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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2007.08.034.

(Accepted 31 August 2007)
(Available online 21 September 2007)

ELSEVIER

Brain & Development xxx (2008) xxx–xxx

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Common behavioral influences of the ErbB1 ligands transforming growth factor alpha and epiregulin administered to mouse neonates

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Received 31 October 2007; received in revised form 16 January 2008; accepted 24 January 2008

Abstract

Ligands for epidermal growth factor (EGF) receptor (ErbB1), such as EGF, transforming growth factor alpha (TGFα), and epiregulin, are enriched in body fluids and blood and regulate development of various peripheral organs. It remains however how such circulating polypeptide growth factors influence brain development and function. Here, we performed peripheral injections of TGFα and epiregulin to mouse neonates and evaluated immediate physical and neurochemical development and later behavioral consequences. Subcutaneous administration of TGFα and epiregulin increased phosphorylation of brain ErbB1, suggesting their effects on brain development. Repeated their injections similarly enhanced physical development of eyelid opening and tooth eruption during early postnatal stage and resulted in abnormal behavioral traits in the adult stage. Acoustic startle responses of mice treated with these growth factors as neonates were enhanced and prepulse inhibition was decreased without an apparent correlation between prepulse inhibition level and startle intensity. Locomotor activity and fear-learning performance with tone and context cues were not altered, however. These results suggest that circulating ErbB1 ligands in the periphery of neonates have some common influences on later behavioral traits. Abnormal ErbB1 ligand production at neonatal and potentially prenatal stages might therefore associate with neurodevelopmental disorders such as schizophrenia.

Keywords: EGF; Cytokine; Body weight; Tooth eruption; Eyelid opening; Sensorimotor gating; Animal behavior; Neurodevelopmental disorder

1. Introduction

ErbB1 receptor tyrosine kinase binds to various peptide ligands including epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), heparin-binding EGF-like factor (HB-EGF), amphiregulin, betacellulin, epigen, and epiregulin [1–4]. These ligands are synthesized as membrane-anchored precursors in a large variety of peripheral tissues, such as, kidney, blood cells, placenta, etc., and liberated to urine, blood, and amniotic fluid [5–9]. As all these ligands have a cell mitotic activity and thus are implicated in growth regulation of peripheral organs as well as in cancer progression [9–12]. Our recent studies on brain development indicate that exogenously supplied EGF in the periphery can penetrate the developing brain–blood barrier (BBB) of neonatal rats and mice to perturb phenotypic development of brain neurons [13]. Therefore, such growth

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doi:10.1016/j.braindev.2008.01.006

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factors in blood stream have latent but profound impact on brain development during early prenatal and postnatal stages [14]. It is unknown, however, how endogenous growth factors reach the brain to perturb brain development and function.

ErbB1 is expressed in many types of brain cells, but is especially enriched in midbrain dopaminergic neurons as well as neural stem cells [15,16]. Our previous studies suggest that EGF efficiently penetrates the immature blood–brain barrier during early rodent development and later perturbs dopamine-associated animal behaviors, such as sensorimotor gating, social interaction, exploratory motor activity and amphetamine sensitivity [13,17,18]. These findings have indicated that not only EGF but also other ErbB1 ligands in some human fetuses or neonates potentially influence brain development and future psychobehavioral traits. However, it remains to be determined which ErbB1 ligands contribute to the establishment of psychiatric disease or traits.

In the present investigation, we evaluated the biological significance of the ErbB1 ligands, TGFα, and epiregulin, in determining behavioral traits in mice. Mice were subjected mainly to the behavioral tests that are often used to evaluate schizophrenia models. Their effects on behaviors and physical development were compared with the rodent model established by neonatal exposure to EGF [13,17].

2. Materials and methods

2.1. Animal protocols

Four strains of neonatal mice (C57BL/6N, 12 litters total; Nihon Charles River, Yokohama, Japan) were purchased together with their dams. Recombinant human epiregulin (1.0 μg/g; Taisho Pharmaceutical Inc., Ltd, Japan) or TGFα (1.0 μg/g; Peprotech Inc., Ltd, UK) was administered subcutaneously to half of individual litters daily during postnatal days (PND) 2–10. Control littermates of the other half received saline injections. After PND 20, mice were separated according to gender and raised separately (3–4 mice per cage; 13.6L × 20.8W × 11.5H cm). All mice 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 (17:00–19:00 h). All animal experiments were authorized by the Animal Use and Care Committee of Niigata University and performed during the day cycle (10:00–19:00 h). Animals (TGFα-, epiregulin- or saline-treated mice, $n = 10$–17 per group) were assigned to three behavioral tests to minimize the effects of the preceding behavioral test; locomotor test followed by prepulse inhibition (PPI) and then 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 h before testing.

2.2. Immunoblot analysis

Using an independent set of animals, TGFα or epiregulin (1.0 μg/g) or saline was injected subcutaneously into mouse pups (PND2) to examine the acute effect on ErbB1 activation in the brain. Brains were removed 15–240 min after injection and homogenized by sonication with 1× sample loading buffer (10 mM Tris–HCl, 150 mM NaCl, 2% SDS, 20 mM NaF, 1 mM Na_3VO_4). To confirm subchronic influences of these factors on brain neurons, alternatively, tissue lysate was prepared from the frontal cortex of mice (PND11) one day after repeated administration (PND2–PND10). Protein (50 μg/lane) was subjected to a 7.5% 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, USA). Alternatively, immunoblots were probed with anti-GluR1 receptor (1:1000), anti-GluR2/3 (1:1000), and anti-β actin (1:1000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was visualized with chemiluminescence reaction (Western Lighting, Perkin-Elmer, Tokyo, Japan).

2.3. 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, USA). Mice were placed in an automated activity monitor equipped with infrared photosensors. Horizontal activity for every 5 min was measured as beam crossings during the initial 60 min and analyzed by using fully automated tracking system (Activity Monitor, Med Associates).

2.4. Measurement of acoustic startle response and prepulse inhibition

Mice were placed in a plastic cylinder and fixed in an automated startle chamber (SR-Lab Systems, San Diego, CA, USA). After a 5-min acclimation period with 70-dB-background noise (white noise), an 75-, 80-, 85-, 90-, 100-, 110-, or 120-dB white noise stimulus (40 ms duration) was given 8 times to each mouse in the same pseudo-random order at 15 s-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

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in the startle chamber (SR-Lab) and initially acclimatized for 5 min 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-ms 120-dB noise burst presented alone (S), (ii–v) a 40-ms 120-dB noise burst following prepulses by 100 ms (20-ms noise burst) that were 3, 6, 9, or 12 dB above background noise (i.e., 73-, 76-, 79-, or 82- 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 8 times in a pseudorandom order to give 48 trials. The inter-trial interval was 15 s. 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) [19,20]

2.5. Contextual conditioning

The test paradigm of contextual conditioning was based on a work by Tohmi et al. [17]. Mice were placed in a shock chamber with a stainless steel grid floor (10W × 10D × 10H cm box; Ohara Medical Industry Inc., Tokyo, Japan) for 2 min to monitor baseline movement/freezing and then exposed to 0.8-mA electric shocks (2-s duration, 3 times at an interval of 90 s) all together with 30-s tone cues (60-dB, 10 kHz). One day after conditioning, mice were returned to the same chamber. The time spent freezing was recorded and averaged every 30 s. After 3 h, mice were moved to a different chamber with a flat floor (10W × 10D × 10H cm box) and the time spent freezing was recorded and scored for 3 min before and after the tone cue. Freezing behavior was automatically monitored by a video camera during all sessions and analyzed with the aid of an imaging software (Ohara Medical Industry Inc.).

2.6. Statistical analysis

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

3. Results

3.1. ErbB1 activation following peripheral injection of TGF-α and epiregulin

We have previously reported that peripheral administration of EGF activates ErbB1 in the neonatal brain, presumably penetrating the immature blood–brain barrier [13,21]. In the present study, we examined whether peripheral administration of the EGF homologs, TGF-α, and epiregulin, similarly triggers ErbB1 phosphorylation in the brain (Fig. 1). A single subcutaneous injection of TGF-α to neonatal C57BL/6N mice immediately increased phosphorylation of ErbB1. There was a marked increase in immunoreactivity for phosphorylated ErbB1 at the 15-min period. The increase was disappeared within 2 h. In contrast to TGF-α, epiregulin produced a more modest and prolonged increase in phosphorylated ErbB1 levels. β-Actin levels (control) were not significantly altered ($F[5,11] = 0.393$, $p = 0.063$ for TGF-α; $F[5,11] = 3.397$, $p = 0.062$ for epiregulin).

We previously found that EGF and TGF-α attenuate the developmental increase of AMPA receptor expression in the neocortex [22,23]. To control the present experimental procedure, we examined whether the administration of epiregulin mimics the effect of TGF-α in neonatal mice (Fig. 2). Epiregulin and TGF-α were daily administered to neonatal mice until PND10 and protein levels for AMPA receptors (GluR1 and GluR2/3) were examined in the neocortex by immunoblotting. The both EGF homologs attenuated the protein expression of GluR2/3 ($F[2,12] = 12.69$, $p = 0.001$ after normalization with actin levels) (Fig. 2B). There was no significant influence of those ErbB1 ligands on GluR1 protein levels, however ($F[2,12] = 0.862$, $p = 0.445$ after normalization with actin levels).

3.2. Effects of TGF-α and epiregulin treatment on physical development of mice

Repeated administration of EGF to mouse newborn pups accelerates eyelid opening and tooth eruption [10]. To compare the peripheral effects of TGF-α and epiregulin, we similarly administered these factors or saline subchronically into mouse pup and monitored these physical indices (Table 1). TGF-α treatment mark-

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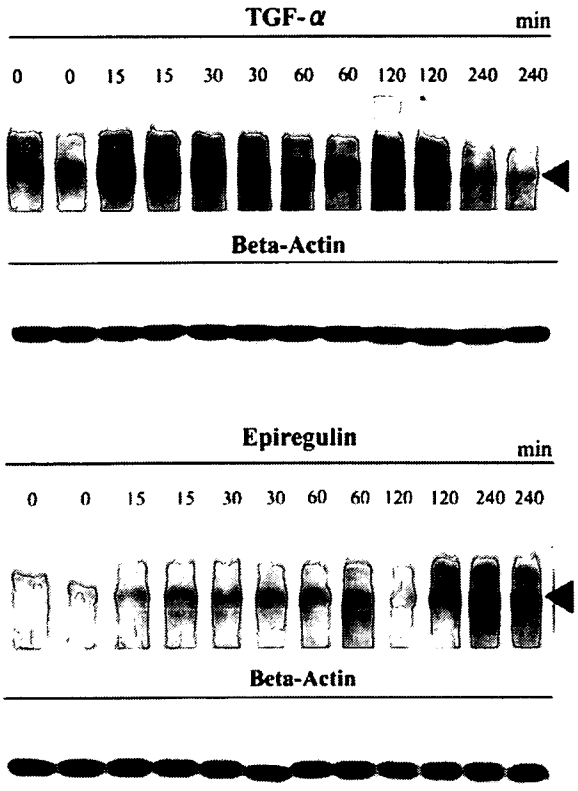

Fig. 1. Brain ErbB1 phosphorylation induced by subcutaneously administered TGF-α and epiregulin in mouse pups. Time courses of ErbB1 phosphorylation in the brain were determined after TGF-α or epiregulin injection at postnatal day (PND) 2. Solution of TGF-α or epiregulin (1.0 μg/g) was subcutaneously injected into neonatal C57BL/6N mice ($n = 12$ mice total for each group). The whole brain was dissected 0 (no injection), 15, 30, 60, 120, and 240 min after injection, and protein extracts were subjected to immunoblotting for anti-phospho ErbB1 antibody (two mice for each time point) and anti-β-actin antibody (control).

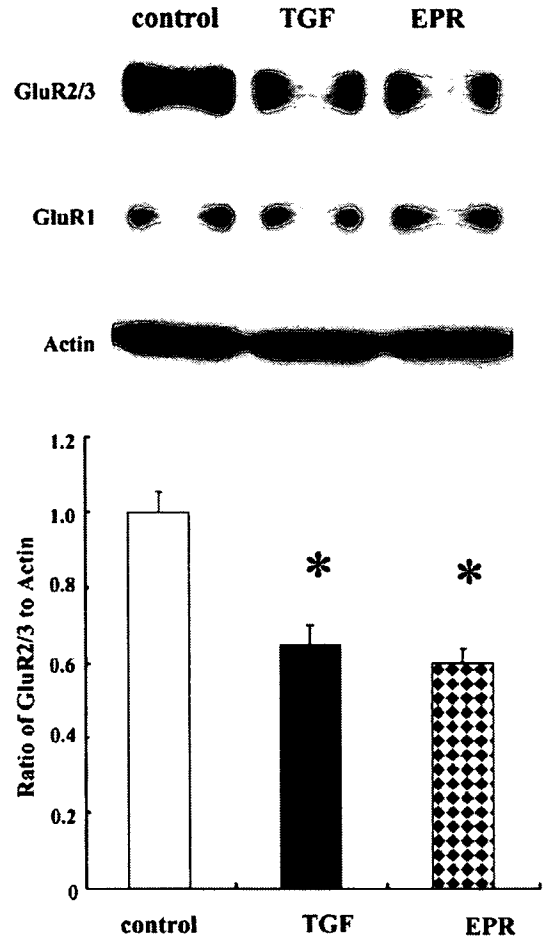

Fig. 2. Effects of repeated injections of TGF-α and epiregulin on AMPA receptor expression in the neocortex. Neonatal mice were daily treated with TGF-α, epiregulin (EPR), or saline from PND2 to PND10 and brain tissues were dissected out at PND11 when 24 h passed after the last injection was performed ($n = 5$ mice each). Protein was extracted from the neocortex and examined by immunoblotting with the antibodies directed against GluR1, GluR2/3, and β-actin. Immunoreactivity for GluR1 and GluR2/3 was normalized with that for β-actin in each lane. Mean protein level of control mice was set to 1.0. $^{*}p < 0.05$

edly stimulated eyelid opening (*two-tailed t-test*, $p < 0.001$) and tooth eruption (*two-tailed t-test*, $p < 0.001$). Similarly, epiregulin accelerated eyelid opening (*two-tailed t-test*, $p < 0.001$) and tooth eruption (*two-tailed t-test*, $p < 0.001$). The magnitude of the epiregulin-triggered acceleration for tooth eruption was less marked than that induced by TGF-α. The magnitudes of the TGF-α-triggered acceleration for physical development were, however, comparable to those induced by neonatal treatment with EGF (i.e., −5.2 day for eyelid, −2.1 day for tooth eruption) [17].

We also monitored the influence of TGF-α and epiregulin administration on body weight (Table 1). We compared mean body weight on PND10 when the last treatment was done as well as on PND60 when the mice grew to adults. In the postnatal stage, there was no significant alteration triggered by TGF-α or epiregulin treatment. In the adult stage, however, their effect on body weight emerged. A two-way ANOVA revealed a significant interaction between gender and TGF-α treatment ($F[1,21] = 8.527$, $p = 0.008$). Post hoc analysis found that neonatal treatment with TGF-α specifically elevated body weight of male mice. Epiregulin-treated male animals exhibited a similar trend in body weight that was not statistically significant ($F[1,25] = 2.638$, $p = 0.117$ for the interaction).

3.3. Influence of neonatal ErbB1 ligands on acoustic startle response and prepulse inhibition

Neonatal challenges of EGF are known to alter acoustic startle response and sensorimotor gating of rats and mice at their post-pubertal stages. Sensorimotor

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Table 1
Effects of TGF-α and epiregulin challenges on physical development in mice

	Control	TGF-α
TGF-α		
Tooth eruption (PND)	10.80 ± 0.12	8.86 ± 0.09***
Eyelid opening (PND)	13.20 ± 0.12	9.85 ± 0.09***
Body weight (g)		
PND10	5.89 ± 0.09	5.59 ± 0.15
PND60 (male)	18.96 ± 0.75	21.47 ± 0.31*
PND60 (female)	18.13 ± 0.20	17.95 ± 0.42
	Control	Epiregulin
Epiregulin		
Tooth eruption (PND)	9.93 ± 0.16	8.80 ± 0.10***
Eyelid opening (PND)	13.0 ± 0.00	8.93 ± 0.15***
Body weight (g)		
PND10	5.86 ± 0.13	5.81 ± 0.11
PND60 (male)	20.91 ± 0.34	21.99 ± 0.28*
PND60 (female)	18.36 ± 0.41	18.08 ± 0.53

We monitored postnatal days (PND) when the tooth eruption and eyelid opening were first detected in mouse littermates receiving saline and TGF-α or those receiving saline and epiregulin. Body weight was also measured on PND10 and PND60 and compared between groups.
* $p < 0.05$.
*** $p < 0.001$.

gating is the neural process in which inhibitory neural pathways filter multiple stimuli to allow attention to be focused on one stimulus [19]. Sensorimotor gating can be assessed as PPI of startle, which is the modulation of the startle response, following a weak prepulse [20]. PPI magnitudes are decreased in several human neuropsychiatric disorders, including schizophrenia, obsessive–compulsive disorder, Huntington's disease, and Tourette's syndrome, suggesting that sensorimotor gating defects are a common neural dysfunction contributing to these psychiatric disorders [19,20].

We examined the effects of TGF-α and epiregulin on acoustic startle response in the presence or absence of four levels of prepulse tones at the young adult stage and separately analyzed data for TGF-α or epiregulin treatment as control littermates were set for each treatment (Fig. 3). Pulse-alone startle was significantly elevated at the higher ranges of acoustic stimuli. A multiple ANOVA for TGF-α employed the factors of treatment (2), gender (2), and sound intensity (7, repeated) and revealed significant main effects of TGF ($F[1,23] = 10.8$, $p = 0.003$) but not gender ($F[1,23] = 0.296$, $p = 0.592$) without an interaction between treatment and gender ($F[1,23] = 0.913$, $p = 0.349$) (Fig. 3A). Post hoc detected that TGF-α treatment increased pulse-alone startle at 110- and 120-dB tones. Similarly, neonatal epiregulin treatment elevated pulse-alone startle amplitudes ($F[1,25] = 6.56$, $p = 0.017$) without an interaction between treatment and gender ($F[1,25] = 1.302$, $p = 0.265$) (Fig. 3B). Comparisons with the basal startle levels for 75-dB tone revealed that treatment with TGF-α or epiregulin did not alter minimum thresholds of acoustic startle. These results suggest that the magnitudes or threshold levels of sound startle were not associated with the increases in startle amplitudes. Thus, neonatal treatment with TGF-α and epiregulin similarly enhances pulse-alone startle responses to 120-dB sound stimuli.

We simultaneously measured effects on PPI at the adult stage (Fig. 4). In TGF-α group, a multiple ANOVA with factors of gender (2), treatment (2), and prepulse intensity (4, repeated) yielded significant main effects for treatment ($F[1,23] = 10.79$, $p = 0.003$) and prepulse intensity ($F[1,23] = 69.2$, $p > 0.001$) but not gender ($F[1,23] = 0.549$, $p = 0.466$) (Fig. 4A). There was a significant interaction between treatment and gender ($F[1,23] = 6.29$, $p = 0.020$). There were significant main effects of TGF-α treatment at all prepulse levels ($F[1,23] = 4.46$–13.27, $p = 0.001$–0.046) and significant gender interactions only at 73- and 76-dB prepulses ($F[1,23] = 6.31$ and 5.28, $p = 0.019$ and 0.031, respectively). Post hoc revealed that PPI levels of saline-treated control males exhibited significantly higher PPI levels than control females at 73-dB prepulse ($p = 0.005$). In the epiregulin-treated mice, we observed the similar activity of epiregulin decreasing PPI levels. A multiple ANOVA revealed significant main effects for treatment ($F[1,25] = 6.56$, $p = 0.017$) and prepulse intensity ($F[1,25] = 135.1$, $p < 0.001$) but not gender ($F[1,25] = 1.22$, $p = 0.280$) without an interaction between treatment and gender ($F[1,25] = 1.302$, $p = 0.265$) (Fig. 4B). Post hoc detected the significant decrease in PPI levels at 76-dB and 82-dB prepulses ($p = 0.016$ and 0.008, respectively).

As neonatal treatments with TGF-α and epiregulin both simultaneously affected the pulse-alone startle and percentage PPI, interpreting the PPI decrease may be controversial and require evaluation [20]. To test the possibility that the increase in pulse-alone startle responses was responsible for the decrease in PPI, individual animal data for either TGF-α or epiregulin treatment were re-analyzed by the Pearson's correlation analysis (Fig. 5). When the percent PPI levels for 82-dB prepulse were plotted against the magnitude of the pulse-alone startle for each animal, there were no significant correlations between these values in TGF-α-treated group as well as in saline-treated controls ($p = 0.580$ for controls, $p = 0.911$ for TGF-α group) (Fig. 5A). The slope of the linear regression curve for the TGF-α-treated group was almost horizontal ($r = -0.033$). Both mathematical evaluations suggest that the TGF-α-triggered increase in pulse-alone startle is unlikely to contribute to the reduction PPI. Similar statistical results for the correlation between pulse-alone startle and %PPI in the TGF-α-treated group were obtained for the other prepulse levels ($p = 0.434$–0.682, $r = -0.121$–0.227).

We similarly applied the Pearson's correlation analysis to data of epiregulin-treated animals and their con-

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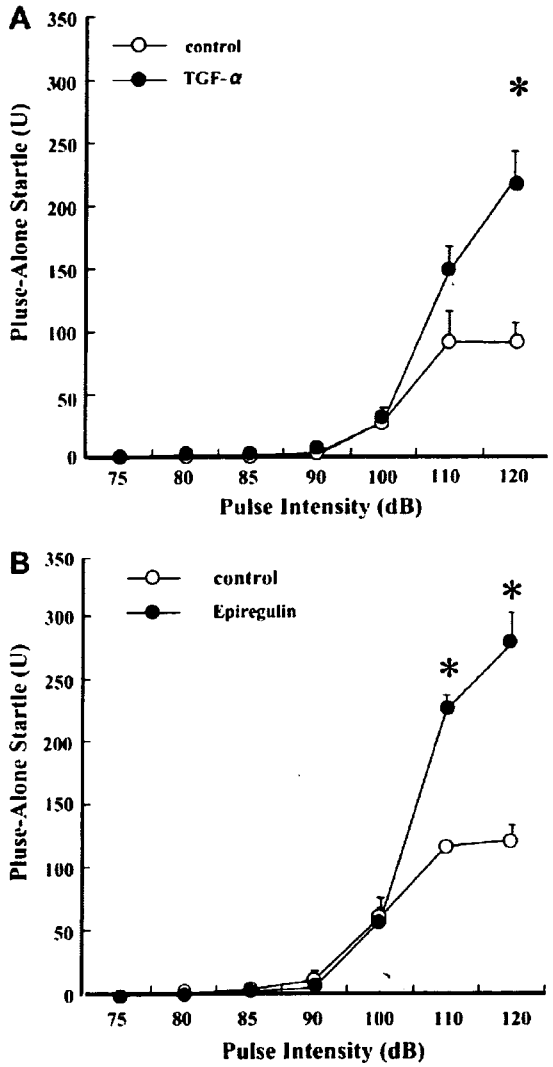

Fig. 3. Comparison of acoustic startle reactions. Neonatal mice were given TGF-α, epiregulin or saline ($n = 10$–17 for each group) during the early postnatal period as described above. At the adult stage (PND56-62), relative amplitudes of the startle response to 75-, 80-, 85-, 90-, 100-, 110-, and 120-dB white noise were measured and compared between littermates receiving TGF-α or saline (A) or receiving epiregulin or saline (B). Data represent mean ± SEM (arbitrary units). $^{*}p < 0.05$, a single startle level compared with saline-injected control littermates or $^{+}p < 0.05$ that with the background startle at 75-dB noise by Fisher LSD. *Note:* The changes in startle amplitude were indistinguishable between males and females (data not shown).

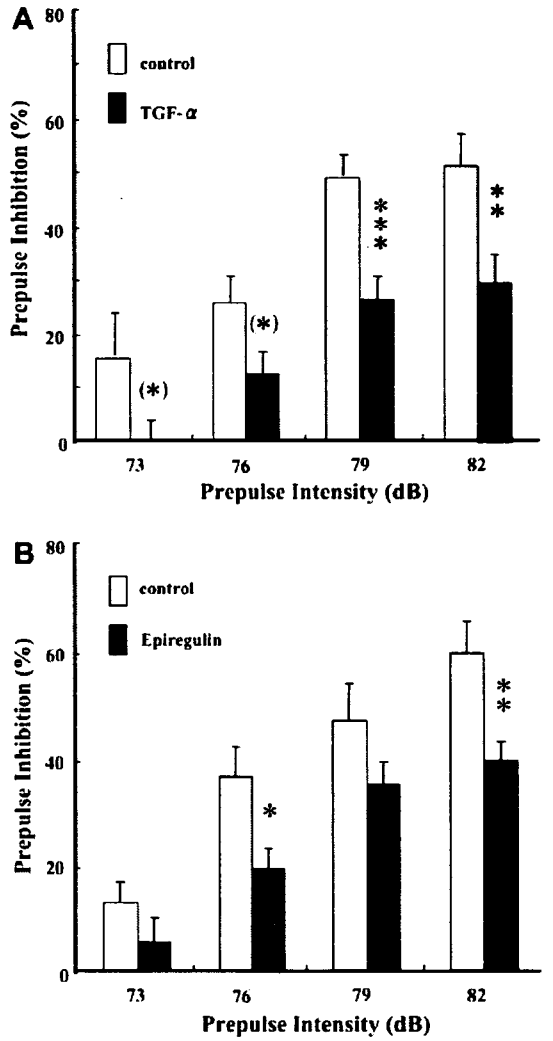

Fig. 4. Effects of neonatal administration of TGF-α and epiregulin on prepulse inhibition. Prepulse inhibition (PPI) was measured with 73-, 76-, 79-, and 82-dB prepulse stimuli in mice treated neonatally with TGF-α (A) or epiregulin (B) and compared with control littermates receiving saline ($n = 12$–17 for each group). Data represent mean ± SEM (% inhibition of main pulse responses). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, a single PPI level compared with saline-injected control littermates by Fisher LSD. (*) There were gender × treatment interactions at 73- and 76-dB prepulse levels; Male control mice exhibited significantly higher PPI levels; 65 ± 9% for male and 56 ± 8% for female at 73-dB prepulse, and 75 ± 9% for male and 65 ± 7% for female at 76-dB prepulse.

trols to estimate the potential effects of pulse-alone startle on %PPI. There was no significant correlation between pulse-alone startle and %PPI for all prepulses ($p = 0.130$–0.565 for controls and $p = 0.243$–0.755 for epiregulin group) (Fig. 5B). The regression curves for epiregulin-treated mice all displayed positive correlation coefficient ($r = 0.088$–0.321). Thus, we statistically conclude that epiregulin-triggered decreases in %PPI are not attributed to the increase in pulse-alone startle.

3.4. Influences of neonatal TGF-α and epiregulin treatments on exploratory motor behavior in adult mice

The effect of TGF-α and epiregulin on locomotor activity was assessed in a novel environment at young adult stage more than 6 weeks following their final injections (Fig. 6). In the experiment of TGF-α treatment, a multiple ANOVA with factors of treatment (2) × gender (2) × test duration (12, repeated) revealed no significant main effect of treatment ($F[1,23] = 0.00$, $p = 0.984$) and

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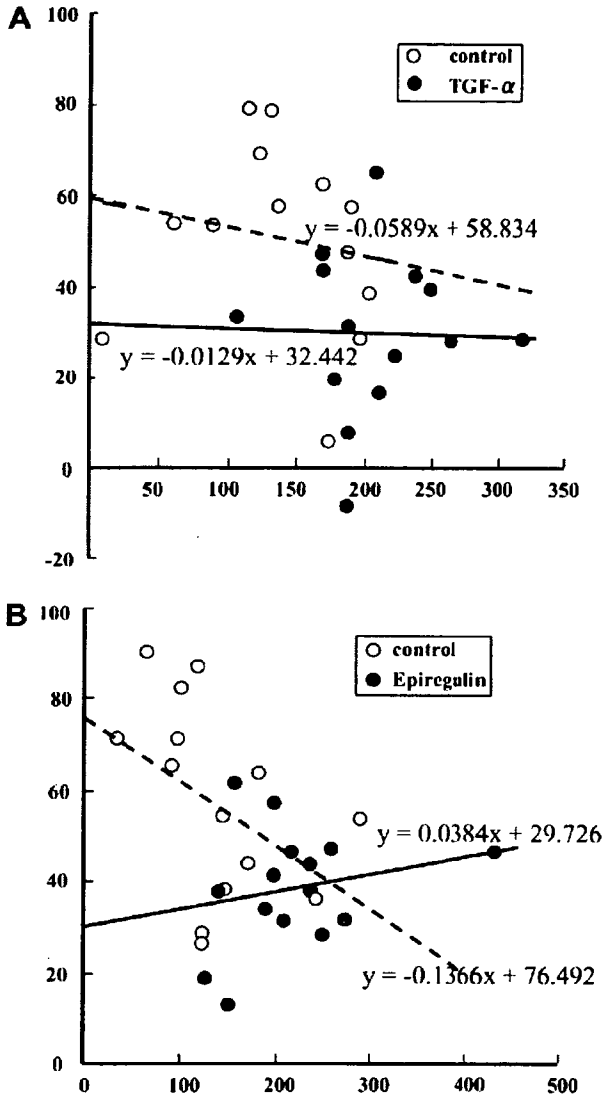

Fig. 5. Correlation analyses between %PPI and pulse-alone startle. (A) Percentage of PPI of the mice treated with vehicle (open circle, $n = 15$) and TGF-α (closed circle, $n = 15$) as neonates and plotted against pulse-alone startle. The data correspond to Fig. 4A. (B) Percentage of PPI of the mice treated with vehicle (open circle, $n = 15$) and epiregulin (closed circle, $n = 15$) was similarly plotted against the pulse-alone startle. The data correspond to Fig. 4B. A linear regression lines was estimated for each group and indicated with an equation. *Note:* There was no trend towards a decrease in the percentage of PPI versus absolute pulse-alone startle in either TGF-α-treated or epiregulin-treated animals.

gender ($F[1,23] = 1.630$, $p = 0.214$) without an interaction between treatment and gender ($F[1,23] = 1.167$, $p = 0.291$). Test duration had a significant overall effect ($F[1,23] = 268$, $P < 0.001$) without any factorial interactions. There was no significant effect of epiregulin on locomotor activity ($F[1,25] = 0.425$, $p = 0.521$) without an interaction between gender and treatment ($F[1,25] = 0.635$, $p = 0.433$). Thus, neonatal treatment with these ErbB1 ligands has no effect on exploratory locomotion at the adult stage.

3.5. Effects of ErbB1 ligands on contextual and auditory conditioning in mice

The effect of the ErbB1 ligands 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 (Fig. 7). There was no significant difference in acoustic startle sensitivity with less than 100-dB sound stimuli (see Fig. 3). Freezing levels were compared separately for each treatment as well

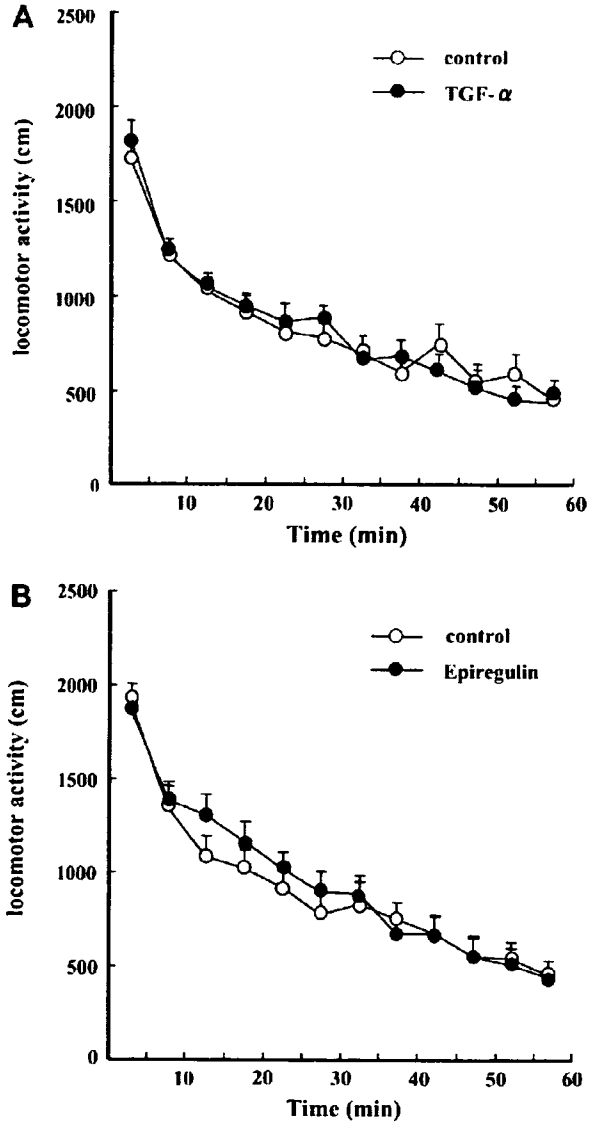

Fig. 6. Exploratory locomotor activity of mice treated with TGF-α and epiregulin. (A) We measured locomotor activity of mice (PND54-60) treated with TGF-α or saline ($n = 13$–17 each) as neonates in a novel environment. (B) Mouse littermates treated with epiregulin or saline ($n = 13$–17 each) were subjected to the same test at the adult stage. Horizontal activity in a novel open field was measured every 5 min and was plotted on the ordinate. Note: The changes in locomotor activity were indistinguishable between males and females (data not shown).

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as in each testing session with ANOVA [treatment × gender × test duration (repeated, 6 points over the initial 3 min)]. In the TGF-α experiment, there were no significant main effects of TGF-α treatment on freezing time coupled with the context cue ($F[1,23] = 0.000$, $p = 0.996$) and the tone cue ($F[1,23] = 0.018$, $p = 0.896$) (Fig. 7A). Neonatal treatment with epiregulin did not alter learning performance in either paradigm ($F[1,27] = 0.351$, $p = 0.559$ for context and $F[1,25] = 0.070$, $p = 0.793$ for tone) (Fig. 7B). In both experiments, there were no significant differences in freezing rate during conditioning between ErbB1 ligand-treated mice and saline-treated controls, suggesting no influence of neonatal TGF-α or epiregulin treatment on shock sensitivity ($F[1,23] = 1.166$, $p = 0.291$ for TGF-α and $F[1,25] = 3.118$, $p = 0.090$ for epiregulin). There was no gender effect or no gender × treatment interaction in the above learning performance, either (data not shown). Thus, neonatal exposure to neither TGF-α or epiregulin influences shock-triggered fear-learning ability.

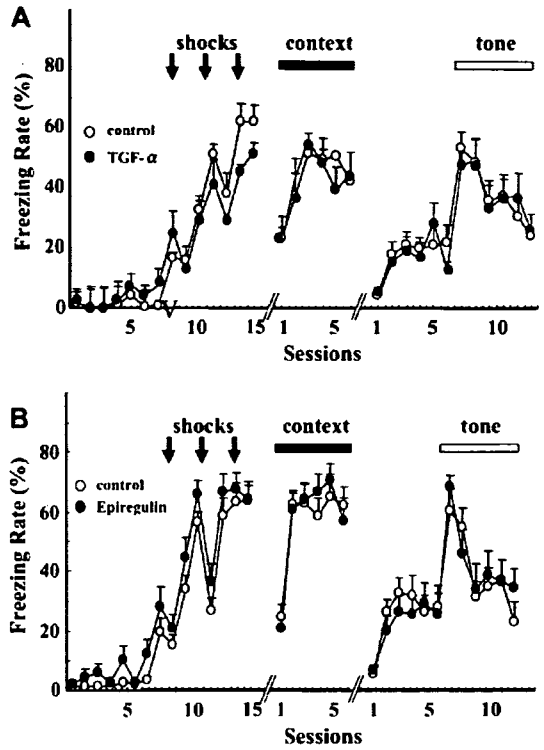

Fig. 7. Tone-dependent and context-dependent learning in TGF-α- and epiregulin-treated mice. (A) TGF-α- and saline-treated mice ($n = 12$–16 each) were subjected to shock-paired contextual conditioning with a tone cue at PND63-70 and, after 1 d, their learning performance was measured with the contextual cue and the tone cue. Mean percentage (±SEM) of freezing time of TGF-α-treated mice (closed circle) was scored every 30 s during conditioning as well as during testing periods (3 min) and compared with that of control littermates receiving saline as neonates (open circle). (B) Learning performance of epiregulin-treated mice was determined with the same protocol. Arrows mark the timing of electric shock given. Open and closed bars indicate the timing of the context cue given and that of the tone cue given during test sessions, respectively. *Note:* Freezing time was indistinguishable between males and females in all test sessions (data not shown).

4. Discussion

Our previous studies demonstrated that peripherally-administered inflammatory cytokines influence development of immature neurons in postnatal brain and later produce a variety of behavioral and cognitive abnormalities [13,24,25]. Adult endophenotypes of behavioral abnormalities vary depending upon cytokine species administered as well as on animal strains [17,26]. In the present study, however, we learned that the distinct cytokines, acting on the same ErbB1 receptor, can produce the same behavioral phenotypes. Neonatal administration of TGF-α and epiregulin both increased acoustic startle response and decreased PPI levels at the adult stage. These ErbB1 ligands both failed to affect exploratory movement and fear-learning ability, however. Thus, the observed behavioral impairments induced by these ErbB1 ligands considerably resembled those triggered by neonatal challenge of EGF [17], although there were differences in the magnitudes of brain ErbB1 phosphorylation as well as in those of acceleration of physical development among the ErbB1 ligands. As the transgenic mice carrying the EGF expression vector also a similar behavioral abnormality (HN unpublished data), it is unlikely that a minor compound(s) in the samples of TGF-α and epiregulin might produce the behavioral deficits.

Considering the fact that serum, milk and amniotic fluids contain effective concentrations of various ErbB1 ligands such as EGF, TGF-α, amphiregulin and betacellulin [6–9,27], the present study demonstrates that all ErbB1 ligands in the peripheral tissues or fluid of neonates may have common latent effects on brain development and synergistically influence adult behavioral traits, irrespective ErbB1 ligand spices. In the case of the rat model established with interleukin-1 injection as neonates, there is a clear time window for both cytokine penetration through the blood–brain barrier and later behavioral consequences [28]. In this context, we assume that the permeability of TGF-α and epiregulin to the blood–brain barrier of mouse neonates similarly has crucial impact on later behavioral traits.

Transgenic mice carrying TGF-α transgene and natural TGF-α hypomorphic mutant mice (*wa-1*) have been analyzed previously [29–32]. The male transgenic mice are reported to be aggressive in the resident-intruder test and depressive in the enforced swim test [30]. The behavioral deficits are ameliorated by administration of serotonin uptake inhibitors [29]. Similarly, male mice carrying *wa-1* allele exhibited impaired auditory and contextual fear learning with a decrease in hippocampal

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volume [31]. Again, there were significant gender differences in monoamine metabolism of *wa-1* mutants [32]. In contrast to these reports, our procedure of the neonatal TGF-α administration did not produce gender-specific alterations in behaviors, although there was a significant gender × treatment interaction in adult body weight. In addition, there was no significant effect of TGF-α and epiregulin on fear-learning performance in the present study. As we found the modest change of EGF-challenged rats in monoamine metabolism but not that in brain structures [13,18], the peripheral challenge of TGF-α and epiregulin in the neonatal period of time may also produce an influence on brain monoamine metabolism at the adult stage, leading to the behavioral changes.

Neonatal treatment with TGF-α and epiregulin both facilitated eyelid opening and tooth eruption, as that with EGF does [10]. There was difference in the magnitude of acceleration in physical development, however. Epiregulin exhibited a lower potency of accelerating tooth eruption than TGF-α at the same dose. In agreement with this finding, the receptor phosphorylation following epiregulin injection was also delayed and less pronounced, compared with animals receiving TGF-α injection. TGF-α triggered phosphorylation of ErbB1 in the brain peaked and disappeared within 240 min after injection while EGF administration to rat neonates results in sustained phosphorylation of ErbB1 [13]. In spite of the differences in receptor activation and physical development, the subchronic influences of TGF-α and epiregulin on AMPA receptor expression as well as on adult behaviors were almost comparable. The discrepancy in peripheral vs. central or acute vs. chronic effects of these ErbB1 ligands awaits future investigations.

Maternal infection during pregnancy, birth complications and maternal care of neonates are all suggested to influence later psychobehavioral traits or a risk for schizophrenia or other neurodevelopmental disorders [32–34]. ErbB1 ligands appear to be potential candidate molecules that mediate these pathological processes following infection, inflammation, and psychological stimuli. Endogenous expression of ErbB1 receptor ligands (EGF, TGFα, HB-EGF, and amphiregulin) is induced by neuronal activity, inflammation, and injury [35–38]. Rearing conditions also affect the production of TGFα in the neonatal brain [39]. As patients with schizophrenia exhibited abnormal expression of EGF in the brain, we previously focused on EGF among many ErbB1 ligands and characterized acute and intensively investigated chronic influences of EGF on behaviors. The present results, however, indicate that EGF is not the sole player leading to the behavioral deficits involving ErbB1 signaling, although there is no direct evidence that abnormal expression of any ErbB1 ligand in human subjects results in schizophrenia or other neurodevelopmental disorders.

These ErbB1 ligands exert common biological activities on various types of neurons. EGF, HB-EGF, and TGF-α have strong trophic activity on midbrain dopaminergic neurons [15,40–42]. These ErbB1 ligands also have a de-differentiation activity on GABAergic neurons [22] and an inhibitory action on growing excitatory synapses [43]. Our recent studies indicate that ErbB1 stimulation with EGF or TGF-α has an activity to down-regulate AMPA receptor expression [22,23]. The finding contrasts with the other report showing that the inhibition of neuregulin-1/ErbB4 signaling similarly down-regulates AMPA receptors [44]. Although it remains unknown which biological activity of ErbB1 signals play a main pathological role in the behavioral impairments, these activities appear to all match the schizophrenia hypotheses for the GABAergic, dopaminergic, and glutamatergic systems proposed previously. We hope that investigations on ErbB1-triggered behavioral impairments will help elucidating psychiatric pathology and etiology, leading to novel therapeutic inventions.

Acknowledgements

This work was supported by a grant-in-aid for Basic Scientific Research (B), CREST Grant from JST Corporation, a grant-in-aid from the Ministry of Welfare and Labor, Japan, Research Grant from Astrazeneka Pharmaceuticals Inc. and the Grant for the Promotion of Niigata University Research Projects. We thank Ms. Katsumoto for technical assistances.

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Behavioral/Systems/Cognitive

A Cyclooxygenase-2 Inhibitor Ameliorates Behavioral Impairments Induced by Striatal Administration of Epidermal Growth Factor

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Consistent with the hypothesis that neuroinflammatory processes contribute to the neuropathology of schizophrenia, the protein levels of epidermal growth factor (EGF) and its receptor ErbB1 are abnormal in patients with schizophrenia. To evaluate neuropathological significance of this abnormality, we established an animal model for behavioral deficits by administering EGF into the striatum and evaluated the effects of cyclooxygenase-2 (Cox-2) inhibitor celecoxib. Intracranial infusion of EGF into the striatum of adult male rats activated ErbB1 and induced neurobehavioral impairments observed in several schizophrenia models. Unilateral EGF infusion to the striatum lowered prepulse inhibition (PPI) in a dose-dependent manner and impaired latent learning of active shock avoidance without affecting basal learning ability. Bilateral EGF infusion similarly affected PPI. In contrast, EGF infusion to the nucleus accumbens did not induce a behavioral deficit. Intrastriatal EGF infusion also increased Cox-2 expression, elevated tyrosine hydroxylase activity, and upregulated the levels of dopamine and its metabolites. Subchronic administration of celecoxib (10 mg/kg, p.o.) ameliorated the abnormalities in PPI and latent learning as well as normalized dopamine metabolism. We conclude that this EGF-triggered neuroinflammatory process is mediated in part by Cox-2 activity and perturbs dopamine metabolism to generate neurobehavioral abnormalities.

Key words: inflammation; cyclooxygenase; EGF; schizophrenia; dopamine; prostaglandin

Introduction

In the CNS, epidermal growth factor (EGF) and structurally related EGF-like peptides (ErbB1 ligands) enhance survival and neurite outgrowth of midbrain dopaminergic neurons and are implicated in dopamine (DA)-related brain diseases such as Parkinson's disease and schizophrenia (Casper et al., 1994; Farkas and Krieglstein, 2002). EGF content is decreased and EGF receptor (ErbB1) levels are increased in the striatum of schizophrenia patients (Futamura et al., 2002). Genetic linkage studies may also support the contribution of EGF to schizophrenia etiology or pathology (Anttila et al., 2004; Hanninen et al., 2007). EGF and other EGF homologs were isolated as cell growth factors and implicated in the progression of cancer (Ackerman et al., 2004; Slice et al., 2005; Liao et al., 2006). Binding of EGF to ErbB1 enhances the expression of inducible prostaglandin synthetase [cyclooxygenase 2 (Cox-2)] and triggers a variety of inflammatory processes (Slice et al., 2005; Liao et al., 2006). Therefore, EGF and other homologs are implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis. Subsequently produced prostaglandins bind G-protein-coupled receptors and stimulate production of EGF or other ErbB1 ligands by ectodomain shedding (Pai et al., 2002; Han et al., 2006). This feedforward loop between ErbB1 ligands and Cox-2 expression promotes cancer cell proliferation and inflammatory progression (Huh et al. 2003). In contrast, the central actions of EGF or other ErbB1 ligands on the neuroinflammatory processes are poorly understood.

Neuroinflammation is implicated in etiology or neuropathology of not only neurodegenerative diseases but also a number of psychiatric disorders (Das and Khan, 1998; Heleniak and O'Desky, 1999). Patients with schizophrenia often exhibit impaired autoimmune responses and abnormal levels of cytokines (Licinio et al., 1993; Lin et al., 1998; Toyooka et al., 2003). Interestingly, there is also a reverse correlation between neuroinflammation and psychiatric symptoms. Various psychiatric symptoms develop during or after cytokine therapy for cancer, viral infection, and anemia (Denicoff et al., 1987; McDonald et al., 1987). Thus, cytokine-mediated inflammatory reactions may in certain circumstances evoke psychiatric symptoms. Based on this hypothesis, preclinical trials of nonsteroidal anti-inflammatory drugs on patients with schizophrenia are underway (Muller et al., 2002, 2004; Riedel et al., 2005). Tetracycline, an antibiotic that possesses anti-inflammatory activity, improves the positive and negative syndrome scale (PANSS) of schizophrenia patients (Miyaoka et al., 2007). Thus, anti-inflammatory medication aug-

Received Jan. 18, 2007; revised July 9, 2007; accepted July 31, 2007.

This work was supported by a grant-in-aid from the Health and Labor Sciences Research Grants, a grant-in-aid for Basic Scientific Research B, Core Research for Evolutional Science and Technology from the Japan Science and Technology Corporation, and a grant for Promotion of Niigata University Research Projects. We thank Dr. K. Kobayashi for biochemical advice.

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DOI:10.1523/JNEUROSCI.2368-07.2007

 0270-6474/07/2710116-12$15.00/0

ments therapies for neuropsychiatric conditions, although the pharmacological mechanisms underlying their antipsychotic actions are not fully characterized.

To study a potential neuroinflammatory role of the striatal EGF signal in a schizophrenia model, we examined the neurobehavioral consequences of striatal EGF administration as well as its effects on Cox-2 expression in rats. Given the biological activity of EGF on dopaminergic neurons, we focused on the neurochemical markers and animal behaviors related to dopaminergic dysfunctions and/or schizophrenia, prepulse inhibition (PPI) of startle, and latent inhibition (Braff et al., 2001; Geyer et al., 2001; Jeanblanc et al., 2003; Peterschmitt et al., 2005). We also evaluated the effects of acute and subchronic inhibition of Cox-2 in conjunction with dopamine metabolism and behavioral performance.

Materials and Methods

Subjects. Male Sprague Dawley rats (7–8 weeks old, 300–380 g body weight, 190 rats in total) were purchased from SLC (Shizuoka, Japan) and maintained under a 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) with access to food and water *ad libitum*. Before testing, rats were habituated to experimental rooms and experimenters with daily handling for at least 1 week. Surgical operation and behavioral tests were performed during the day cycle. Recombinant human EGF (Higeta Syoyu, Chiba, Japan) was dissolved in saline and intracranially administered from an osmotic minipump (see below). All of the animal experiments described here were performed in accordance with the Animal Use and Care Committee guidelines of Niigata University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Surgical procedure and subchronic EGF administration. Rats (8–9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Dainippon Pharmaceutical, Suita, Osaka, Japan). After confirming the deep anesthesia, each rat was mounted on a stereotaxic apparatus (Narishige, Tokyo, Japan) with the upper incisor bar set 3.0 mm below the interaural line. The skull was exposed and a hole drilled for unilateral placement of intracerebral cannula guides into either the striatum or nucleus accumbens. A cannula (30 gauge; Terumo, Tokyo, Japan) was implanted in the striatum (0.5 mm posterior and 3.0 mm right lateral measured from the bregma, 5.5 mm below the skull) or in the nucleus accumbens (1.6 mm anterior and 2.0 mm right lateral measured from the bregma, 7.0 mm below the skull), glued to the skull, and connected to an Alzet osmotic minipump (model 2002, a 2 week type; Alza, Palo Alto, CA) by medical-grade vinyl tubing. For bilateral EGF infusion, cannulas were implanted in both hemispheres of the striatum (0.5 mm posterior and ±3.0 mm lateral measured from the bregma, 5.5 mm below the skull) and connected to two minipumps. A minipump was filled with human recombinant EGF (0.005, 0.05, or 0.15 mg/ml; 0.2 ml total) or 0.9% saline (vehicle) and implanted subcutaneously in the nape of the neck. Either saline or EGF was administered continuously at a rate of 0.5 μl/h from the minipump. Unless the dose of EGF is otherwise specified, each minipump contained 30 μg of protein and EGF was infused at a rate of 75 ng/h. The scalp incision was closed with surgical staples and treated with a topical antiseptic, Cefmetazon (50 mg/d; Sankyo Pharmaceuticals, Tokyo, Japan). The cannula position and EGF content in a pump were confirmed after completion of behavioral tests.

Schedule of behavioral testing, drug treatment, and dissection. Rats were subjected to behavioral tests after a recovery period at least 7 d in length after minipump implantation but before the minipump was depleted of EGF (14 d after surgery). The saline- or EGF-infused rats were additionally treated with the Cox-2 inhibitor celecoxib. Celecoxib (10 mg/kg; Pfizer, New York, NY) was dissolved in saline and administered once a day orally with the aid of oral zonde for rats (Natusme Seisakusho, Tokyo, Japan). The celecoxib treatment was performed daily 3–4 d after pump implantation to days 11–12 after surgery. Behavioral tests followed this treatment regimen. To minimize the acute influences of celecoxib, examinations were performed at least 20 h after treatment unless it is otherwise specified.

To avoid interactions between independent behavioral tests, rats were subjected to one of the scheduled tests only once. The sole exception to this testing strategy was that rats that had been subjected to PPI testing were also tested for context learning. After behavioral evaluation, most of rats were killed, and tissue was harvested for neurochemical analysis or histochemistry (see below). Tissue samples for basal monoamine turnover were harvested from rats examined for effects of EGF doses on PPI. The effects of celecoxib on monoamine turnover were examined with tissue from animals tested for latent learning. The rats receiving EGF in the nucleus accumbens were fixed for histochemistry after PPI testing. To minimize the influences of the behavioral tests on the levels of monoamines, their extraction was performed at least 6 h after the end of the test. All other tissue samples were prepared from the rats that did not receive behavioral testing. Animals that exhibited any symptoms of infection around the cannula or the incision site were removed from study. In total, eight experimental groups representing 180 rats were analyzed in the present study.

Histochemistry. Rats receiving EGF infusion were transcardially perfused with 4% paraformaldehyde in a 0.1 M PBS, pH 7.4. Coronal sections (15 μm thick) were cut from frozen brains and immunostained with antibodies directed against human EGF (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or Cox-2 (1:500; Cayman Chemical Ann Arbor, MI). The immunoreactivity was visualized with biotinylated anti-rabbit Ig antibody coupled with the ABC method (Vector Laboratories, Burlingame, CA).

Immunoblot analysis. Rat receiving EGF or saline were killed by carbon dioxide exposure, and the brains were dissected out. Samples of striatum were homogenized by ultrasonication in 2× sample loading buffer (100 mM Tris-HCl buffer, pH 6.8, 4% SDS, 100 mM dithiothreitol, 20% glycerol, and 0.0001% bromophenol blue). Protein extracts (5 or 50 μg per lane) were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes by electrophoresis. Membranes were probed with antibodies directed against ErbB1 (1:1000; Santa Cruz Biotechnology). Alternatively, immunoblots were probed with anti-phosphorylated ErbB1 (1:1000), anti-ErbB2 (1:2000), anti-D_2 receptor (1:1000), anti-dopamine transporter (DAT) (1:1000), anti-Cox-2 (1:1000; all from Santa Cruz Biotechnology), anti-D_1 receptor (1:1000; Sigma, St. Louis, MO), anti-synaptophysin (1:1000; NeoMarkers, Fremont, CA), or anti-tyrosine hydroxylase (1:1000; Chemicon, Temecula, CA) antibodies. The immunoreactivity on the membrane was detected by chemiluminescence (ECL kit; GE Healthcare, Little Chalfont, UK).

The activity of tyrosine hydroxylase. Striatal tissue was homogenized in lysis buffer [0.32 M sucrose, 20 mM Tris-HCl, pH 7.3, 1 mM dithiothreitol, and protease inhibitor cocktail (Complete Mini; Roche Diagnostic, Mannheim, Germany)]. Protein homogenates (20 μl/tube) were incubated with 180 μl of reaction buffer [0.2 mM L-tyrosine, 0.2 M sodium acetate, 0.1 M 2-mercaptoethanol, 0.5 mM ferrous sulfate heptahydrate, 1 mM 6-methyl-5,6,7,8-tetrahydropterin (Sigma), 0.2 mg/ml catalase (Roche Diagnostic), and 50 μM *s*(−)-carbidopa (Wako Chemical, Tokyo, Japan)] at 37°C for 10 min. The enzyme reaction was terminated on ice with a stop solution (250 μl) [1 M perchloric acid, 0.2 M EDTA, and 1 mM α-methyldopa (an internal standard)]. After 15 min incubation on ice, 150 μl of 1 M K_2CO_3 and 1 ml of 0.1 M Tris-HCl, pH 8.5, were added, and the supernatant was collected. The product of 3,4-dihydroxy-L-phenylalanine (L-DOPA) was absorbed with alumina (aluminumoxide 90 active acidic; Merck, Darmstadt, Germany) and then eluted with 0.5 M HCl (0.8 ml/tube). Concentrations of L-DOPA were determined by HPLC with electrochemical detection ECD (see below) (mobile phase: 50 mM trisodium citrate, 25 mM NaH_2PO_4, 100 μM EDTA, and 1% methanol, pH 2.8).

Measurement of acoustic startle and prepulse inhibition. Acoustic startle and PPI responses were measured in a startle chamber (SR-Lab Systems; San Diego Instruments, San Diego, CA) adapted for rats (Braff and Geyer, 1990; Swerdlow and Geyer 1998; Swerdlow et al., 2001). The chosen paradigm was adapted and modified from Braff and Geyer (1990) and used to assess startle amplitude, habituation, and PPI response with acoustic stimuli of 120 dB, a single prepulse interval (100 ms), and three

Table 1. Effects of striatal EGF infusion on monoamine contents and turnover

	Saline (0 μg)	3 μg of EGF	10 μg of EGF	30 μg of EGF
5-HIAA	326 ± 69	428 ± 105	480 ± 62	498 ± 103
5-HT	17,200 ± 4200	25,800 ± 7610	24,600 ± 2250	20,200 ± 3320
DA	109,000 ± 2690	109,000 ± 37,900	157,000 ± 12,300	184,000 ± 5780*
DOPAC	1220 ± 213	1230 ± 373	1850 ± 152	2020 ± 152*
HVA	2400 ± 580	3440 ± 1140	5580 ± 423**	5030 ± 502**
EP	47 ± 5	43 ± 4	47 ± 3	47 ± 3
NE	404 ± 62	590 ± 159	394 ± 54	490 ± 35

Different doses of EGF were unilaterally administered to rat striatum from a minipump for 10 d, and monoamines were extracted from the ipsilateral hemisphere of the striatum. The levels of DA, DOPAC, HVA, serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in the striatum were determined by HPLC-ECD as described previously (Futamura et al., 2003). The total amounts of EGF in a minipump were given in this table. Data represent means ± SEM (picomoles per gram of wet tissue, $n = 5$–6 animals each). *$p < 0.05$, **$p < 0.01$ by Fisher's LSD. EP, Epinephrine, NE, norepinephrine.

different prepulse intensities [5, 10, and 15 dB above background noise (white noise, 70 dB)]. Each rat was placed in the startle chamber and initially acclimatized for 5 min with background noise alone. The rat was then subjected to 50 startle trials, each trial consisting of one of five conditions: (1) a 40 ms, 120 dB noise burst presented alone; (2–4) a 40 ms, 120 dB noise burst after prepulses by 100 ms (20 ms noise burst) that were 5, 10, or 15 dB above background noise (i.e., 75, 80, or 85 dB prepulse, respectively); or (5) no stimulus (background noise alone), which was used to measure baseline movement in the chamber. In PPI tests, these five trial types (1–5) were each repeated eight times in a pseudorandom order to give 40 trials. Each trial type was presented once within a block of five trials. At the beginning and end of the PPI test, five consecutive trials of condition 1 were presented to assess habituation during sessions. The intertrial interval was 15 s. Analysis of PPI was based on the mean of the eight trials for each trial type. The percentage PPI of a startle response was calculated as follows:

$$\mathrm{PPI} = 100 - \frac{(\text{prepulse and pulse stimulus trials} - \text{no stimulus trials}) \times 100}{\text{pulse-alone trials} - \text{no stimulus trials}}.$$

Active-avoidance learning and latent inhibition. Rats were given 60 trials of two-way active-avoidance conditioning (10 trials per block) (Salmi et al., 1994; Futamura et al., 2003). Active-avoidance testing was conducted in an automated shuttle box (Muromachi-kiki, Tokyo, Japan) subdivided into two virtual compartments with independently electrified stainless-steel bars as floors. One trial consisted of a buzzer tone [conditioning stimulus (CS)] and an electric shock [unconditioning stimulus (US)]. The CS was an 80 dB tone for 10 s. The US was a 2 s positive half-wave constant current of 0.5 mA intensity. When the CS was on, the animals had to cross to the other side of the shuttle box apparatus (avoidance response) to turn the CS off and to avoid the US. The US was initiated if the animal failed to make an escape response. The intertrial schedule had a variable interval (10–90 s). Animals in non-preexposed (NPE) group were directly subjected to the above conditioning. One day before the conditioning, rats in the preexposed (PE) group were placed in the automated shuttle box and exposed to the buzzer tones (CS) with the above protocol but without receiving electric stimuli (US). Preexposed to CS were followed by the final treatment with celecoxib at an interval of 1 h.

Context fear learning. The test paradigm of contextual conditioning was based on a work by Matus-Amat et al. (2004). Rats were transported to the laboratory at least 30 min before fear conditioning. Rats were placed in a shock chamber with a stainless-steel grid floor (21.5 cm width × 20.5 cm depth × 30 cm height box; Ohara Medical Industry, Tokyo, Japan) for 2 min to monitor baseline movement/freezing and were then exposed to 0.8 mA electric shocks (2 s duration, twice at an interval of 1 min). One day after conditioning, rats were returned to the same chamber. The time spent freezing was recorded by a video camera and averaged every 30 s with the aid of an imaging software (Ohara Medical Industry). The final treatment with celecoxib was done 1 d before conditioning.

Quantification of dopamine and its metabolites. The levels of DA, L-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin, 5-hydroxyindoleacetic acid, epinephrine, and norepinephrine were determined by HPLC-ECD as described previously (Futamura et al., 2003). Frontal cortex and striatum were dissected out from rats, weighed, and then immediately homogenized by ultrasonication in 0.5 ml of 0.1 M perchrolic acid containing 0.1 mM EDTA and 250 nM isoproterenol as an internal standard. The homogenate was placed on ice for 30 min and then centrifuged at 10,000 × *g* for 10 min. The HPLC-ECD system consisted of a pump (model LC-10ADVP; Shimadzu, Kyoto, Japan), an automatic sample injector (model SIL-10ADVP; Shimadzu), a C18 column (model CA-5ODS, 4.6 × 150 mm; Eicom, Kyoto, Japan), and an electrochemical detector with a glassy carbon-working electrode (model ECD-300; Eicom). The mobile phase consisted of 50 mM trisodium citrate, 25 mM NaH_2PO_4, 0.03 mM EDTA, 10 mM diethylamine, 3 mM octanesulfonic acid sodium salt, 6% methanol, and 1% dimethylacetamide, pH 3.2. The current produced was monitored using an EPC-300 (Eicom).

Statistical analysis. Results were expressed as means ± SEM. Statistical differences were determined by ANOVA. When univariate data were obtained only from two groups, a two-tailed *t* test was used for comparison. Behavioral scores were initially analyzed using multiple ANOVA with EGF administration (two levels or four doses), celecoxib treatment (two levels), and/or preexposure to CS (two levels) as between-subject factors and prepulse magnitude (three levels) or block (six) as a within-subject factor. Interaction of a within-subject factor with between-subject factors was estimated by analysis of covariance and multivariate analysis of variance with Pillai compensation. Because the initial analyses yielded significant factorial interaction, the data were separated to avoid the interaction for the final analyses. Subsequently, a Fisher's least significant difference (LSD) test was applied to absolute behavioral values as a *post hoc* test of multiple comparisons. A p value <0.05 was regarded as statistically significant. Statistical analysis was performed using Statview software (SAS Institute, Cary, NC). n values in parentheses represent the number of animals used.

Results

Effects of intrastriatal EGF administration on monoamine metabolism and sensorimotor gating

Peptides in the EGF family exert neurotrophic effects on dopaminergic neurons both *in vitro* and *in vivo* (Casper et al., 1994; Farkas et al., 2002; Futamura et al., 2003; Iwakura et al., 2005) and increase the activity of tyrosine hydroxylase (Halegoua and Patrick, 1980; Anastasiadis et al., 1997). To investigate the central actions of EGF, we unilaterally administered various concentrations of EGF to the striatum of rats with an osmotic minipump and then determined the levels of monoamines and their metabolites in brain tissue. Subchronic EGF infusion to the striatum increased the levels of dopamine ($F_{(3,17)} = 3.48$; $p = 0.039$) and its metabolites DOPAC and HVA ($F_{(3,17)} = 4.03$, $p = 0.025$ for DOPAC and $F_{(3,17)} = 5.27$, $p = 0.009$ for HVA) in the striatum in a dose-dependent manner (Table 1). *Post hoc* comparisons revealed that only the highest dose of EGF (30 μg/pump, 1.8 μg/d) significantly increased the levels of dopamine, DOPAC, and HVA. In contrast to the effects on the striatum, EGF did not alter

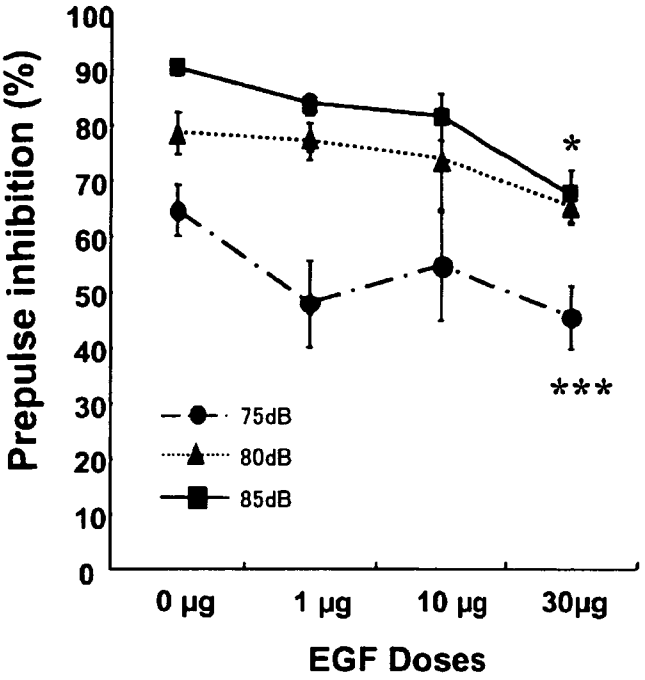

Figure 1. EGF dose dependency of PPI deficits. Different doses of human EGF (0, 1, 10, and 30 μg/pump, equivalent to 0, 0.06, 0.6, and 1.8 μg/d) were administered to the striatum of male adult rats from an osmotic minipump for 10 d. PPI with 75, 80, and 85 dB prepulse stimuli was measured and compared between doses. Values indicate means ± SEM (n = 7–10 each). $^*p < 0.05$, $^{***}p < 0.001$ compared with vehicle-infused controls by Fisher's LSD.

the concentrations of dopamine and its metabolites in frontal cortex (data not shown).

To evaluate the neurobehavioral consequences of intrastriatal EGF infusion, we also monitored the dose-dependent effects of EGF on PPI (Fig. 1). A two-way repeated ANOVA for PPI revealed significant main effects of EGF dose ($F_{(3,30)} = 5.58$; $p = 0.0036$) and prepulse amplitude ($F_{(2,60)} = 58.8$; $p < 0.0001$) that were independent. *Post hoc* analysis indicated that the maximum dose of EGF (30 μg/pump) significantly reduced PPI with 75 and 85 dB prepulse tones. Therefore, EGF administered to the striatum elevated dopamine turnover and reduced PPI in a dose-dependent manner. In subsequent experiments, rats received the highest dose of EGF (30 μg/pump, 1.8 μg/d).

Intrastriatal administration of EGF induces Cox-2 immunoreactivity

After subchronic EGF infusion, distributions of EGF in the brain were examined by immunohistochemistry with an anti-EGF antibody. EGF appeared to diffuse through the striatal region efficiently. There was marked EGF immunoreactivity in the ipsilateral striatum as well as in the somatosensory neocortex along the route of the cannula (Fig. 2*A*). No immunoreactivity for EGF was detected in animals receiving an infusion of saline (data not shown). Because EGF is a potent Cox-2 inducer (Slice et al., 2005; Liao et al., 2006), we also examined Cox-2 immunoreactivity in EGF-infused rats. EGF increased the immunoreactivity of Cox-2 in the striatum and around the ventricular wall, relative to vehicle-infused animals (Fig. 2*B–E*).

Effects of EGF depletion and bilateral EGF administration on PPI

To examine the reversibility of the effect of EGF on PPI, first we confirmed its effects by administering rats EGF for 7 d and then

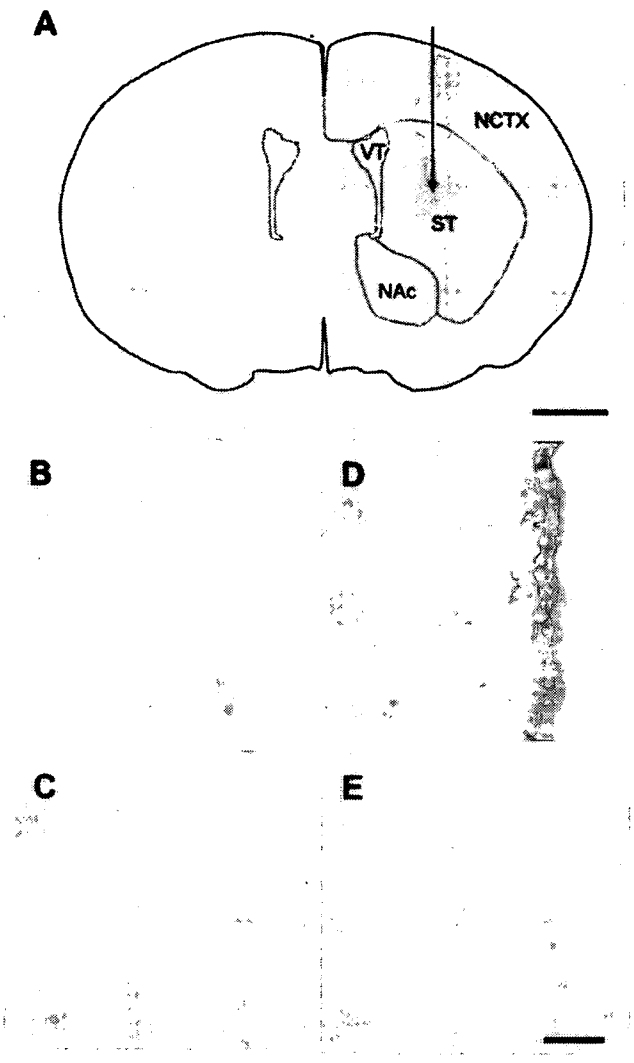

Figure 2. Distributions of EGF and Cox-2 immunoreactivity after subchronic striatal administration of EGF. ***A***, The efficacy of EGF infusion was examined by immunohistochemistry. EGF was administered to the striatum of adult male rats from an osmotic minipump (30 μg/pump) for 8 d. Coronal sections along the cannula route were immunostained with an anti-human EGF antibody. ***B–E***, The distribution of Cox-2 immunoreactivity was examined in vehicle-infused (***B***, ***C***) and EGF-infused (***D***, ***E***) animals. The most marked increase in Cox-2 immunoreactivity was observed around the lateral ventricle (***D***) and in the striatum (***E***) of the ipsilateral hemisphere. NCTX, Neocortex; ST, striatum; VT, lateral ventricle; NAc, nucleus accumbens. Scale bars: ***A***, 250 μm; ***B–E***, 30 μm. An arrow indicates the cannula position.

monitoring acoustic startle responses to 120 dB noise and PPI (Fig. 3). EGF infusion did not alter the startle response to 120 dB noise when compared with vehicle-infused animals (two tailed t test, $p = 0.072$) (Fig. 3*A*). We confirmed the significant main effects of EGF ($F_{(1,28)} = 6.59$; $p = 0.016$) on PPI (Fig. 3*C*). *Post hoc* analysis detected a significant decrease in PPI levels with the 85 dB prepulse tone. Ten days after cessation of EGF administration, there was no significant difference in startle amplitudes and PPI levels between EGF-treated and vehicle-treated animals, demonstrating that the effects of EGF are reversible (Fig. 3*B*,*D*).

To examine whether the effect on PPI were a consequence of unilateral infusion of EGF, we assessed bilateral effects of EGF infusion on PPI. We subchronically administered EGF to the striatum in both hemispheres from two osmotic minipumps and measured acoustic startle responses to 120 dB noise (Fig. 4*A*,*B*).

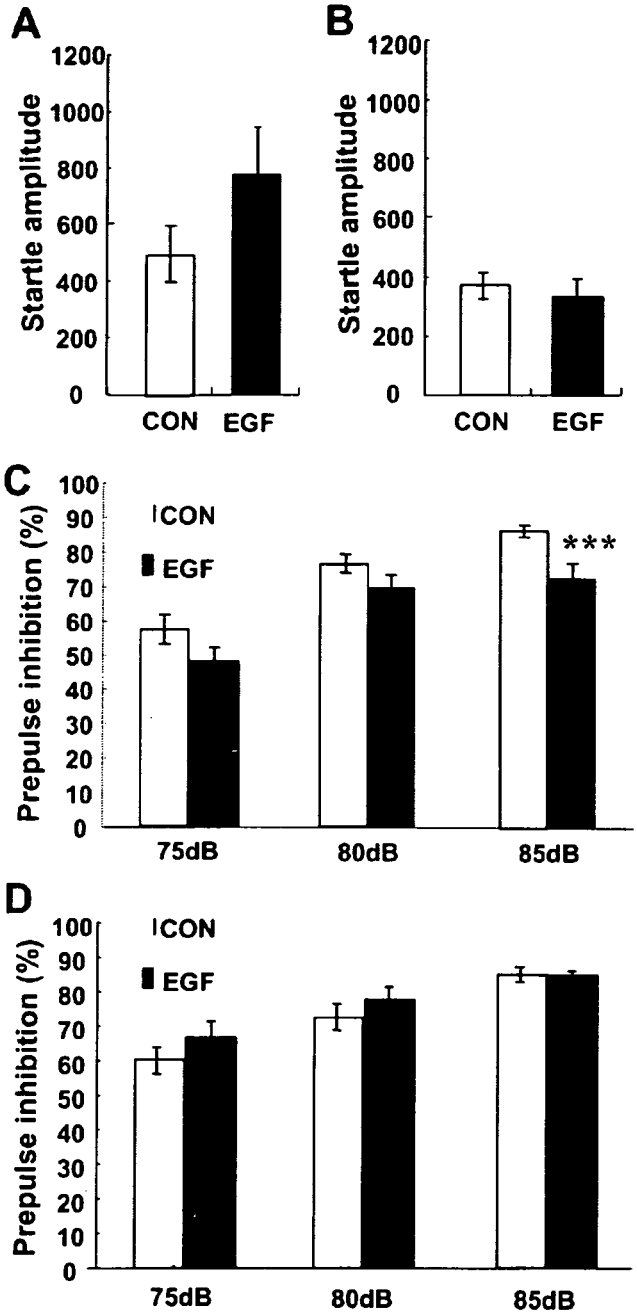

Figure 3. Effects of EGF depletion on acoustic startle response and prepulse inhibition. ***A***, ***B***, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured on day 8 of EGF administration (30 μg/pump) (***A***) and 10 d after completion of EGF administration (***B***). ***C***, ***D***, Simultaneously, PPI of vehicle-infused control (open box) and EGF-infused (filled box) animals with 75, 80, and 85 dB prepulse stimuli was measured during EGF administration (***C***) and after completion of EGF administration (***D***). Error bars indicate means ± SEM (n = 15 each). ***$p < 0.001$ by Fisher's LSD.

EGF infusion to the striatum in both hemispheres did not significantly alter the startle response to 120 dB noise when compared with vehicle-infused animals. A two-way repeated ANOVA revealed significant main effects of bilateral EGF infusion ($F_{(1,10)}$ = 7.58; p = 0.020) and prepulse amplitude ($F_{(2,20)}$ = 19.5; $p <$ 0.001) on PPI without interaction. *Post hoc* analysis identified significant decreases in PPI with the 75 and 80 dB prepulse tones.

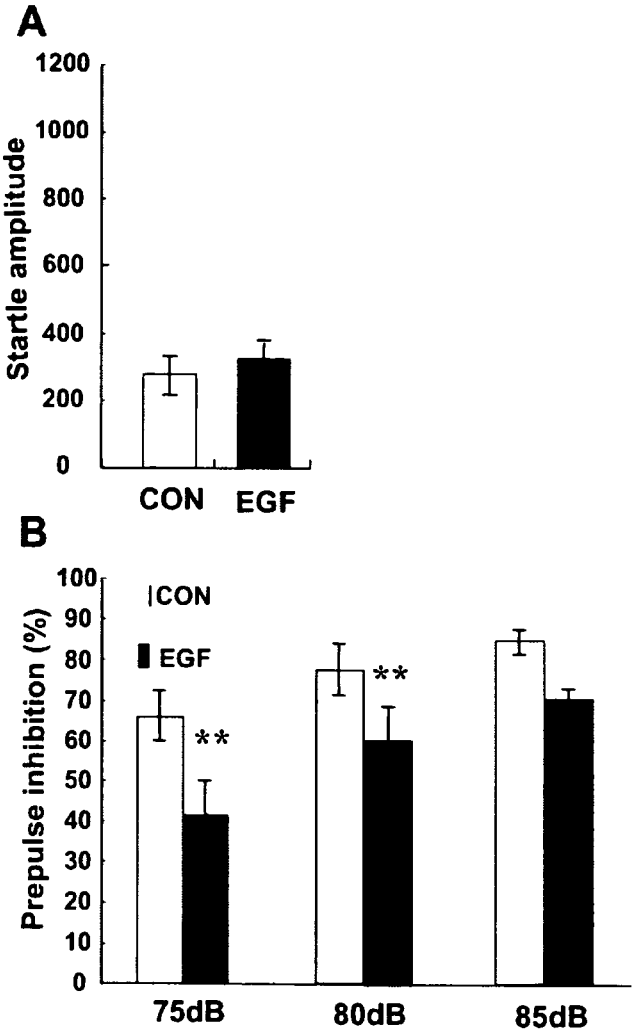

Figure 4. Effects of bilateral EGF infusion on acoustic startle response and PPI. EGF was administered to both hemispheres of the striatum of adult rats from two osmotic minipumps (30 μg/pump × 2). ***A***, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured 8 d after bilateral EGF administration was initiated. ***B***, Simultaneously, PPI of vehicle-infused control (open box) and bilaterally EGF-infused (filled box) animals with 75, 80, and 85 dB prepulse stimuli was measured. Error bars indicate means ± SEM (n = 6 each). **$p < 0.01$ by Fisher's LSD.

Effects of EGF administration to the nucleus accumbens on prepulse inhibition

We also monitored the subchronic effects of EGF administration to the nucleus accumbens, a locus most implicated in PPI regulation (Swerdlow et al., 1990, 2001; Swerdlow and Geyer, 1998). Immunohistochemistry revealed that EGF immunoreactivity was predominantly localized around the nucleus accumbens as well as along the cannula route (Fig. 5*A*). There was no significant difference in acoustic startle responses between saline- and EGF-infused rats (Fig. 5*B*). A two-way repeated ANOVA of PPI scores revealed no main effect of EGF treatment but a significant effect of prepulse amplitude ($F_{(2,28)}$ = 47.0; $p < 0.0001$) without their interaction (Fig. 5*C*). Thus, the administration of EGF to the nucleus accumbens failed to influence PPI levels.

Effects of intrastriatal EGF infusion on neurochemical markers

To assess the neurochemical consequences of striatal EGF infusion, we examined molecular markers for EGF signaling as well as

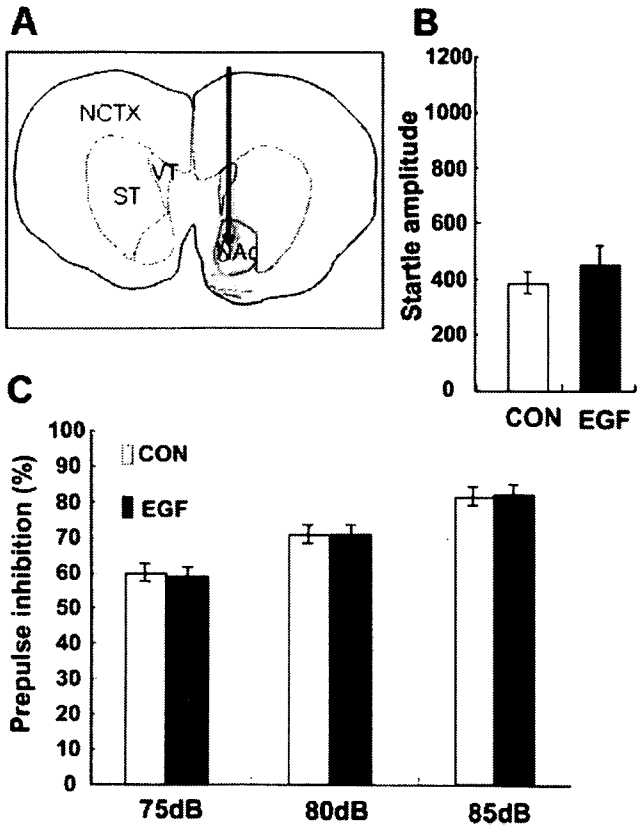

Figure 5. Effects of EGF infusion into the nucleus accumbens on PPI. EGF was administered to the nucleus accumbens of adult male rats from an osmotic minipump (30 μg/pump) for 8 d. *A*, The efficacy of EGF infusion to the nucleus accumbens was examined by immunohistochemistry. Coronal sections along the cannula route were immunostained with an anti-human EGF antibody. *B*, Acoustic startle response of vehicle-infused control (CON; open box) and EGF-infused (filled box) rats was measured before fixation for the above immunohistochemistry. PPI of vehicle-infused control and EGF-infused animals was determined with 75, 80, and 85 dB prepulse stimuli (*C*). Error bars indicate means ± SEM (n = 11 each). NCTX, Neocortex; ST, striatum; VT, lateral ventricle; NAc, nucleus accumbens. An arrow indicates the cannula position.

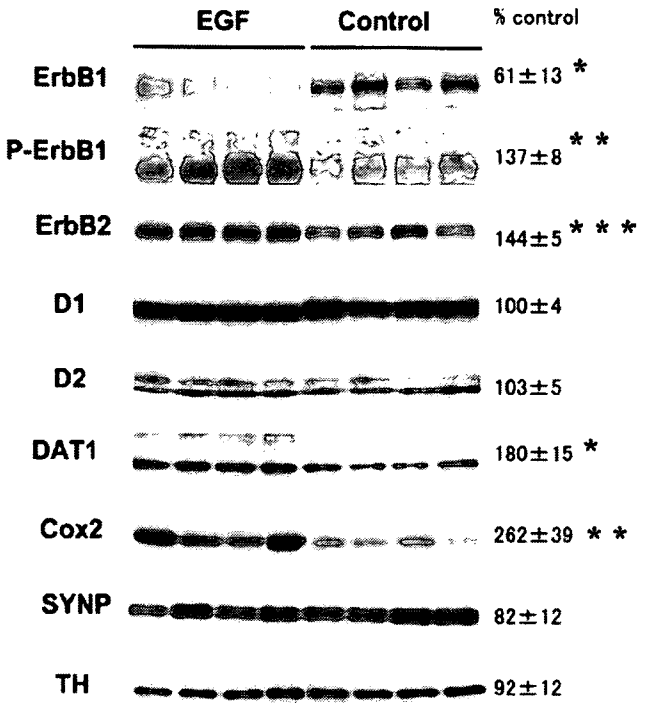

Figure 6. ErbB1 phosphorylation and neurochemical markers induced by EGF administration. EGF (30 μg/pump) or saline was administered into the striatum of adult rats (n = 4 animals each). On day 12 of the EGF infusion, protein extract was prepared from the ipsilateral striatum and subjected to immunoblotting for antibodies directed against ErbB1, phosphorylated ErbB1, ErbB2, DAT, D_1 receptor, D_2 receptor, synaptophysin (SYNP), tyrosine hydroxylase (TH), and Cox-2. Immunoreactivity was measured by densitometric analysis, and its ratio to that in vehicle-infused control rats (mean ± SEM) is presented. *$p < 0.05$, **$p < 0.01$, ***$p <$ 0.001 by two-tailed t test (n = 4 animals each).

those for dopamine signaling in the striatum (Fig. 6). EGF administration significantly elevated phosphorylation levels of ErbB1 (to 137%; two-tailed t test, $p < 0.001$) and conversely downregulated total protein levels of ErbB1 (to 61%; $p = 0.039$). As indicated in the above immunohistochemistry, EGF administration upregulated total protein levels of Cox-2 (to 262%; $p =$ 0.006). In parallel, DAT levels were increased (179%; $p = 0.041$), although there were no significant changes in the expression of dopamine receptors, the synaptic marker synaptophysin, or tyrosine hydroxylase (Fig. 6).

A Cox-2 inhibitor ameliorates the EGF-induced deficit of prepulse inhibition

To evaluate the contribution of Cox-2 induction to the PPI deficit, rats were simultaneously given a Cox-2 inhibitor, celecoxib (10 mg/kg, p.o.). Prepulse inhibition of vehicle-infused and EGF-infused animals was examined 1 h and 7 d after celecoxib or saline treatment was initiated (Fig. 7). In the acute paradigm of celecoxib administration, the effect of the Cox-2 inhibitor on PPI was analyzed by three-way ANOVA using between-subject factors of EGF [in its absence or presence (+/−)] and celecoxib (+/−) and a within-subject factor of prepulse intensity (Fig. 7*A*). Celecoxib failed to exhibit a main effect on PPI levels, although main effects of EGF ($F_{(1,26)} = 18.9$; $p < 0.001$) and prepulse intensity ($F_{(2,52)} =$ 63.7; $p < 0.001$) were significant. There were no factorial interactions. Similarly, the subchronic effects of celecoxib administration were evaluated by three-way ANOVA. Although there were significant main effects of EGF ($F_{(1,17)} = 8.59$; $p = 0.009$) and celecoxib ($F_{(1,17)} = 9.39$; $p = 0.007$), ANOVA detected a significant interaction between EGF and celecoxib ($F_{(1,17)} = 11.2$; $p =$ 0.004), suggesting that the therapeutic effects of celecoxib significantly differ between EGF-infused and vehicle-infused animals. Accordingly, the effects of celecoxib were separately evaluated in either the vehicle-infused or EGF-infused group. ANOVA revealed no significant main effect of celecoxib in the vehicle-infused group (Fig. 7*B*). In contrast, celecoxib administration to EGF-infused animals improved PPI scores significantly ($F_{(1,13)} =$ 2.61; $p < 0.001$) (Fig. 7*C*).

Effects of celecoxib treatment on fear learning and its latent inhibition of EGF-infused rats

We also evaluated the effects of striatal EGF infusion and celecoxib on fear learning and latent inhibition. EGF- and vehicle-infused rats were subjected to an active-avoidance test with a two-way shuttle chamber in the presence or absence of preexposure to a buzzer tone (CS) (Salmi et al., 1994). Initial four-way ANOVA using between-subject factors of EGF infusion (+/−), celecoxib administration (+/−), and preexposure to CS (+/−) and a within subject factor of test block (six) revealed significant interactions between EGF and preexposure ($F_{(1,72)} = 7.65$; $p =$ 0.007) and between pre-exposure and block ($F_{(5,360)} = 5.27$; $p <$

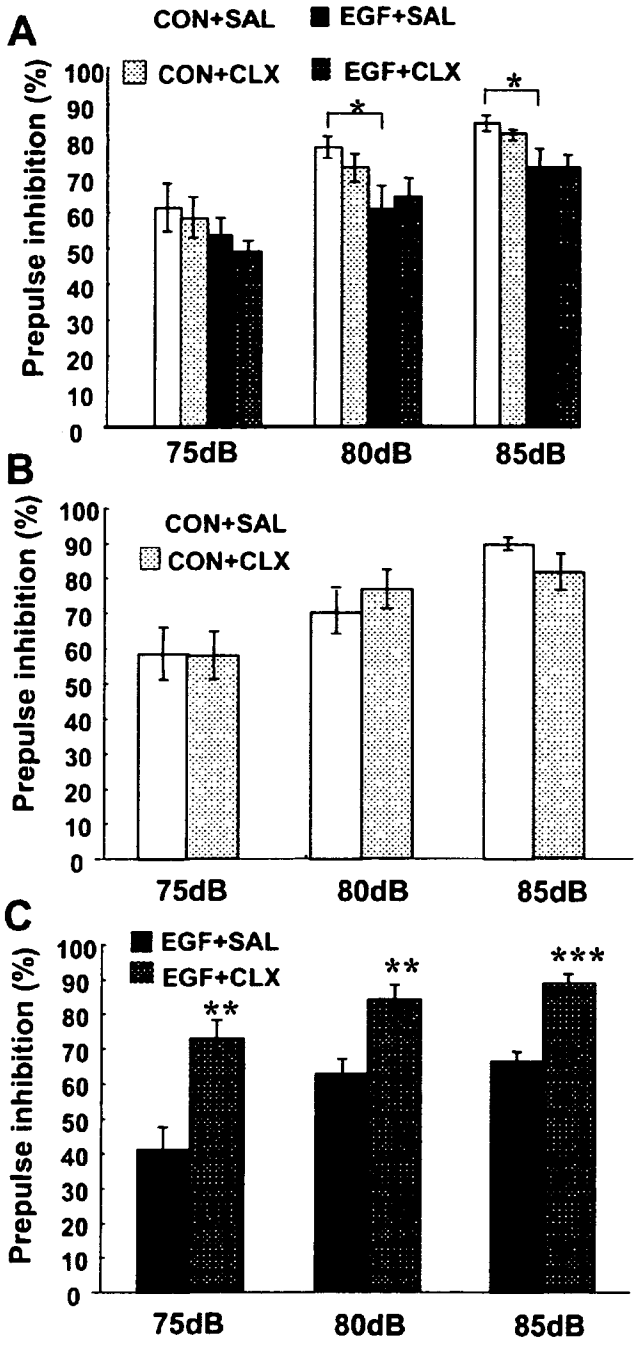

Figure 7. Effects of a Cox-2 inhibitor on the PPI deficits of rats receiving EGF in the striatum. Three days after striatal administration of EGF (30 μg/pump) or vehicle (CON) was initiated, rats were orally given celecoxib (CLX) or saline (SAL) daily. *A*, One hour after the first dose of celecoxib, PPI of the acoustic startle response with 75, 80, and 85 dB prepulse stimuli was measured. *B*, *C*, At 7 d of treatment with celecoxib or saline, PPI was measured in vehicle-infused (*B*) and EGF-infused (*C*) rats. White and black bars represent vehicle-infused and EGF-infused rats that received saline orally. Black dotted and white dotted bars represent vehicle-infused and EGF-infused rats that received celecoxib orally. Error bars indicate means ± SEM for each prepulse intensity ($n = 7–8$ each). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with saline-infused controls (*A*) or EGF-infused rats receiving saline (*C*) by Fisher's LSD. Note that the subchronic effects of celecoxib were measured at least 20 h after the last treatment with celecoxib.

0.001). No other interactions were observed among between-subject factors. The statistical interactions indicated that EGF differentially affected the latent learning scores and that the preexposure effects varied during test sessions. Accordingly, behavioral scores were separated into EGF-infused and vehicle-infused groups and subjected to statistical analysis independently.

A three-way-ANOVA of learning data from the vehicle-infused group revealed a significant main effect of preexposure ($F_{(1,36)} = 22.9$; $p < 0.001$) without any interaction of between-subject factors. The significant interaction between preexposure and block indicated that the effect of preexposure differed among blocks ($F_{(5,180)} = 3.79$; $p = 0.027$) (Fig. 8*A*). *Post hoc* comparisons detected a significant effect of preexposure on learning regardless of celecoxib treatment in blocks 4–6 (Fig. 8*A*). In contrast, the same analysis for EGF-infused group revealed a significant main effect of preexposure ($F_{(1,36)} = 10.7$; $p = 0.023$), marginal interactions between preexposure and celecoxib ($F_{(1,36)} = 3.31$; $p = 0.077$), and marginal interactions between preexposure and session ($F_{(5,180)} = 1.98$; $p = 0.083$). Pillai compensation for repeated measures confirmed statistical significance of the interaction between celecoxib and block ($F_{(5,180)} = 2.693$; $p = 0.039$). *Post hoc* comparisons revealed that celecoxib treatment significantly improved learning scores of the preexposure group but not the non-preexposure group in block 6 compared with the vehicle-treated non-preexposure group (Fig. 8*B*). Neither EGF infusion nor celecoxib treatment appeared to alter locomotion as monitored by the number of intershuttle movements during the intertrial periods (see details in legend of Fig. 8). We focused on behavioral performance during the last test session and calculated the decrease in learning score that was caused by preexposure to the test chamber without a shock (Fig. 8*C*). A two-way ANOVA with subject factors of EGF administration (+/−) and celecoxib treatment (+/−) revealed significant interactions between EGF and celecoxib for this latent inhibition score ($F_{(1,18)} = 13.1$; $p = 0.002$). *Post hoc* comparisons confirmed that striatal EGF infusion disrupted the latent inhibition of fear learning ($p = 0.002$), and subchronic treatment of celecoxib ameliorated the abnormal decrease in latent inhibition ($p = 0.002$).

To assess the effect of EGF infusion on basal fear learning performance, behavioral data were separated into non-preexposed and preexposed groups to avoid the statistical interaction. In non-preexposed group, three way-ANOVA using between-subject factors of EGF infusion (+/−) and celecoxib administration (+/−) and a within-subject factor of test block (six) revealed that there was not a significant main effect of EGF or celecoxib, and there was no interaction of between-subject factors.

To confirm the ineffectiveness of EGF and celecoxib on basal learning performance and shock sensitivity, we performed another fear learning test (Fig. 9). EGF- and vehicle-infused rats, which were treated with saline or celecoxib, were subjected to contextual conditioning with electric shocks (CS) and environmental context (US). Two-way ANOVA failed to detect a significant difference in shock sensitivity among groups without interaction (Fig. 9*A*). Learning performance was also indistinguishable among groups (Fig. 9*B*). Thus, we conclude that neither striatal EGF infusion nor celecoxib treatment influence basal learning performance or shock sensitivity.

EGF activates dopamine synthesis and turnover

Deficits in PPI and latent inhibition are proposed to involve abnormal dopaminergic neurotransmission (Swerdlow et al., 2001; Jeanblanc et al., 2003; Smith et al., 2007). To evaluate the contribution of Cox-2 induction to EGF-enhanced dopamine turnover, we also examined the effects of the Cox-2 inhibitor celecoxib on striatal concentrations of dopamine and its metabolites (Fig. 10). A two-way ANOVA with subject factors of EGF admin-

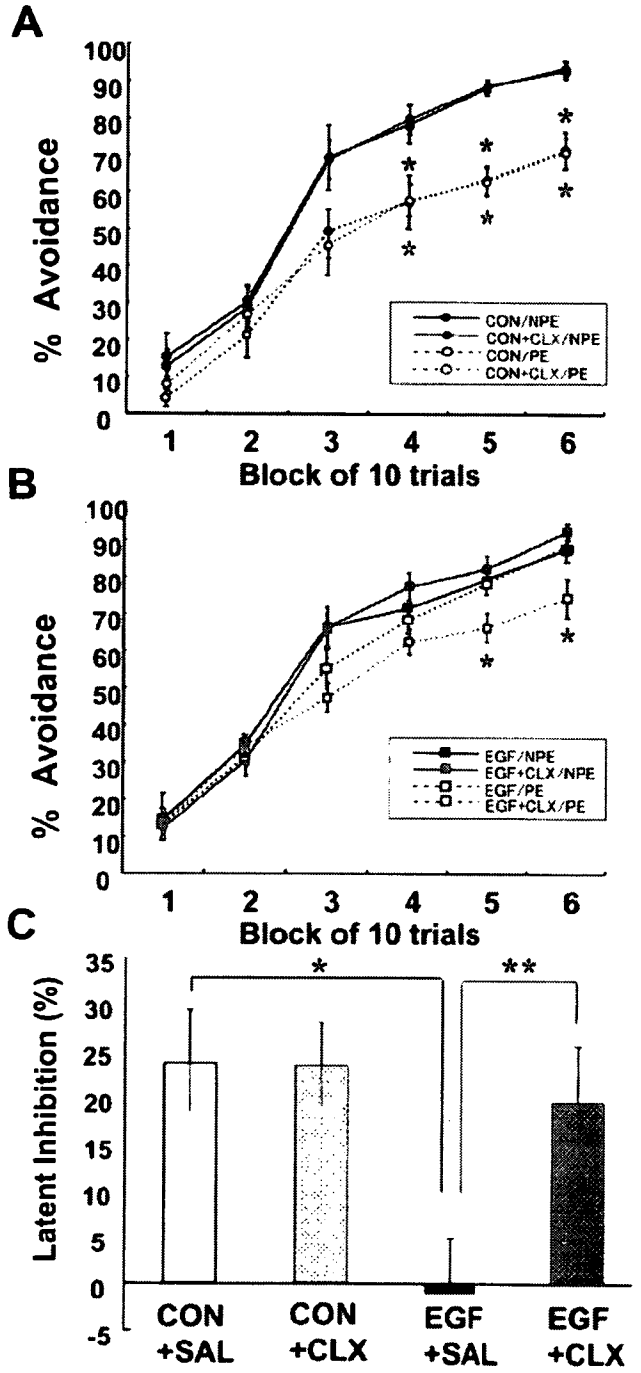

Figure 8. The effects of celecoxib on active-avoidance learning and latent inhibition in EGF-infused rats. Learning ability and latent inhibition of EGF-infused rats (EGF; 30 μg/pump) was determined with an active-avoidance test (10 trials per block, 6 blocks total) and compared with that of vehicle-infused controls (CON). Simultaneously, these animals were daily treated with celecoxib (CLX) or saline (SAL). Detailed schedules of the EGF infusion and celecoxib are described in Materials and Methods. The ability of vehicle- and EGF-infused rats to avoid the electrical shock paired with a tone was defined as learning performance and improved significantly during training sessions. Before the conditioning, rats in the PE group had been preexposed to the same tone cues without shock. Rats in the NPE group were directly subjected to the active-avoidance test. Random shuttle movement of the NPE group during intertrial periods revealed no effects of EGF by ANOVA or celecoxib or factorial interaction. ***A***, ***B***, Because the initial four-way ANOVA revealed factorial interaction between EGF infusion and preexposure, data were separated into vehicle-infused (***A***) and EGF-infused (***B***) groups and then analyzed separately. ***C***, Learning performance at the last block (6th) of the PE groups was compared with that of the NPE group and latent inhibition scores were calculated as follows:

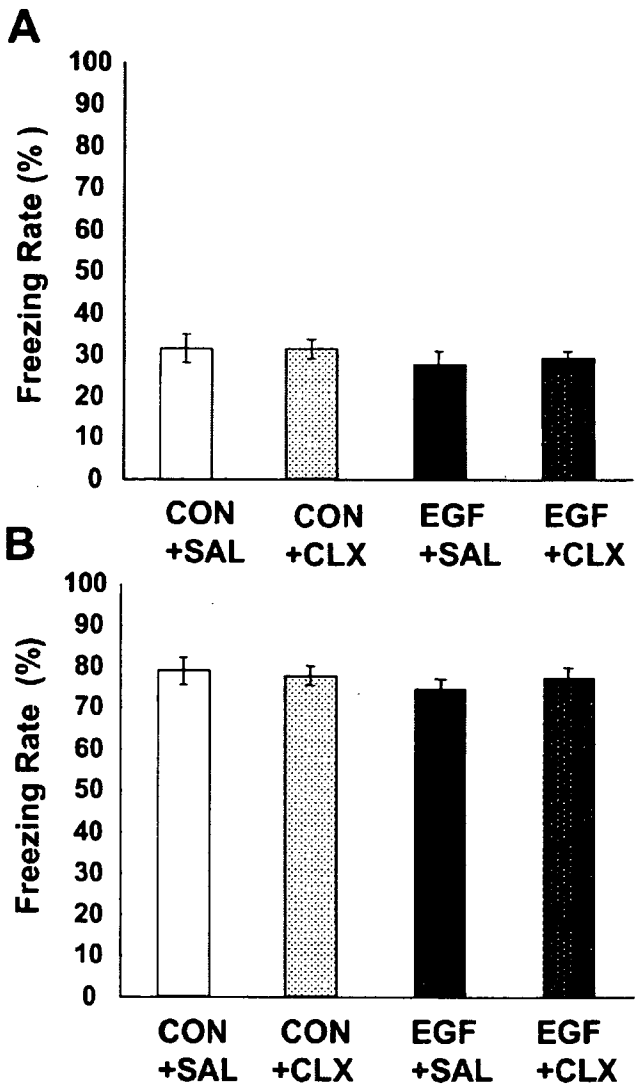

Figure 9. Effects of EGF and celecoxib on shock sensitivity and context learning. Three days after striatal administration of EGF (30 μg/pump) or vehicle (CON) was initiated, rats were orally given celecoxib (CLX) or saline (SAL) daily for 7 d and subjected to conditioning. ***A***, Immobilizing rate (freezing) of rats was calculated for 2 min after the second shock of conditioning. ***B***, Contextual fear learning was evaluated with freezing rates from 1 to 3 min after placing rats in the same chamber 1 d after conditioning. White and black bars represent vehicle-infused and EGF-infused rats that received saline, respectively. Black dotted and white dotted bars represent vehicle-infused and EGF-infused rats that received celecoxib, respectively. Error bars indicate means ± SEM ($n = 10$ each). Note that conditioning was performed 24 h after the last treatment with celecoxib.

istration (+/−) and celecoxib treatment (+/−) revealed significant and marginal interactions between EGF and celecoxib for dopamine and its metabolites, respectively ($F_{(1,17)} = 4.43$, $p = 0.050$ for dopamine; $F_{(1,17)} = 3.99$, $p = 0.062$ for DOPAC; $F_{(1,17)} = 11.2$, $p = 0.039$ for HVA). *Post hoc* comparisons revealed that elevated dopamine and HVA contents after EGF administra-

←

$$\text{Latent inhibition score} = 100 - \frac{(\text{performance of each PE rat}) \times 100}{\text{mean performance of NPE group}}.$$

Error bars indicate means ± SEM ($n = 10$ each). $^{*}p < 0.05$, $^{**}p < 0.01$ by Fisher's LSD. Note that, to minimize acute effects of celecoxib treatment, the active-avoidance test was performed at least 20 h after the last treatment with celecoxib.