Neurobehavioral deficits of epidermal growth factor-overexpressing transgenic mice: Impact on dopaminergic metabolism

Takeyoshi Edaa, Makoto Mizunoa, Kazuaki Arakia, Yuriko Iwakuraa, Hisaaki Nambaa, Hidekazu Sotoyamaa, Akiyoshi Kakitab, Hitoshi Takahashib, Hiroshi Satohc, Siu-Yuen Chand, Hiroyuki Nawaa,*

a Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan
b Department of Pathology, Brain Research Institute, Niigata University, Niigata, Japan
c Division of Pharmacy, Niigata University Medical & Dental Hospital, Niigata, Japan
d Department of Paediatrics and Adolescent Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong, China

HIGHLIGHTS

- We established the transgenic mice overexpressing EGF.
- EGF-transgenic mice exhibited the behavioral deficits relevant to schizophrenia.
- EGF-transgenic mice were hypersensitive to repeated cocaine administration.
- Overexpression of EGF is associated with altered dopaminergic metabolism in a region-specific manner.

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ABSTRACT

Epidermal growth factor (EGF) and its family member neuregulin-1 are implicated in the etiology of schizophrenia. Our recent pharmacological studies indicate that EGF injections to neonatal and adult rats both induce neurobehavioral deficits relevant to schizophrenia. We, however, did not evaluate the genetic impact of EGF transgene on neurobehavioral traits. Here we analyzed transgenic mice carrying the transgene of mouse EGF cDNA. As compared to control littermates, heterozygous EGF transgenic mice had an increase in EGF mRNA levels and showed significant decreases in prepulse inhibition and context-dependent fear learning, but there were no changes in locomotor behaviors and sound startle responses. In addition, these transgenic mice exhibited higher behavioral sensitivity to the repeated cocaine injections. There were neurochemical alterations in metabolic enzymes of dopamine (i.e., tyrosine hydroxylase, dopa decarboxylase, catechol-O-methyl transferase) and monoamine contents in various brain regions of the EGF transgenic mice, but there were no apparent neuropathological signs in the brain. The present findings rule out the indirect influence of anti-EGF antibody production on the reported behavioral deficits of EGF-injected mice. These results support the argument that aberrant hyper-signals of EGF have significant impact on mouse behavioral traits and dopamine metabolism.

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1. Introduction

We have been investigating etiologic and pathologic contributions of epidermal growth factor (EGF) and its family member neuregulin-1 in the etiology or neuropathology of schizophrenia [18]. Both cytokines activate ErbB receptors and influence dopaminergic, GABAergic, and glial development and/or neurotransmission [10]. The gene targeting of ErbB receptors and ligands result in the dysfunction of these cells [2,19,20,25]. Our studies on EGF and ErbB1 started with our initial findings on an increase in ErbB1 levels in the forebrain regions of patients with schizophrenia [5]. Several genetic studies also indicate an association of this illness with the functional SNP promoting EGF transcription [6,13]. Based on these observations, we attempted to establish animal models for this disease by administering EGF and other peptides in the EGF family to rodent pups or adults [5,11,16,22–24]. The animals receiving EGF developed behavioral and cognitive deficits; most of which can be ameliorated by subchronic treatment with atypical antipsychotics [5]. In addition, these animal models exhibited hypersensitivity to psychostimulants [11,17]. There are
significant alterations in dopamine synthesis, metabolism and/or neurotransmission in these EGF-treated animals [3,8,9,21,22]. The phenotypic influences of EGF on GABAergic neurons or glial cell populations appear to be limited [1,5].

One possible drawback in the previous studies is repeated injections or continuous administration of EGF might result in the production of the anti-EGF antibody that neutralizes endogenous EGF and down-regulates EGF signaling. This argument might be supported by clinical studies as schizophrenia patients often contain lower levels of EGF in their serum [5,7]. To avoid the production of anti-EGF antibody, we established EGF-overexpressing mice by genetic manipulation. We explored various behavioral traits of these transgenic mice and examined their neurochemical alterations in dopaminergic markers.

2. Materials and methods

2.1. Animals

All transgenic mice were housed in a plastic cage (200 mm × 300 mm × 140 mm) and given free access to food and water. Each cage contained 2–4 mice and was kept in a temperature-controlled colony room (22 ± 1.0 °C) under a 12-h light–dark cycle (8:00 on–20:00 off). We used both male and female mice following studies (total n = 80). All of the animal experiments described here were approved by the Animal Care and Use Committee of Niigata University and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (NIH, USA).

2.2. EGF transgene

The promoter from cytomegalovirus (CMV) was inserted into the 5′-flanking region of the mouse EGF cDNA which was joined to an SV40 poly (A). Transgenic mice were generated by pronuclear injection of the DNA fragment into fertilized mouse eggs (FVB/N) [15] and were bred by crossing with wild-type mice (strain: FVB/N, CLEA Japan Inc., Tokyo, Japan). Heterozygous offspring were used in this study. Mice were genotyped by PCR using primers corresponding to the 5′-flanking region of the transgene (ATGCTGAGTTGCTGAAGGTG), the 3′-flanking region of the transgene (GGCTGCAAGGTACCACTATG), and EGF cDNA (CTCC-CACCATCTGGATCTCTC). Relative mRNA expression levels for EGF and glucose-6-phosphate dehydrogenase (G6PDH) were estimated by reverse transcription-polymerase chain reaction (RT-PCR) (see details in Supplemental Information).

2.3. Immunoblotting

Tissue protein (20 or 30 μg protein/lane) was subjected to 7.5–10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane [11]. Immunoblots were probed with anti-tyrosine hydroxylase (1:2000, Chemicon International, Temecula, CA, USA), anti-DOPA decarboxylase (1:2000, Sigma, St. Louis, MO, USA), anti-dopamine transporter (DAT) (1:500, Santa Cruz), anti-catechol-O-methyltransferase (COMT) (1:4000, BD Bioscience, San Jose, CA, USA) or anti-actin (1:4000, Chemicon) antibodies. Immunoreactivity was detected using an anti-immunoglobulin antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratory, West Grove, PA, USA) followed by a chemiluminescence reaction and exposure to X-ray films. Film images carrying a liner range of darkness were subjected to densitometric quantification (Image J; National Institutes of Health, USA).

2.4. Behavioral tests

Behavioral traits of the mice (postnatal day 56–70) were assessed as described previously [11]. Locomotor activity was assessed with an automated activity apparatus (MED Associates, St. Albans, VT, USA). Acoustic startle response and prepulse inhibition were measured by an automated startle chamber (SR-Lab Systems, San Diego, CA, USA) [11]. Performance of context-dependent fear learning was monitored in a shock chamber with a grid floor (Obara Medical Ltd., Tokyo, Japan) combined with the exposure to 0.8 mA electric shocks (see details in Supplemental Information).

2.5. Quantification of dopamine and its metabolites

We measured the tissue contents of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptamine (5-HT) as described previously [22]. Brain tissues were homogenized in monoamine extraction buffer [0.1M perchloric acid, 0.1 mM EDTA, 250 mM isoproterenol (internal standard)]. Monoamine levels were analyzed by high performance liquid chromatography (HPLC) electrochemistry (model LC-10ADVP; Shimadzu, Kyoto, Japan) as described previously [11].

2.6. Histopathological examination

Adult mice were deeply anesthetized through inhalation of halothane and perfused with 4% paraformaldehyde plus 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. The tissues were dehydrated with ethanol and embedded in paraffin wax. Serial sections (4 μm) were cut from paraffin block and processed for the Klüver–Barrera stain as well as immunostaining for tyrosine hydroxylase [11].

2.7. Statistical analysis

Behavioral scores were initially analyzed using a two-way analysis of variance (ANOVA) with genotype (two levels) and time or prepulse (three levels) as the within-subjects factors. In all the behavioral tests, a gender ratio was adjusted to a constant (7 males and 3 females for both groups, or all males) to avoid gender interaction. Univariate data from two groups were analyzed using an unpaired two-tailed t test. For post hoc testing, Fisher’s LSD was used to detect differences. These statistical analyses were performed using Statview software (SAS Institute, Cary, NC).

3. Results

3.1. Effects of EGF transgene on mouse behavioral traits

We assessed sound startle responses and prepulse inhibition of the EGF transgenic mice at the stage of young adult. The EGF transgenic mice and their wild-type littermates exhibited indistinguishable strengths of startle responses to various levels of tone stimuli (Fig. 1A). This contrasts with the fact that mice challenged with EGF as neonates have higher startle responses [23]. The transgenic mice showed significantly lower levels of prepulse inhibition at all prepulse levels [F(1,18) = 9.05, p = 0.008] (Fig. 1B). When the EGF transgenic mice received intraperitoneal administration of risperidone for a week, there was no significant difference between transgenic and wild-type mice (Fig. 1C).

We also assessed their ability to learn the pair of context (US) and electric shock (CS) and compared the results with that of wild-type littermate (Fig. 1D). Although there was no change in freezing rates during the conditioning period of electric shock, we found a mild but significant decrease in freezing rates in the test period
Fig. 1. Basal behavioral deficits of EGF transgenic mice. (A) Relative amplitudes of startle responses of EGF transgenic (Tg) mice and wild-type littermates (WT) were measured with 80–120 dB tones. (B) PPI was measured with 74, 78, and 82 dB pre-pulse stimuli and 120 dB main pulses. (C) Effects of subchronic treatment with risperidone (daily 0.3mg/kg, 7 days) on PPI. (D) Mice were subjected to shock-paired contextual fear conditioning. After 1 day, their learning performance was measured with the contextual cue (test). (E) Locomotor activity of mice was scored for 60 min in a novel environment.* $p < 0.05$, ** $p < 0.01$, by Fisher’s LSD or two-tailed $t$ test ($n = 10–13$ each).

We evaluated locomotor activity of the EGF transgenic mouse in a novel environment (Fig. 1E). As far as we monitored total horizontal distance, vertical activity, stereotypy, and center ambulation during a 60-min test period, there were no significant differences in these indices between transgenic and wild-type mice.

3.2. Phenotypic influences of EGF transgene on dopaminergic markers and metabolism

To control the expression of the mouse EGF transgene, we compared EGF mRNA levels between EGF transgenic and wild-type mice (Supplemental Figs. S1 and S2). RT-PCR products of EGF mRNA in the transgenic mice were more than 10-fold enriched in various brain regions and peripheral tissues at both their postnatal and adult stages. However, EGF mRNA levels in the skin, where basal EGF mRNA levels were absolutely high in wild-type mice, were indistinguishable between groups. G6PDH mRNA levels were similar between the transgenic and wild-type mice in all the regions examined. Thus EGF transgene-derived mRNA was ectopically and continuously expressed in the transgenic mouse although absolute amounts of the mRNA appeared to be lower.

We also compared the protein expression of dopamine-associated molecules (Fig. 2B); tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine transporter (DAT), catechol-O-methyltransferase (COMT) in frontal cortex, striatum, nucleus accumbens, and globus pallidus between the transgenic and wild-type mice (Fig. 2A–D). We found a significant decrease in TH levels in the striatum of the transgenic mice. In contrast, the levels of dopamine metabolic enzymes, DDC, and COMT were elevated in several brain regions of the transgenic mice.

To estimate the impact of the EGF transgene on brain structures, we performed neuropathological examination using a conventional dye staining and immunostaining for TH. The Klüver–Barrera staining of the EGF transgenic mice revealed no apparent differences in the lamination and structure of the cortex, hippocampus, or striatum, compared with wild-type littermates (Fig. 3A and B). There were no structural abnormalities in other brain regions, either (data not shown). TH immunostaining failed to detect any neurodegenerative signs in dopaminergic neurons of the EGF transgenic mice (Fig. 3C and D).
We also determined dopamine metabolism in the basal ganglia regions of the EGF transgenic mice (Table 1). We found significant increases in dopamine and DOPAC content in nucleus accumbens of the transgenic mice. Although the alteration of dopaminergic phenotypes was apparent in the EGF transgenic mice, the details of the changes were diverse in individual brain regions.

### Table 1

<table>
<thead>
<tr>
<th>Region</th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus accumbens</td>
<td>162 ± 15</td>
<td>65.1 ± 4.3</td>
<td>44.1 ± 11.2</td>
</tr>
<tr>
<td>EGF transgenic</td>
<td>246 ± 35</td>
<td>102 ± 10</td>
<td>35.8 ± 5.5</td>
</tr>
<tr>
<td>P value</td>
<td>0.040*</td>
<td>0.005**</td>
<td>0.51</td>
</tr>
<tr>
<td>Striatum</td>
<td>552 ± 19</td>
<td>98.7 ± 5.3</td>
<td>19.7 ± 5.2</td>
</tr>
<tr>
<td>EGF transgenic</td>
<td>504 ± 22</td>
<td>94.1 ± 7.0</td>
<td>18.5 ± 3.0</td>
</tr>
<tr>
<td>P value</td>
<td>0.12</td>
<td>0.60</td>
<td>0.83</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>74.6 ± 12.2</td>
<td>34.8 ± 4.4</td>
<td>19.3 ± 2.0</td>
</tr>
<tr>
<td>EGF transgenic</td>
<td>63.2 ± 12.6</td>
<td>34.4 ± 5.5</td>
<td>17.8 ± 2.2</td>
</tr>
<tr>
<td>P value</td>
<td>0.52</td>
<td>0.95</td>
<td>0.62</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>3.35 ± 0.29</td>
<td>2.16 ± 0.21</td>
<td>2.57 ± 0.27</td>
</tr>
<tr>
<td>EGF transgenic</td>
<td>2.63 ± 0.23</td>
<td>2.02 ± 0.26</td>
<td>2.31 ± 0.20</td>
</tr>
<tr>
<td>P value</td>
<td>0.068</td>
<td>0.68</td>
<td>0.45</td>
</tr>
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</table>

The levels of dopamine (DA), DOPAC, and HVA, were determined by HPLC-ECD [5]. Data represent mean ± SEM (pmol/mg protein; n = 10 each). *p < 0.05 and **p < 0.01, compared to WT littermates by unpaired two-tailed t-test.

### 3.3. Behavioral responses to repeated injections of cocaine

EGF hypersignal enhances behavioral sensitization to methamphetamine [17]. We challenged the EGF transgenic mice with cocaine and measured drug-induced locomotor activities daily (Fig. 4). On day 1, the dose of cocaine (20 mg/kg, ip) similarly elevated several locomotor indices in both EGF transgenic mice and wild-type littermates; distance, $F(14,252)=2.18$, $p=0.090$; vertical movement, $F(14,252)=3.87$, $p<0.001$, and center ambulation, $F(14,252)=2.33$, $p=0.005$. The repeated injections of the dose of cocaine induced behavioral sensitization only in the EGF transgenic mice. EGF transgenic and wild-type mice exhibited significant differences in horizontal movement and ambulation levels on days 3 and 5 of cocaine injection [$F(1,18)=6.40$, $p=0.021$ for horizontal movement; $F(1,18)=7.98$, $p=0.011$ for ambulation].

### 4. Discussion

To investigate the neurobehavioral consequences of continuous EGF hyper-signaling, we obtained the transgenic mouse line carrying mouse EGF cDNA driven by the ubiquitous transcription promoter of CMV. These transgenic mice are known to exhibit hair follicle deficits and thin fur with the EGF hyper-signaling [15]. In agreement, there were marked increases in EGF mRNA levels in various tissues of the transgenic mice. As indicated by the neurotrophic action of EGF on dopamine neurons [8,9], the transgenic mice exhibited the increase in dopamine metabolism. The decrease in TH in the striatum of the transgenic mice was, however, observed beyond our expectation. Heterozygous EGF transgenic mice carrying FVB genetic background displayed various neurobehavioral abnormalities; marked reduction in prepulse inhibition, learning deficits, and higher sensitivity to repeated cocaine treatments. In contrast, there were no
significant differences in acoustic startle amplitudes, exploratory movement, and shock sensitivity. These behavioral features of the EGF transgenic mice appear to indicate their normal motor function or sensory abilities in a limited degree.

The neueregulin-1 transgenic mice exhibited similar types of neurobehavioral abnormalities to the EGF transgenic mice; learning deficits in context-fear conditioning, a trend toward decreasing prepulse inhibition, and reduced social interactions [12]. The neurochemical abnormalities of neueregulin-1 transgenic mice also resembled those of the EGF transgenic mice; a decrease in TH levels [12]. Considering the colocalization and potential signal crosstalk of EGF receptor (Erbb1) and neueregulin-1 receptor (Erbb4) in the midbrain dopamine neurons [10], the similarity in neurobehavioral impact is corroborated by the common neurotrophic action on this neuronal population [1,10].

We have been studying neonate and adult rodent animals transiently challenged with EGF as schizophrenia models [4,16,23]. With the given pharmacological procedure, we might induce the production of anti-EGF antibody in the host animals, which might neutralize the action of endogenous EGF. In the present transgenic mouse model, however, the production of the anti-EGF antibody was unlikely because of immune tolerance for self antigens. Some of the observed behavioral differences between EGF-injection model and EGF transgenic mice might stem from the difference in their mouse strains [23].

There is one technical limitation of the present study on the EGF transgenic mice. We only explored one line of the EGF transgenic mice and cannot rule out the possibility that the EGF transgenic mice appear to indicate their normal motor function or sensory abilities in a limited degree.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.04.055.

References


