Neuroscience 158 (2009) 1731–1741

EPIDERMAL GROWTH FACTOR ADMINISTERED IN THE PERIPHERY INFLUENCES EXCITATORY SYNAPTIC INPUTS ONTO MIDBRAIN DOPAMINERGIC NEURONS IN POSTNATAL MICE

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Abstract—Epidermal growth factor (EGF) has a neurotrophic activity on developing midbrain dopaminergic neurons. We investigated developmental effects of peripheral EGF administration on dopaminergic neurons in midbrain slice preparations containing ventral tegmental area (VTA). Subcutaneous EGF administration to mouse neonates triggered phosphorylation of EGF receptors (ErbB1 and ErbB2) in the midbrain region, suggesting its penetration through the blood–brain barrier. We repeated EGF administration in postnatal mice and examined synaptic transmission in the VTA with electrophysiological recordings. Subchronic EGF treatment increased the amplitude of field excitatory postsynaptic potentials evoked by stimulation of the anterior VTA. To analyze the EGF effect at a single cell level, dopaminergic neurons were identified by their characteristic hyperpolarizing activated currents in whole cell recording. In these dopaminergic neurons, EGF effects the amplitude of spontaneous miniature excitatory postsynaptic currents (mEPSCs) without affecting their frequency. In agreement, EGF also enhanced the AMPA/NMDA ratio of evoked EPSCs in the dopaminergic neurons. In contrast, EGF effects on mEPSCs of neighboring neurons not exhibiting hyperpolarizing activated currents were modest or insignificant. Thus, these results suggest that circulating EGF substantially influences the physiological properties of developing midbrain dopaminergic neurons in perinatal and postnatal mice.

Key words: neurotrophic factor, dopamine, ventral tegmental area, AMPA, ErbB1.

Epidermal growth factor (EGF) and EGF homologues have the neurotrophic activity that promotes survival and neurite elongation of midbrain dopaminergic neurons (Casper et al., 1991; Casper and Blum, 1995; Alexi and Hefti, 1993; Ferrari et al., 1991; Iwakura et al., 2005; Pezzoli et al., 1991). Repeated injection of EGF into rat neonates or subchronic infusion into the striatum of adult rats increases dopamine metabolism and/or tyrosine hydroxylase activity *in vivo* (Futamura et al., 2003; Tohmi et al., 2005; Mizuno et al., 2007). Epidermal growth factor receptor (ErbB1) mRNA is widely expressed in the CNS including midbrain regions (Kornblum et al., 1997; Fox and Kornblum, 2005). Previous *in situ* hybridization studies suggest that the rat ventral tegmental area (VTA) and substantia nigra compacta (SNc) express ErbB1 mRNA (Seroogy et al., 1994; Kornblum et al., 1997). In addition, a null mutation for an ErbB1 ligand, transforming growth factor α, reduces the number of dopaminergic neurons in the SNc (Blum, 1998). Thus, EGF signaling in the midbrain is implicated in regulation of dopaminergic function or development.

EGF is synthesized in many peripheral organs, including the kidney, liver and pituitary gland; it is released into the bloodstream and crosses the blood–brain barrier (Kastin et al., 1999; Pan and Kastin, 1999). Transforming growth factor α and heparin-binding EGF-like growth factor are, however, produced endogenously in the CNS and contribute to the activation of ErbB1 as well (Birecree et al., 1991; Schaudies et al., 1989; Lazar and Blum, 1992; Piao et al., 2005). Although EGF mRNA and protein are also expressed endogenously in restricted regions of the brain, their levels are relatively low in comparison with EGF concentrations in blood (Futamura et al., 2002). Concentrations of EGF in human blood are relatively high with a picomolar range and altered in several brain diseases of developmental origin (Futamura et al., 2002; Ikeda et al., 2008). Presumably, circulating EGF could have strong impact on development of the CNS, especially in the fetus where the blood–brain barrier is not fully established (see review: Plata-Salaman, 1991). In this context, it is unclear how influential circulating EGF is in brain development or function. In particular, evidence for the neurotrophic effects of peripheral EGF on dopaminergic development is very limited.

Here we assessed subchronic effects of EGF administered to the periphery on postnatal dopaminergic neurons in the VTA, focusing on the excitatory synapses formed on these neurons. Strength of excitatory inputs to midbrain dopaminergic neurons is regulated in a plastic manner (White, 1996) and the plasticity is often implicated in the regulation of dopaminergic function (Giorgetti et al., 2001). Thus, we analyzed presynaptic and postsynaptic properties of the excitatory inputs to dopaminergic neurons,

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Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; AP, alkaline phosphatase; CNQX, 6-cyano-7-nitoroquinoxaline-2,3-dione; DIG, digoxigenin; EGF, epidermal growth factor; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; ErbB1, epidermal growth factor receptor; fr, fasciculus retroflexus; GFAP, glial fibrillary acidic protein; I_h, hyperpolarizing activated current; K-S, Kolmogorov-Smirnov; mEPSC, miniature excitatory postsynaptic currents; MT, medial terminal nucleus of the accessory optic tract; NaPB, sodium phosphate buffer; NSE, neuron-specific enolase; P, postnatal day; SDS, sodium dodecyl sulfate; SNc, substantia nigra compacta; TBS, Tris-buffered saline; VTA, ventral tegmental area.

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doi:10.1016/j.neuroscience.2008.10.057

employing field potential recordings and slice patch-clamp recordings. Protein levels of ionotropic glutamate receptor subunits were measured by immunoblotting and compared with the electrophysiological results. Physiological and pathologic implication of circulating EGF was discussed.

EXPERIMENTAL PROCEDURES

Animal protocols

Neonatal mice at postnatal day 2 (P2) or pregnant mice at 15 gestation days of C57BL/6 strain, were purchased from SLC (Shizuoka, Japan). Recombinant human EGF (0.875 μg/g body weight, Higeta Shoyu, Chiba, Japan) was administered s.c. to half of the pups in newborn litters daily for 2 weeks (P2–15) (Tohmi et al., 2005; Nagano et al., 2007). The dose of EGF does not impair physical growth of mice (Tohmi et al., 2005). Control littermates received a saline injection. All postnatal mice were housed with a dam until weaning (a litter 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. All animal experiments were authorized by the Animal Use and Care Committee of Niigata University and were carried out in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

Biotinylation of human EGF and injection

To visualize permeation of EGF through the blood–brain barrier, human recombinant EGF was biotinylated with the following procedure. EGF (35μg/μl, Higeta Shoyu) was incubated with EZ-Link sulfo-NHS-LC-biotin (No. 21335, Pierce, Rockland, IL, USA) at a molar ratio to EGF of 50:1 overnight at 4 °C. The reaction mixture was dialyzed for two overnight sessions to remove an uncoupled biotinylation reagent (Slide-A-Lyzer 3.5K, Pierce No. 66330). Biotinylated EGF was s.c. injected (0.8 μg/g body weight) to neonatal mice at P2 as described above.

Fixation and tissue preparation

Mice were anesthetized with hypothermia on ice and transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPB), pH 7.4. Brains were post-fixed overnight with the same paraformaldehyde solution. Then, the tissue was immersed in 30% sucrose in 0.1 M NaPB and embedded in OCT compound (Sakura Finetek, Torrance, CA, USA). Coronal or horizontal sections (12–14 μm thick) were prepared for *in situ* hybridization and immunohistochemistry using the cryostat (CM1510, Leica, Nussloch, Germany).

In situ hybridization

cRNA probes were synthesized as follows: A cDNA fragment for ErbB1 mRNA was synthesized from mouse brain RNA using Platinum® Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) and oligoDNA primers. The primers carried DNA sequences matching mouse ErbB1 gene (GenBank: NM_207655) as well as T7 or SP6 promoter sequences (SP6: cgatttaggtgacactatagaatagtgactgtctggtctgccaaaagtt; T7: gtaatacgactcactatagggccttccgaggagcataaaggattg; 850 bp). Digoxigenin (DIG)-labeled sense and anti-sense cRNA probes were made by *in vitro* transcription with SP6 and T7 RNA polymerases, respectively (Roche Diagnostics, Indianapolis, IN, USA) (Young et al., 1991).

In situ hybridization was carried out as previously described (Liang et al., 2000; Watakabe et al., 2006). In brief, sections (14 μm thick) were treated with proteinase K (0.2 μg/ml). After acetylation, sections were hybridized with 1 μg/ml DIG-labeled cRNA probes at 60 °C for 6 h. Sections were washed with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0)/50% formamide/0.1% N-lauroylsarcosine for 20 min at 60 °C and then treated with 20 μg/ml RNase A (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37 °C. To detect hybridization signals, sections were incubated with alkaline phosphatase (AP)–conjugated sheep anti-DIG antibody (1:1000, Roche Diagnostics) for 4 h at room temperature, and then AP activity was visualized with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) as substrates (Roche Diagnostics).

Immunohistochemistry

For immunostaining, sections (12 μm thick) were incubated with 5% bovine serum albumin and 0.3% Triton X-100 in Tris-buffered saline (TBS; 0.1 M Tris–HCl pH 7.4, 150 mM NaCl) for 1 h and then treated with an anti–tyrosine hydroxylase antibody (1/1000, rabbit polyclonal, Chemicon or 1/1000 mouse monoclonal) (Hatanaka and Arimatsu, 1984) overnight. After rinsing with TBS, sections were incubated with biotin-labeled rabbit or mouse secondary antibodies (1/200, Vector Laboratories, Burlingame, CA, USA), followed by incubation with Vectastain ABC elite kit (1:100). Immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB).

Electrophysiology

Mice (P16–18) were anesthetized with halothane and decapitated. Brains were removed and placed in a cold artificial cerebrospinal fluid (ACSF) solution containing the following (in mM): 195 sucrose, 1 NaH_2PO_4, 2.5 KCl, 5 $MgSO_4$, 1.0 $CaCl_2$, 26.2 $NaHCO_3$, 11 D-glucose, 1 ascorbic acid, pH 7.4, and saturated with 95% O_2 and 5% CO_2. For field recordings, horizontal slices (thickness: 500 μm) containing the VTA were prepared with a microslicer (DTK-2000 or Pro7, Dosaka, Kyoto, Japan) and dissected at the midline according to Zheng et al. (2006). Slices were placed in an incubation chamber for at least 1 h at room temperature. The chamber was filled with an ACSF solution containing the following (in mM): 119 NaCl, 1.0 NaH_2PO_4, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 11 D-glucose, and 1 ascorbic acid.

Electrophysiological experiments were all performed at room temperature (24–26 °C). Slices were placed in a recording chamber continuously perfused with normal ascorbic acid-free ACSF at ~4.0 ml/min. The VTA was identified as the region lateral to the fasciculus retroflexus (fr) and medial to the medial terminal nucleus of the accessory optic tract (MT) (Johnson and North, 1992; Zheng et al., 2006). Field recording procedures were modified in accordance with our previous experiments with slices of rat brain (Zheng et al., 2006). A glass microelectrode filled with ACSF (5–8 MΩ) was placed between *fr* and MT. Field potentials were evoked by electrical stimuli (50 μs duration, 0.05 Hz) from the microconcentric electrode (tip diameter; 25 μm, MCE-100, David Kopf Instruments, Tujunga, CA, USA), which was located at the anterolateral position of the recording electrode. The anterior region of the VTA contains glutamatergic fibers possibly originated from prefrontal cortex, hypothalamus, and other brain regions (Geisler et al., 2007). Field potentials were recorded with 0.1 mA increments of stimulus intensity (0.12–0.62 mA) in the presence of 20 μM bicuculline. The amplitude of fiber volley component (N1) was calculated with the program for population spike measurement in Axograph 3.5 (Axon Instruments) to compensate for the stimulation artifacts. To measure the amplitude of the CNQX-sensitive component (N2), field excitatory postsynaptic potential (EPSP), five responses were averaged in each stimulus intensity and the peak difference in amplitude before and after CNQX application was calculated with Clampfit 6 (Axon Instruments, Foster City, CA, USA) (Zheng et al., 2006).

For whole cell recordings, horizontal slices (thickness: 250 μm) were prepared. Individual cells in the medial region of MT were visualized with an upright phase microscope. Whole-cell patch-clamp recordings were made with an Axopatch 200B (Axon Instruments). Dopaminergic neurons were identified by their char-

Table 1. Passive properties of midbrain dopaminergic and non-dopaminergic neurons in EGF-treated mice

	Vm (mV)	Rm (MΩ)	Cm (pF)	Rs (MΩ)	I_h (pA)
DA neuron					
cont (n=13)	−56.8±1.2	410±53	79.5±3.9	16.0±0.7	139±35
EGF (n=13)	−56.9±1.3	375±53	96.9±8.1	14.6±1.0	155±47
Non-DA neuron					
cont (n=9)	−55.4±2.0	698±185	59.5±4.8	21.9±1.4	15±4
EGF (n=9)	−59.6±1.7	701±143	60.0±8.0	21.4±1.4	20±6

Input resistance and series resistance were determined by measuring the current response to a negative 5 mV pulse from a holding potential. Cell capacitance measurements were made by integration of capacitive transients. I_h was measured by 900 ms of hyperpolarizing voltage steps of −70 mV from holding potential at −69 mV. Cells displaying >40 pA of I_h currents were classified to putative dopaminergic (DA) neurons. We confirmed that more than 70% of the cells in this criteria carried tyrosine hydroxylase immunoreactivity (data not shown). Abbreviations: Vm, membrane potential; Rm, input resistance; Cm, membrane capacitance; Rs, series resistance.

acteristic hyperpolarizing activated current (I_h) (more than 40 pA; see Table 1) (Johnson and North, 1992). For miniature excitatory postsynaptic current (mEPSC) analysis, a pipette (3–5 MΩ) was filled with an internal solution containing (in mM), 122.5 $KMeSO_3$, 7.5 KCl, 10 Hepes, 0.2 EGTA, 5 biocytin-Cl, and 4 Mg-ATP and sucrose to adjust the osmolarity to 290 mOsm (pH 7.4). Membrane and holding potentials were compensated with the liquid junction potential (−9 mV). All data were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. Data were acquired using Axoclamp7 (Axon Instruments). Spontaneous mEPSCs were recorded in the presence of 1 μM tetrodotoxin and 10 μM bicuculline. No compensation for whole-cell capacitance or series resistance was made. Series resistance was determined by measuring a current response to a negative 5 mV pulse from the holding potential. Recording with a series resistance of more than 30 MΩ or cases in which the series resistance changed by more than 10% were excluded. Miniature events were detected with a threshold level for detection of 6 pA with the Mini Analysis Program (Jaejin Software, Leonia, NZ, USA). One hundred twenty events were pooled and analyzed in each cell.

To assess the contribution of AMPA receptor-mediated and NMDA-receptor-mediated components to evoked excitatory postsynaptic currents (EPSCs), whole cell recordings were performed with a patch pipette (3–5 MΩ) containing (in mM) 117 $CsMeSO_3$, 2.8 NaCl, 20 Hepes, 0.4 EGTA, 5 TEA-Cl, 2.5 Mg-ATP, and 5 QX314Cl and sucrose to adjust the osmolarity to 290 mOsm (pH 7.4) (Ungless et al., 2001). Holding potentials were compensated with the liquid junction potential (−6 mV). Synaptic currents were evoked by constant electrical stimuli (0.05–0.2 mA, 50 μs duration, 0.067 Hz) with a micro-concentric bipolar electrode (tip-diameter: 25 μm; MCE-100, David Kopf Instruments), placed 100–300 μm antero-lateral to the recording position. AMPA currents were recorded at holding potential of −66 mV in the presence of 10 μM bicuculline. NMDA currents were recorded in the presence of 10 μM 6-cyano-7-nitoroquinoxaline-2,3-dione (CNQX) and 10 μM bicuculline at holding potential of 34 mV. Ten traces of synaptic events in each cell were averaged with Clampfit 6 (Axon Instruments) and the peak amplitude from the reflection point was measured. The ratio of these currents was calculated as the AMPA/NMDA ratio.

Immunoblotting

For immunoblotting analysis, we dissected the VTA region with two different procedures. In the first procedure, one 800-μm thick horizontal slice including VTA was prepared with a micro-slicer and cold high sucrose ACSF (see above). The VTA region, located medially between MT, was dissected with a surgical blade under a stereoscopic microscope with coordinates from the mouse brain atlas (see Fig. 5A) (Paxinos and Franklin, 2004; see also Zheng et al., 2006). Tissues obtained from two animals were pooled. In the second procedure, a coronal slice (1 mm of thickness) was dissected from ventral side with a pair of razor blades and the medial region between MT was dissected. Tissues were homogenized with Laemmli sample buffer (2% sodium dodecyl sulfate (SDS), 62.5 mM Tris pH 6.8). Consistent results were obtained with samples prepared with either procedure.

To detect phosphorylation of ErbB1 and ErbB2, whole brain was dissected 45 min after s.c. administration of EGF (Nagano et al., 2007; Tohmi et al., 2005). A coronal slice of the midbrain (1 mm of thickness) was dissected from the ventral side with a pair of razor blades. Ventral midbrain tissue was quickly homogenized in a 2% SDS buffer containing phosphatase inhibitors (2 mM $NaVO_4$, 10 mM NaF) and a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany) (see also Fig. 1A). After centrifugation, supernatants were denatured at 95 °C in the presence of 5% 2-mercaptoethanol and 10% glycerol. Protein concentrations were measured with the Micro BCA kit (Pierce) using bovine serum albumin as a standard. Protein (5–30 μg/lane) was separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electrophoresis. Primary antibodies were diluted (see below) and incubated with the membrane at 4 °C overnight. Immunoreactivity was detected with goat anti-mouse, anti-rabbit immunoglobulin, or rabbit anti-goat immunoglobulin conjugated to peroxidase (1:10,000; Vector Laboratories), and visualized with a chemiluminescence reaction (ECL kit, Amersham Biosciences, Tokyo, Japan). Primary antibodies were used at the following dilutions: anti-phospho-ErbB1 (Tyr1173; 1/500, sc-12351), anti-ErbB1 (1/500; sc-03), anti-ErbB2 (1/500; Neu (C-18), sc-284) (all from Santa Cruz, Santa Cruz, CA, USA), and anti-phospho-ErbB2 (Tyr1248) (1/2000; #06–229, Upstate, Millipore, Billerica, MA, USA) (Kim et al., 1999). Anti-GluR1, anti-GluR2/3, anti-NR1, anti-NR2A and anti-NR2B antibodies were purchased from Chemicon International (Temecula, CA, USA) and GluR4 (rabbit) was from Upstate. Anti–glial fibrillary acidic protein (GFAP) antibody was from DAKO (Glostrup, Denmark) and anti–neuron-specific enolase (NSE) antibody from Polysciences Inc. (Warrington, PA, USA). Rabbit anti–synapsin I and mouse anti-synaptophysin antibodies were obtained from Dr. E. Miyamoto and Dr. M Takahashi, respectively (Obata et al., 1986; Fukunaga et al., 2002).

Statistical analysis

Results are presented as the means±S.E.M. Electrophysiological data were subjected to parametric analysis by analysis of variance (ANOVA) or univariate analysis with Student's t-test. Data of mEPSCs were also subjected to the non-parametric analysis, Kolmogorov-Smirnov (K-S) test to avoid type 2 statistical errors. To quantify immunoreactivity on blots, the densitometry of bands (arbitrary units) was performed and subjected to t-test. 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).

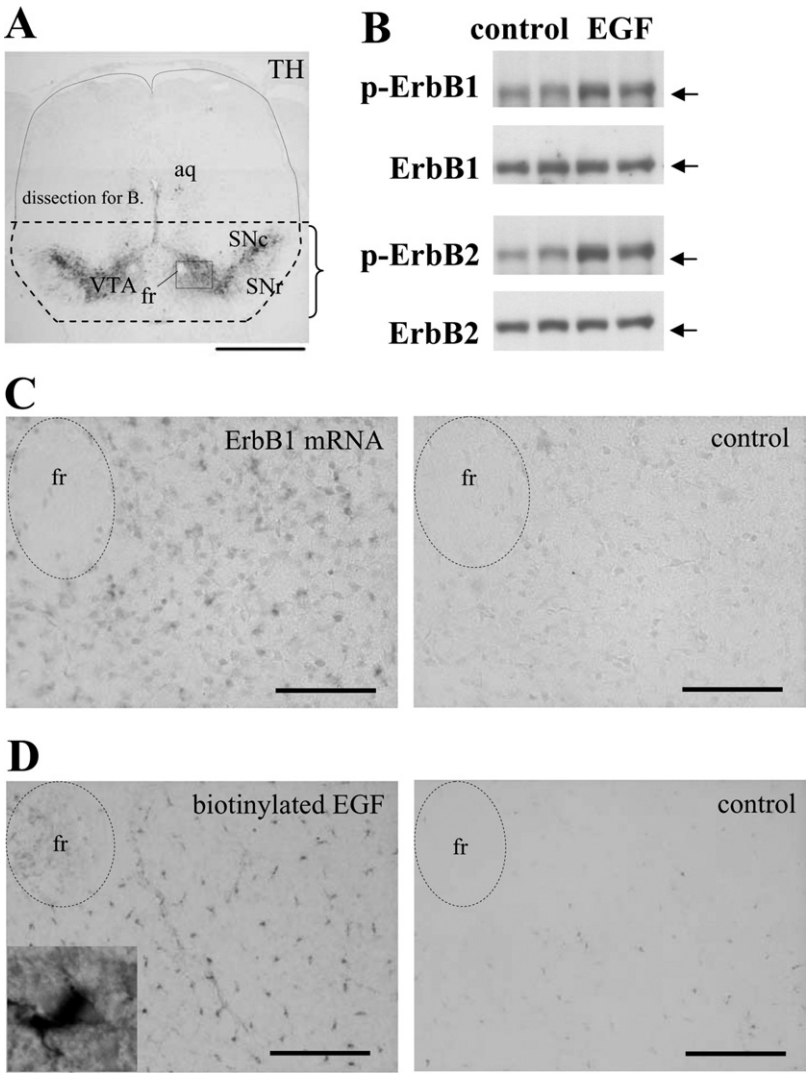

Fig. 1. Acute phosphorylation of ErbB1 and ErbB2 in the ventral midbrain following s.c. administration of EGF. EGF (0.875 μg/g body weight) was s.c. injected into neonatal mice at P2. The midbrain region indicted in A was taken 45 min after administration. Protein lysate (30 μg) was loaded and separated on 7.5% SDS-PAGE gels and transferred to membranes. (A) Typical distributions of tyrosine hydroxylase immunoreactivity of the present slice preparation are displayed; aqueduct (aq), fr, SNc, and substantia nigra reticulata (SNr). (B) Membranes were probed with anti-phospho ErbB1 and anti-ErbB1 antibodies or anti-phospho-ErbB2 and anti-ErbB2 antibodies. No alterations were observed in the total levels of ErbB1 and ErbB2. Arrows indicate the molecular size of 175 kDa. (C) Distributions of ErbB1 mRNA were examined in the midbrain region by *in situ* hybridization with an antisense probe. Control sections were probed with a sense probe for ErbB1 mRNA. Coronal sections containing the VTA were selected with the presence of fr. (D) Biotinylated EGF was similarly injected to mouse neonates. EGF penetration to midbrain through the blood–brain barrier was assessed by the avidin–biotin–complex method. Adjoining serial sections were immunostained with anti-tyrosine hydroxylase antibody as shown in A. The position of a window in A roughly matched that of C and D. The inset in D indicates the accumulation of biotinylated EGF in a cell body. Scale bars=1 mm (A); 100 μm (C), (D).

RESULTS

Peripherally administered EGF penetrated the blood–brain barrier

To estimate the permeability of EGF to the blood–brain barrier, we monitored acute phosphorylation of ErbB1 and its subunit (ErbB2) following s.c. injection of EGF to mouse neonates. Immunoblotting revealed that EGF administration increased the immunoreactivity for phospho-ErbB1 in the ventral midbrain region without changing the levels of total ErbB1 immunoreactivity (Fig. 1A, 1B). The increase in phospho-ErbB1 immunoreactivity was confirmed by the other immunoblot with the different antibody (data not shown). EGF administration also increased phosphorylation of ErbB2. In agreement, *in situ* hybridization detected ErbB1 mRNA in the midbrain region (Fig. 1C). We attempted to confirm that the phosphorylation of ErbB1 resulted from the penetration of administered EGF through the blood–brain barrier but not secondary release of endogenous EGF. In the place of authentic EGF, biotinylated EGF was s.c. injected to mouse neonates and localization of biotin signals was examined using the avidin–peroxidase complex. Significant levels of biotin signals were detected in the cell

bodies in the midbrain region (Fig. 1D). In contrast, the signals in a saline-injected control animal were modest or negligible. These results suggest that EGF circulating in blood can reach midbrain neurons of neonatal mice and influence their function and development.

EGF treatment increased amplitudes of field EPSP in the VTA

We assessed gross physiological influences of the EGF penetration to the VTA with field potential recording (Zheng et al., 2006; Nugent et al., 2008). In the previous study, we found that more than half of the response to stimulation detected by field recordings reflects synaptic inputs onto dopaminergic neurons (Zheng et al., 2006). The field potentials recorded in the presence of 20 μM bicuculline exhibited two negative components, an N1 action potential component and an N2 synaptic component that was sensitive to CNQX (Fig. 2). The amplitude of N1 increased linearly with stimulus intensity. The N1 amplitude was not altered by EGF administration, however (P=0.47; two-way repeated measures ANOVA). In contrast, postnatal EGF administration significantly increased the amplitude of CNQX-sensitive N2 synaptic component (P=0.042). The peak latencies of N1 and N2 were not statistically different between groups at the stimulus intensity of 0.62 mA ([N1] control: 2.0±0.1 ms; EGF: 2.0±0.1 ms, P=0.63; [N2] control: 5.8±0.4 ms; EGF: 5.1±0.2 ms, P=0.16). Thus, EGF administration enhanced the AMPA receptor-mediated component of synaptic transmission in the VTA.

Amplitudes of spontaneous miniature EPSCs were increased in dopaminergic neurons

To determine whether the increase in the field EPSP resulted from increased synaptic inputs onto dopaminergic neurons, we examined these neurons with whole-cell patch clamp recordings. Putative dopaminergic neurons were identified by their characteristic I_h (Johnson and North, 1992). Dopaminergic neurons are known to generate more than 40 pA of currents in response to hyperpolarizing voltage steps (Neuhoff et al., 2002) (Table 1). The frequency of spontaneous mEPSCs was not affected (P=0.22, t-test) (Fig. 3C). However, amplitudes of spontaneous mEPSCs of the putative dopaminergic neurons were significantly increased by EGF administration (P<0.01, t-test). Averaged cumulative histograms of inter-event intervals and amplitudes of mEPSCs also drew the same statistical conclusions (P>0.99 for inter-event interval, P=0.0003 for amplitude, K-S test) (Fig. 3D). In examining the passive electrical properties of recorded neurons, EGF-administration did not affect resting potential or input resistance whereas cell capacitance displayed a non-significant increase (P=0.07) (Table 1). In contrast, EGF effects on mEPSCs of the I_h-negative neuronal population were insignificant or very limited (Fig. 4). The decreasing trend of the EGF-treated group in the frequency was insignificant in the parametric analysis (P=0.49, t-test) but significant in the nonparametric test (P=0.049, K-S test). Thus, these results, at least, suggest that postnatal EGF administration enhances sensitivity of the I_h-positive dopaminergic neurons to an excitatory neurotransmitter in mouse VTA.

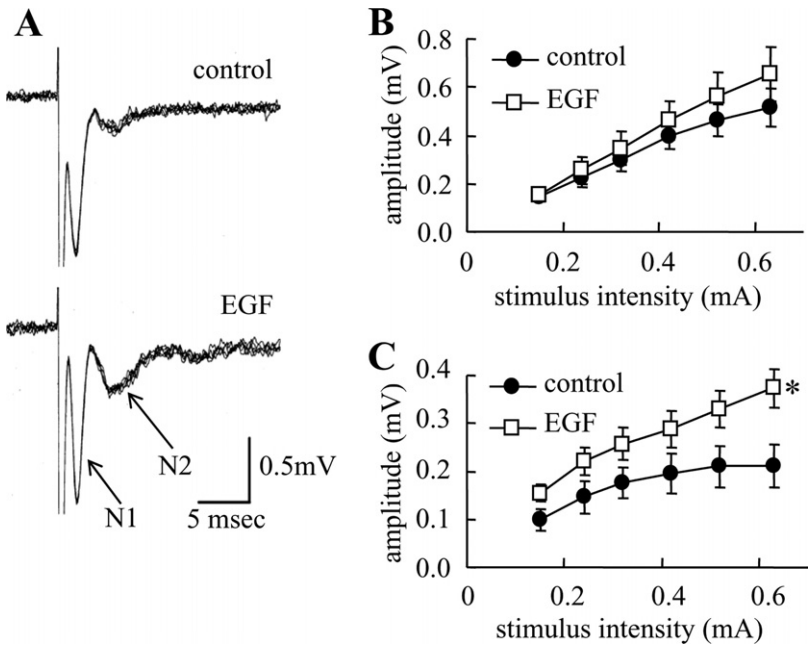

Fig. 2. Amplitude of the EPSP component of field potentials in the VTA. EGF (0.875 μg/g s.c.) was injected daily into the neonatal mice from P2 to P15. Midbrain slices were prepared at P15-17 (A). Representative traces of field potentials recorded in horizontal slices. Two negative components that represent the fiber volley (N1) and synaptic potential (N2), were monitored in the presence of 20 μM bicuculline (Zheng et al., 2006; Nugent et al., 2008). Five responses to electrical stimuli (0.62 mA) are superimposed for display. EGF administration increases the amplitude of the N2 component (C) without affecting the N1 component (B) (control: n=10 slices from six animals; EGF: n=9 slices from five animals). * P<0.05, t-test.

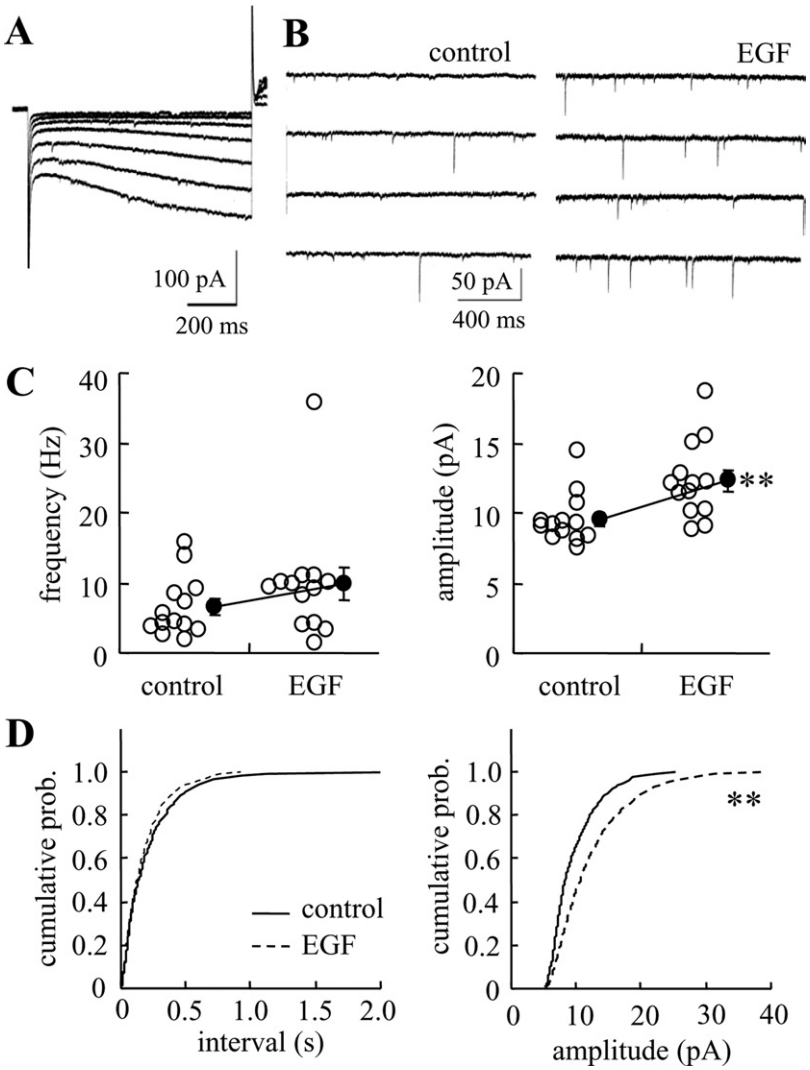

Fig. 3. Amplitudes and frequencies of mEPSCs in putative dopaminergic neurons. (A) Putative dopaminergic neurons were identified by their characteristic I_h (>40 pA). Hyperpolarizing voltage steps were applied with −10 mV increments from −69 mV to −139 mV. I_h-positive neurons in the VTA were subjected to whole cell patch clump recording. (B) Typical traces of mEPSCs for EGF-treated and vehicle-treated mice were displayed. (C) One hundred twenty miniature events were pooled and analyzed for each putative dopaminergic neuron. The mean amplitude and frequency of mEPSCs were calculated in each cell and plotted (control: n=13 cells from seven animals; EGF: n=13 cells from eight animals). ** P<0.001, t-test. (D) Averaged cumulative histograms of inter-event intervals and amplitudes of mEPSCs were calculated and plotted with the same synaptic events for EGF-treated group and for saline-treated controls. ** P<0.001, K-S test.

To estimate the number of functional glutamate receptors at synapses of dopaminergic neurons, we determined the AMPA/NMDA ratio in the I_h-positive neurons (Fig. 5A). Excitatory synaptic currents were evoked by stimulating the anterior region of the VTA. Whole cell recording was carried out at different holding potentials in the presence of 20 μM bicuculline. The AMPA/NMDA ratio was significantly increased in the I_h-positive dopaminergic neurons of EGF-administered animals (P= 0.045, t-test) (Fig. 5B). In addition, we measured the paired pulse ratio to assess alterations in presynaptic function. Consistent with the previous report (Bonci and Malenka, 1999), the majority of electrophysiologically identified dopaminergic neurons exhibited synaptic depression with paired pulse stimulation separated by 20–100 ms intervals (control: six of seven cells; EGF: five of seven cells) (Fig. 5C). The paired pulse ratios were not affected by EGF administration (P=0.3; two-way repeated measures ANOVA) (Fig. 5D). Therefore, EGF administration appeared to influence postsynaptic components rather than presynaptic function of the excitatory afferents to dopaminergic neurons.

EGF administration altered the expression of ionotropic glutamate receptors in the VTA

In the VTA, dopaminergic neurons express mRNAs for the AMPA-type glutamate receptor subunits GluR1 and GluR2/3 as well as the NMDA-type glutamate receptor subunits NR1, NR2A and NR2B (Paquet et al., 1997; Chen et al.,

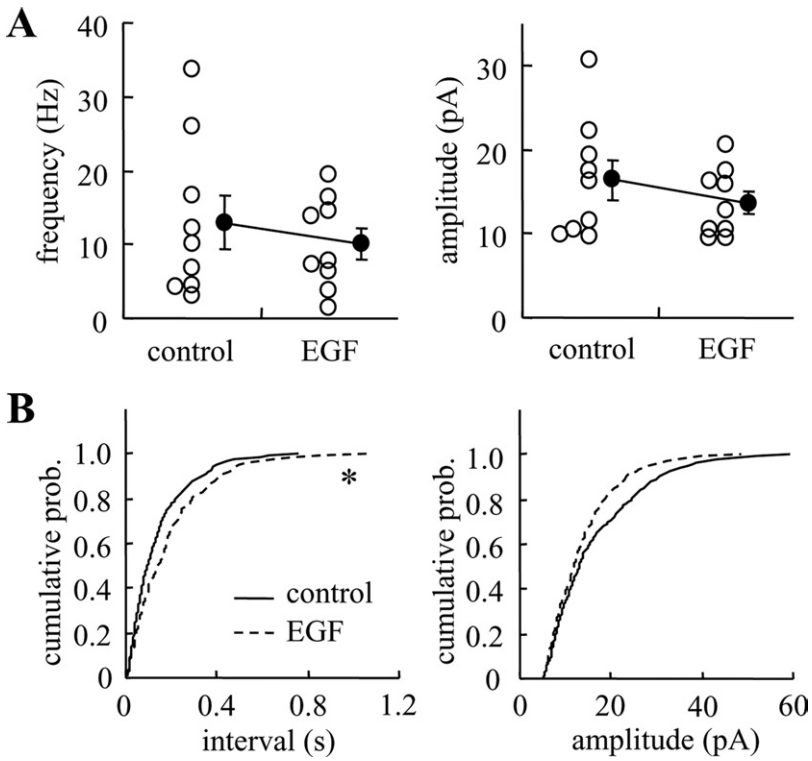

Fig. 4. Amplitudes and frequencies of mEPSCs in I_h-negative cells. One hundred twenty miniature events were pooled and analyzed for each I_h-negative cell. (A) The mean amplitude and frequency of mEPSCs were calculated in each cell and plotted (control: $n=9$ cells from six animals; EGF: $n=9$ cells from seven animals). *t*-test detected no significant differences. (B) Averaged cumulative histograms of inter-event intervals and amplitudes of mEPSCs were calculated and plotted with the same synaptic events for EGF-treated group and for saline-treated controls. * $P<0.05$, K-S test.

2001; Yung, 1998). In contrast, the GluR4 AMPA receptor subunit is mainly expressed in neighboring GABAergic neurons (Paquet et al., 1997).

To examine the possibility that EGF administration influenced the expression of these glutamate receptor subunits, we determined the protein levels of AMPA and NMDA receptor subunits. Tissue containing the whole VTA and medial SNc was dissected from horizontal slices under a stereomicroscope and subjected to immunoblotting. We observed a statistically significant increase in the levels of the GluR1 subunit in animals receiving EGF ($P=0.015$, *t*-test) (Fig. 6). GluR2/3 protein displayed a trend toward greater levels in EGF-treated animals, but did not reach statistical significance ($P=0.067$). In contrast, GluR4 levels were not affected ($P=0.35$). Protein levels of the NR1 NMDA receptor subunit were significantly elevated ($P=0.030$), whereas NR2A and NR2B levels were not affected (NR2A, $P=0.35$; NR2B, $P=0.52$). In addition to the postsynaptic receptors, we examined the presynaptic markers synapsin I and synaptophysin. EGF administration had no effects on protein levels of these presynaptic molecules as well as a neuronal marker, NSE and an astrocyte marker, GFAP. These results suggest that synaptic potentiation following EGF administration might result from an increase in AMPA receptor expression in dopaminergic neurons.

DISCUSSION

In the present study, we investigated subchronic influences of peripherally administered EGF on electrophysiological property of midbrain dopaminergic neurons in postnatal mice. Repeated administration of EGF enhanced excitatory synaptic transmission onto I_h-positive dopaminergic neurons in the VTA. Increases in the amplitude of mEPSCs and the AMPA/NMDA ratio were consistent with an increase in CNQX-sensitive potentials observed in field recording. In addition, we observed an elevation in the protein expression of the AMPA receptor subunit GluR1 and the NMDA receptor subunit NR1 in the ventral midbrain region. The elevation of glutamate receptor expression may underlie the increase in strength of synaptic inputs onto I_h-positive dopaminergic neurons.

In general, neurotrophic polypeptides exhibit distinct activities in acute and chronic phases (Patterson and Nawa, 1993). The acute effects mainly depend on intracellular signaling while chronic effects involve gene expression. Previous studies on hippocampal synaptic plasticity report the rapid synaptic responses to EGF. Acute application of EGF facilitates the induction of long term potentiation or enhances its magnitude (Ishiyama et al., 1991; Abe et al., 1991; Abe and Saito, 1992). Our preliminary study, however, indicated that acute EGF application to midbrain slice preparations failed to affect mEPSC (data not shown). Thus, the observed biological activity of EGF

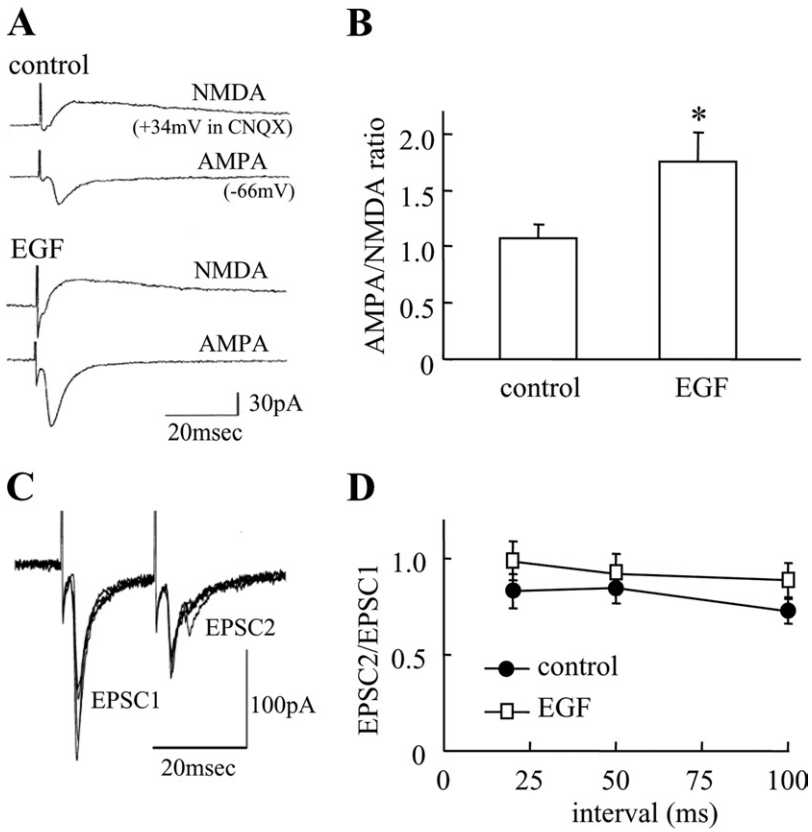

Fig. 5. Effects of EGF on the AMPA/NMDA ratio of EPSCs evoked in putative dopaminergic neurons. (A) Averaged traces for 10 synaptic responses are shown. Synaptic responses were triggered by electrical stimulation (0.05–0.2 mA, 0.067 Hz). AMPA currents were recorded at a holding potential of −66 mV. NMDA currents were recorded at a holding potential of 34 mV in the presence of 10 μM CNQX. (B) The AMPA/NMDA ratio was calculated for each cell and plotted as the means±S.E.M (control: n=7 cells from five animals; EGF, n=8 cells from six animals). * P<0.05, t-test. (C) Typical responses of paired pulse inhibition of evoked EPSCs are shown for display. Four superimposed traces at the interval of 20 ms were recorded from control I_h-positive cells. Paired pulse ratios (peak amplitude of EPSC2/peak amplitude of EPSC1) were calculated from four averaged responses. (D) Paired pulse ratios at each stimulus interval (20, 50, 100 ms) are calculated and plotted as the means±S.E.M (control: n=7 cells from five animals; EGF, n=7 cells from four animals).

presumably represents the slow neurotrophic action of EGF that regulates dopaminergic development.

In our experimental paradigm, dopaminergic neurons were identified by their electrophysiological property of the I_h currents. The recorded cells were localized in the anterior region of VTA in the horizontal slices (Ford et al., 2006). Accumulated evidence suggests that, in this brain region, I_h currents serve as a specific electrophysiological maker for dopaminergic neurons as identified with tyrosine hydroxylase immunoreactivity (Ford et al., 2006; Neuhoff et al., 2002; Johnson and North, 1992; Wanat et al., 2008; but see also Margolis et al., 2006). In agreement, we also found that more than 70% of I_h-positive cells showed tyrosine hydroxylase immunoreactivity in post-fixed slice preparations (data not shown). Although we cannot fully rule out the possibility that some of the I_h-positive cells, in parts, represented non-dopaminergic cells (Margolis et al., 2006), it is likely that the I_h-positive cells not carrying tyrosine hydroxylase immunoreactivity were produced by cell dialysis of a patch pipette during recording. Thus, we considered that the electrophysiological property of I_h-positive cells mainly represents that of dopaminergic neurons in the VTA.

Whole cell recording revealed cell specificity of the postsynaptic action of EGF. Synaptic facilitation triggered by EGF was limited to the dopaminergic neurons exhibiting prominent I_h currents. No significant differences were detected in either the amplitude or frequency of mEPSCs in the I_h-negative neurons. In this context, immunoblotting might support the specificity of the neurotrophic effects of EGF on dopaminergic neurons. In the midbrain, mRNA and protein for GluR1, GluR2/3 and NR1 are detectable in tyrosine hydroxylase–positive dopaminergic neurons, whereas GluR4 is expressed only in GABAergic neurons (Paquet et al., 1997; Chen et al., 2001; Yung, 1998). The evidence that GluR4 levels were not affected in mice receiving subchronic administration of EGF may suggest insensitivity of the GABAergic population to EGF. In contrast, an influence of EGF stimulation on afferent fibers to midbrain dopaminergic neurons was relatively limited. Subchronic EGF administration did not influence mEPSC frequency or the paired pulse ratio in putative dopaminergic neurons. Furthermore,

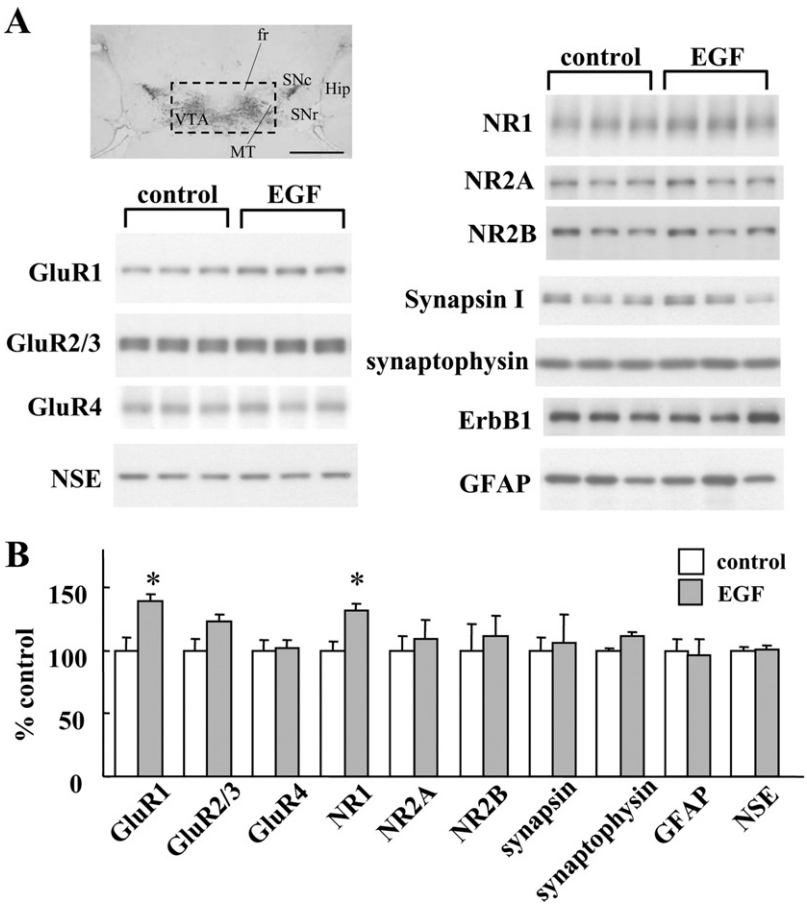

Fig. 6. Protein expression of glutamate receptor subunits in the VTA. After daily injection of saline or EGF, brain tissue around the VTA including SNc was dissected at P15 as shown in the schematic outline of the midbrain immunostained with the anti–tyrosine hydroxylase antibody (A). This inset in A includes fr, MT, hippocampus (Hip), and VTA. Scale bar=1 mm for inset in A. Immunoblots of 5 or 20 μg protein were probed with antibodies raised against AMPA receptor subunits (GluR1, GluR2/3, and GluR4) and NMDA receptor subunits (NR1, NR2A, and NR2B). Immunoreactivity for the presynaptic proteins synapsin I and synaptophysin, the neuronal marker NSE, the astrocyte marker GFAP, and ErbB1, was examined as well. Representative immunoblots are displayed. (B) Levels of immunoreactivity were measured by densitometry (n=5, each represents a pooled sample of two mice). Results are all normalized to protein levels in controls (100%) and plotted. * $P<0.05$, t-test.

the expression of the presynaptic proteins synapsin I and synaptophysin was not affected by EGF. Thus, EGF has no apparent influences on neurotransmitter release from afferent terminals in the VTA.

In situ hybridization reveals the expression of ErbB1 mRNA in rat midbrain (Seroogy et al., 1994; Kornblum et al., 1997). EGF circulating in peripheral blood can cross the blood–brain barrier (Pan and Kastin, 1999; Kastin et al., 1999) and activate ErbB1 in the brain (Futamura et al., 2003). In particular, the blood–brain barrier is leaky during early postnatal stage of rodents when the blood–brain barrier is not fully established (Tohmi et al., 2007). In agreement, biotinylated EGF efficiently penetrated the blood–brain barrier of neonatal mice and reached the midbrain region. Peripherally administered EGF also triggered phosphorylation of ErbB1 as well as that of ErbB2 in the ventral midbrain tissue. It is possible that ErbB1 forms hetero-oligomers with ErbB2 in this brain region (Leahy, 2004) and phosphorylates ErbB2 (Fox and Kornblum, 2005; Gerecke et al., 2001). These results illustrate that circulating EGF must have significant impact on the midbrain dopaminergic system, at least, during early postnatal development.

Rodents treated with EGF as neonates exhibit schizophrenia-like behavioral abnormalities in prepulse inhibition, exploratory motor activity, and social interaction at the adult stages (Futamura et al., 2003; Tohmi et al., 2005; Sotoyama et al., 2007). These behavioral abnormalities induced by EGF may in part result from the alteration in the dopaminergic system (Sotoyama et al., 2007). Consistent with these observations, EGF-treated rats exhibits higher sensitivity to cocaine, which enhances glutamatergic neurotransmission to dopamine neurons (Ungless et al., 2001; Zhang et al., 1997; Mizuno et al., 2004). Cocaine facilitates AMPA receptor-mediated transmission in the VTA (Zhang et al., 1997; Ungless et al., 2001), enhances agonist-induced burst firing, and dopamine release in the nucleus accumbens, leading to behavioral and cognitive impairments (Tong et al., 1995; Giorgetti et al., 2001). The present findings suggest that neonatal EGF treatment may mimic the pharmacological action of cocaine.

The present synaptic effects of EGF on dopaminergic neurons appear not to be persistent, however. At P30, when 2 weeks passed after completion of EGF administration, there were no detectable influences remaining in the synaptic properties of dopaminergic neurons as well as in glutamate receptor expressions in the midbrain (H. Nawa, unpublished observations). In this context, further studies should determine how the change in the synaptic properties of dopaminergic neurons leads to the behavioral abnormalities at the adult stage.

Acknowledgments—This work was supported by the Basic Scientific Research (B), the Health and Labor Sciences Research Grants, a grant for Promotion of Niigata University Research Projects, and Core Research for Evolutional Science and Technology from the JST Corporation.

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(Accepted 31 October 2008)
(Available online 7 November 2008)