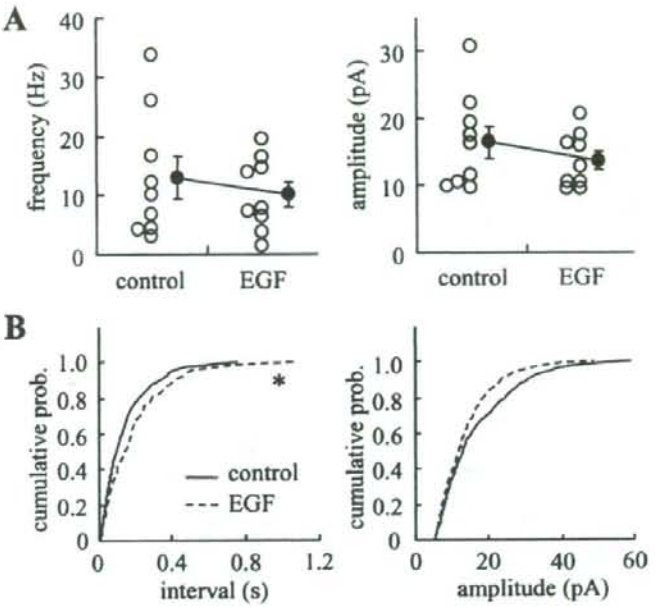

Fig. 4. Amplitudes and frequencies of mEPSCs in I_h-negative cells. One hundred twenty miniature events were pooled and analyzed for each I_h-negative cell. (A) The mean amplitude and frequency of mEPSCs were calculated in each cell and plotted (control: $n=9$ cells from six animals; EGF: $n=9$ cells 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

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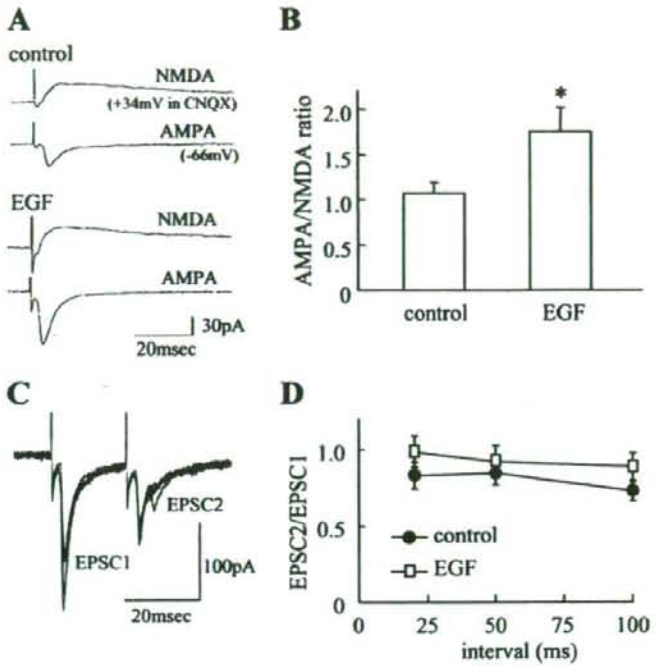

Fig. 5. Effects of EGF on the AMPA/NMDA ratio of EPSCs evoked in putative dopaminergic neurons. (A) Averaged traces for 10 synaptic responses are shown. Synaptic responses were triggered by electrical stimulation (0.05–0.2 mA, 0.067 Hz). AMPA currents were recorded at a holding potential of −66 mV. NMDA 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,

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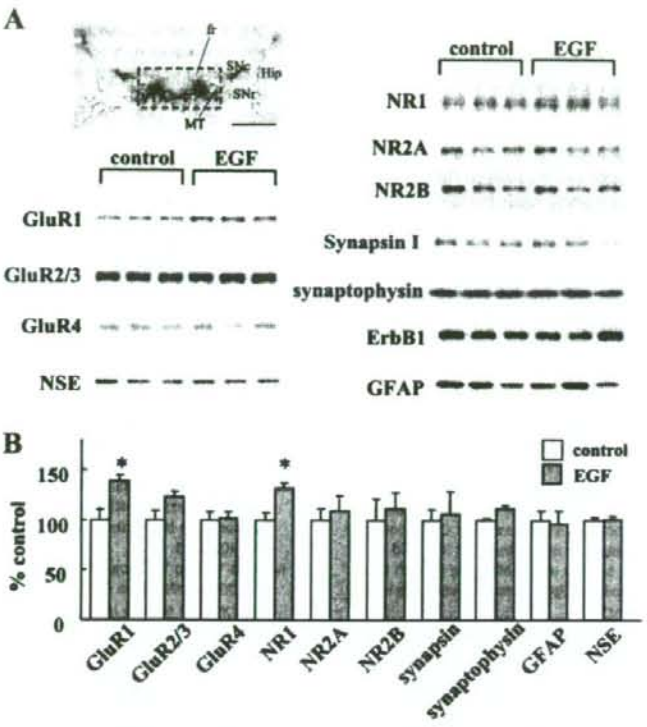

Fig. 6. Protein expression of glutamate receptor subunits in the VTA. After daily injection of saline or EGF, brain tissue around the VTA including SNc was dissected at P15 as shown in the schematic outline of the midbrain immunostained with the anti–tyrosine hydroxylase antibody (A). This inset in A 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.

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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.

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