation in pathophysiologic indices of schizophrenic patients might also involve genetic differences in ErbB1/EGF signaling or other neurotrophic sensitivity.

Previous extensive studies on schizophrenia indicate the importance of both environmental and genetic influences on schizophrenia etiology. Environmental factors include maternal viral infection and birth complications that increase the risk for schizophrenia or impair animal behaviors (Cannon *et al*., 2002; Lipska *et al*., 1998; Narayan *et al*., 1983; O'Callaghan *et al*., 1991, 1992; Salvatore *et al*., 1997; Shi *et al*., 2003). Both brain insults and infections are known to induce a variety of neurotrophic cytokines including EGF (Isono *et al*., 2003; Kawahara *et al*., 1999; Opanashuk *et al*., 1999; Schaudies and Johnson, 1993; Tanaka *et al*., 1999). It is noteworthy that EGF in the amniotic fluids is markedly increased to receptor-stimulating levels in some pregnant women (Hofmann and Abramowicz, 1990; Varner *et al*., 1996). Thus, it is possible that neurotrophic cytokines, such as EGF, mediate the above environmental insults or stress in human embryos and neonates to influence the future risk of schizophrenia (Nawa *et al*., 2000; Petronis, 2004).

A gene(s) is another factor that markedly influences the risk of schizophrenia as well as the behavioral features of its models (Lipska and Weinberger, 1995; Moldin and Gottesman, 1997; Pletnikov *et al*., 2002). In particular, the present results illustrate the strength and complexity of the interaction between genetic (mouse strain) and environmental (neonatal EGF challenge) factors in behavioral development, although many questions regarding their interactions remain unanswered. Genetic background influences whether a particular set of behavioral changes occur, but it also determines the direction of the behavioral change following exposure to EGF. We assume that, if abnormal EGF/ErbB1 activity occurs in human fetuses or neonates with different genetic backgrounds, the resulting behavioral traits will be diverse and possibly distinct in pathophysiology. The present results indicate that the biological interaction between genetic background and neurotrophic conditions during development determines later psychobehavioral traits in animals, possibly including human beings.

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