ELSEVIER

MCN Molecular and Cellular Neuroscience

www.elsevier.com/locate/ymcne
Mol. Cell. Neurosci. 26 (2004) 418–428

Disruption of EphA/ephrin-A signaling in the nigrostriatal system reduces dopaminergic innervation and dissociates behavioral responses to amphetamine and cocaine

Beth-Anne Sieber,[a,1] Alexander Kuzmin,[b] Josep M. Canals,[c] Anne Danielsson,[a] Gustavo Paratcha,[a] Ernest Arenas,[d] Jordi Alberch,[c] Sven Ove Ögren,[b] and Carlos F. Ibáñez[a,*]

[a] *Division of Molecular Neurobiology, Department of Neuroscience, Karolinska Institute, Stockholm, Sweden*
[b] *Division of Neurophysiology and Behavior, Department of Neuroscience, Karolinska Institute, Stockholm, Sweden*
[c] *Departament de Biologia Celular i Anatomia Patològica, Facultat de Medicina, IDIBAPS, Universitat de Barcelona, Barcelona, Spain*
[d] *Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden*

Received 7 March 2004; accepted 25 March 2004
Available online 28 May 2004

We have investigated functional roles of EphA/ephrin-A signaling in the development and function of the nigrostriatal system by overexpressing a soluble, broad-range EphA receptor antagonist in the central nervous system of transgenic mice. Adult transgenic mice showed a 30–40% reduction in the total volume of the substantia nigra (SN) without detectable differences in the number of dopaminergic neurons. Using fluorogold retrograde tracing from the striatum, we detected a 40–50% reduction in the number of dopaminergic neurons that could be traced from this structure in transgenic mice, suggesting that, a lower proportion of these cells were able to reach the striatum after disruption of EphA/ephrin-A signaling. In spite of this, total dopamine content in the striatum of transgenic mice was comparable to wild type. Analysis of locomotor activity and its regulation by pharmacological treatments that stimulate dopaminergic transmission revealed an unexpected dissociation of the behavioral responses to amphetamine and cocaine. In particular, transgenic mice were relatively insensitive to amphetamine while retaining normal responsiveness to cocaine, which, to the best of our knowledge, represents the first report of a dissociation of the behavioral responses to these two psychostimulants. Together, these results reveal an unexpected role for EphA/ephrin-A signaling in the normal connectivity and function of midbrain dopaminergic neurons.
© 2004 Elsevier Inc. All rights reserved.

Introduction

During development of the nervous system, stereotypic patterns of axonal growth and target innervation are generated under the influence of specific signals from the extracellular environment. An array of soluble and immobilized molecular cues are deployed in highly refined spatiotemporal patterns to guide and direct axonal growth, target invasion and connectivity. Many of the same signals persist at later stages of nervous system development and into adulthood, suggesting additional roles in maintenance and plasticity.

The Eph receptor tyrosine kinases and their ligands, the ephrins, play prominent roles in the control of axon guidance and target innervation (Dodelet and Pasquale, 2000; Flanagan and Vanderhaeghen, 1998; Frisen et al., 1999; Holder and Klein, 1999; Lemke, 1998; O'Leary and Wilkinson, 1999; Wilkinson, 2001). Fourteen Eph receptors and eight ephrins are known in vertebrates, making this one of the most diversified ligand–receptor systems and therefore well suited to provide specificity during axon guidance. Eph receptors and ephrins comprise two major specificity subclasses: EphA receptors bind GPI-anchored ephrin-A ligands, and EphB receptors bind transmembrane ephrin-B ligands. Both receptor and ligand need to be membrane anchored for transmembrane signaling to occur, a property that allows Eph/ephrins to provide local guidance cues through direct cell–cell contact. In fact, soluble forms of either ligand or receptor lack intrinsic biological activity and have been shown to function as antagonists (Krull et al., 1997; Gao et al., 1998; Lackmann et al., 1998). Eph receptors and ephrins are often, although not always, expressed in complementary graded patterns in different regions of the nervous system (Gale et al., 1996; Zhang et al., 1996). The presence of complementary ligand–receptor gradients is believed to encode positional information for topographic connectivity such as in the retinotectal (Cheng et al., 1995; Drescher et al., 1995) and hippocamposeptal (Gao et al., 1996) systems. Although most studies have so far highlighted the ability of Eph/ephrins to promote short-range repulsive interactions, a few examples of their ability to enhance cell adhesion have also been reported (Holmberg and Frisen, 2002). Interestingly, analyses of loss-of-function animal models of Eph and ephrin signaling have identified both cell-autonomous and non-cell-autonomous functions for receptors and ligands (Davy et al., 1999; Holland et al., 1996; Klein, 1999; Knoll and Drescher, 2002). Combined with biochemical studies, this

* Corresponding author. Division of Molecular Neurobiology, Department of Neuroscience, Karolinska Institute, Berzelius vag 1, 17177, Stockholm, Sweden. Fax: +46-8-33-95-48.
E-mail address: carlos.ibanez@neuro.ki.se (C.F. Ibáñez).
[1] Present address: National Institute of Mental Health, MCN/DNBBS, Bethesda, MD, USA.
Available online on ScienceDirect (www.sciencedirect.com.)

1044-7431/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2004.03.009

work has demonstrated that Eph/ephrin interactions trigger bidirectional signaling in the two interacting cells, suggesting a complex web of intercellular communication underlying axonal pathfinding via this signaling system.

In addition to its effects on axon guidance and migration, recent evidence suggests a role for Eph/ephrin signaling in the regulation of neurotransmission and neuronal plasticity. Using a soluble EphA5 construct as a receptor antagonist and clustered ephrin-A5 as an agonist, Gao et al. (1998) showed that EphA/ephrin-A signaling is required for the induction of long-term potentiation (LTP) in hippocampal slices. Blockade of EphA signaling affected LTP induction but not basal synaptic transmission. Intriguingly, however, activation using clustered ephrin-A5 induced a sustained increase in normal synaptic transmission that mimicked LTP (Gao et al., 1998). More recently, Dalva et al. (2000) and Takasu et al. (2002) reported a physical and functional interaction between EphB and NMDA glutamate receptors with important consequences for the excitability and function of hippocampal neurons. These and other studies have raised the possibility that Eph/ephrin signaling contributes not only to the development but also to the function of the nervous system by regulating synaptic plasticity.

Ventral midbrain dopaminergic neurons of the substantia nigra and ventral tegmental area project to the forebrain via several pathways. Substantia nigra dopaminergic neurons project to the caudate putamen (dorsolateral striatum), whereas ventral tegmental neurons project to the nucleus accumbens (ventromedial striatum) and to the cerebral cortex. The molecular mechanisms directing the development and maintenance of these pathways are still not defined. Within the Eph/ephrin system, expression of two Eph receptors, EphA5 and EphB1, has been observed in dopaminergic neurons of the ventral mesencephalon. Expression of EphB1 appears to be predominant in substantia nigra dopaminergic neurons but low or absent in cells of the ventral tegmental area (Yue et al., 1999). In dissociated cultures, ephrin-B2 (a ligand of EphB1) behaved as a negative regulator of neurite outgrowth and neuronal survival of substantia nigra neurons (Yue et al., 1999). The preferential expression of ephrin-B2 in the nucleus accumbens, from which substantia nigra axons are normally excluded, indicated that this ligand/receptor pair may help to confine ascending dopaminergic pathways from the substantia nigra to the caudate putamen. EphA5 expression has been detected in dopaminergic neurons of both the substantia nigra and ventral tegmental area (Maisonpierre et al., 1993; Yue et al., 1999), while expression of ephrin-A5 (a ligand of EphA5) has been detected in cells of the caudate putamen (Renping Zhou, personal communication). The possible roles of EphA5 and ephrin-A ligands in the development and function of the nigrostriatal system still remain unexplored.

In this study, we set out to investigate functional roles of EphA/ephrin-A signaling in the development and function of the nigrostriatal system using a soluble EphA5 receptor construct as a broad-range antagonist of this subclass of ephrins. Since EphA5 is able to interact with all ephrin-A ligands (Gale et al., 1996), overexpres-

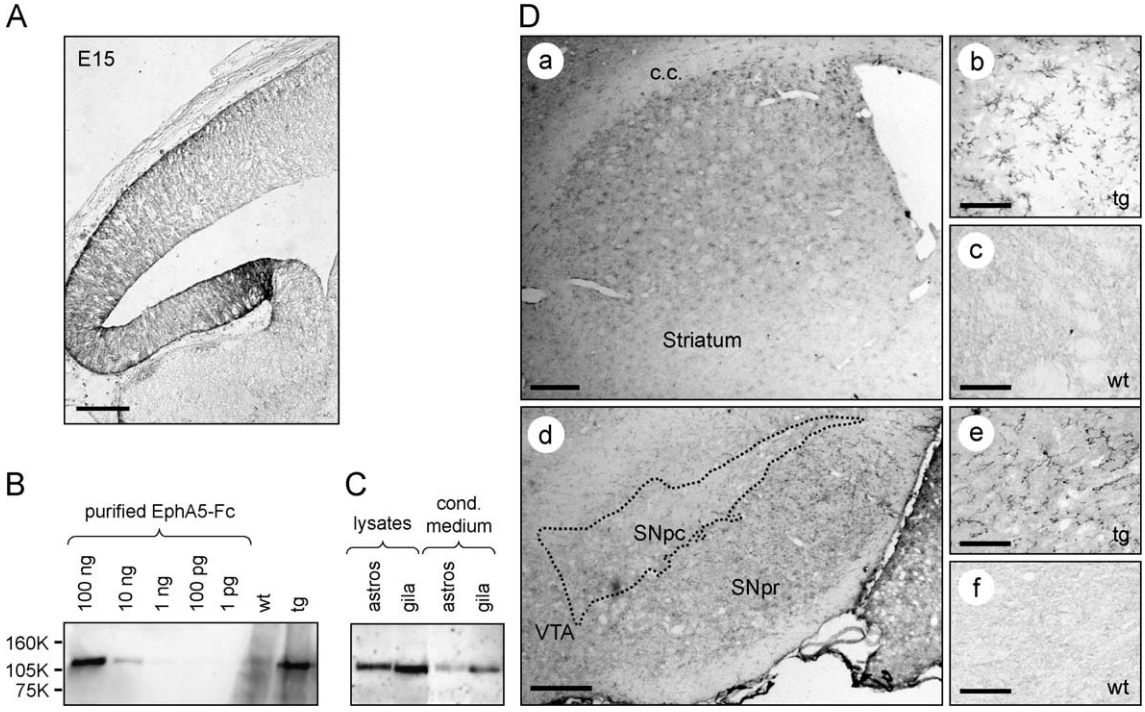

Fig. 1. Characterization of the expression of the GFAP-EphA5Fc transgene. (A) Expression of the EphA5Fc transgene in the brain of an embryonic day 15 (E15) transgenic mouse embryo. The sagittal section shows strong immunostaining for the product of the transgene in the tectum. (B) Immunoblot of total brain extracts of wild type (wt) and transgenic (tg) mice compared to standards of purified recombinant EphA5-Fc protein. Lanes 1–5, purified EphA5-Fc; lane 6, 1 mg wild-type postnatal day 3 (P3) brain extract; lane 7, 1 mg transgenic line 3083 P3 brain extract. The EphA5-Fc fusion protein runs at about 100 K. (C) Immunoblot of lysates and conditioned medium of total glial and purified astrocyte cell cultures extracted from P1 transgenic brains. (D) Detection of EphA5Fc expression on adult brain of transgenic mice. (a) Immunoreactivity is observed in the striatum of transgenic animals. (b) At high magnification, the cells show an astrocytic morphology. (c) No signal is observed in wild-type animals. (d) In the substantia nigra (SN), higher levels of expression are observed in the pars reticulata (SNpr) than in the pars compacta (SNpc) subregions. (e) High magnification showing immunoreactive cells with astrocytic morphology. (f) Wild-type animals showed no immunoreactivity. Scale bars: a, d, 200 μm; b–c, e–f, 50 μm.

sion of soluble EphA5 is expected to have a broad antagonistic effect on the activities of all members of the A subclass of ephrins. We found that this manipulation affected the pattern of striatal innervation by substantia nigra dopaminergic neurons and caused an unexpected dissociation of behavioral responses to the psychostimulants amphetamine and cocaine.

Results

Generation of transgenic mice overexpressing a soluble form of the EphA5 receptor and characterization of transgene expression

Broad CNS-specific overexpression of a transgene encompassing the extracellular domain of the rat EphA5 receptor fused to the Fc domain of human IgG (EphA5Fc) was obtained using the promoter and regulatory sequences of the human glial fibrillary acidic protein (GFAP) gene, a specific astrocytic marker. Previous studies in transgenic mice using the *lacZ* reporter gene have shown that this promoter becomes activated at embryonic day (E) 13 of development. Its activity increases throughout gestation and early postnatal stages, and levels off in adult stages at relatively high levels (Brenner and Messing, 1996; Brenner et al., 1994). Several independent lines of EphA5Fc overexpressors were obtained based on analyses of transgene mRNA expression by RNase protection assay (data not shown). Two of these, termed 3083 and 3114, respectively, were subsequently bred to obtain mice that were homozygous for the transgene. Transgene mRNA expression could first be detected during the last week of embryonic development (Fig. 1A), with increasing levels during postnatal and adult stages. EphA5Fc protein expression was estimated by Western blotting to be in excess of 100 ng per milligram of total brain protein for the 3083 line (Fig. 1B). Somewhat lower levels were produced by the 3114 line (data not shown). EphA5Fc protein was present in total cell lysates of mixed glial cultures and purified Type 1 astrocytes prepared from cortices of postnatal day (P) 1 transgenic pups (Fig. 1C). Abundant expression was also seen in the conditioned medium of 3-day-old cultures (Fig. 1C), indicating release of the EphA5Fc fusion protein by Type 1 astrocytes. Using antibodies directed against the human Ig portion of the overexpressed protein, EphA5Fc immunoreactivity could be detected in both striatum and substantia nigra (Figs. 1Da and d) on cells with astrocytic morphology (Figs. 1Db and e). No immunoreactivity was observed in sections from wild-type mice (Figs. 1Dc and f).

The overall level of activation of EphA5 receptors in wild type and transgenic mice was assessed by immunoprecipitation from lysates of synaptic membranes followed by immunoblotting with anti-phosphotyrosine antibodies. Decreased levels of tyrosine phosphorylation of EphA5 receptors could be observed in the brain of transgenic mice (line 3114) at both postnatal day (P) 4 and P14 compared to wild-type littermate controls (Figs. 2A and B). This result indicated that overexpression of the EphA5Fc protein reduced signaling by endogenous EphA5 receptors in the brain of transgenic mice.

Reduced volume but normal number of dopaminergic neurons in the substantia nigra of GFAP-EphA5Fc transgenic mice

Dopaminergic neurons in the substantia nigra pars compacta (SNpc) of adult transgenic mice were examined by tyrosine hydroxylase (TH) immunohistochemistry (Figs. 3A–C). A reduction in the volume of the SNpc was observed in both lines of transgenic mice. Analyses of serial sections revealed that SNpc volume was reduced by 28% and 42% in the 3083 and 3114 transgenic lines, respectively, compared to wild-type controls (Fig. 3D). Despite this reduction in volume, no difference could be observed in the number of TH+ dopaminergic neurons in the SNpc of adult transgenic mice compared to wild type (Fig. 3E), indicating that the reduction in volume was not due to loss of dopaminergic neurons, but may have been caused by a reduction in the number of non-dopaminergic neurons or glial cells.

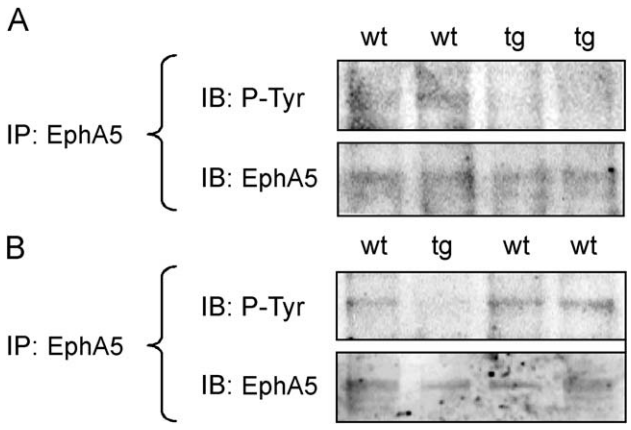

Fig. 2. Decreased tyrosine phosphorylation of endogenous EphA5 receptors in the brain of transgenic mice. Lysates of synaptic membranes were immunoprecipitated (IP) with anti-EphA5 antibodies and subsequently immunoblotted (IB) with anti-phosphotyrosine antibodies. Extracts were obtained from total brains of P4 (A) and P14 (B) transgenic mice from line 3114 (tg) and littermate controls (wt). The panels below show comparable levels of EphA5 in the different samples.

Abnormal dopaminergic innervation of the striatum in GFAP-EphA5Fc transgenic mice

We next investigated whether overexpression of soluble EphA5 affected the pattern of striatal innervation by dopaminergic neurons of the substantia nigra. To this purpose, we injected fluorogold in the striatum of adult wild type and GFAP-EphA5Fc transgenic mice. Retrograde transport of fluorogold was observed and quantified in the substantia nigra. A reduction of fluorogold transport, measured as the number of fluorogold-positiveneurons in the substantia nigra, was observed in both transgenic lines (Fig. 4A). Analyses of serial sections revealed that fluorogold retrograde transport was reduced to 57.7% and 56% of the level of wild type in the 3083 and 3114 transgenic lines, respectively (Fig. 4A). No significant difference could be seen between the two transgenic lines. Double labeling with fluorogold and TH immunohistochemistry demonstrated that only a fraction of dopaminergic neurons were able to transport fluorogold from the striatum in transgenic mice compared to wild type (Fig. 4B).

Although a lower number of dopaminergic neurons appeared to be able to reach the striatum in transgenic mice, total dopamine content was not different from wild type in this brain region. Levels of dopamine in the striatum of adult wild type and GFAP-EphA5Fc transgenic mice (line 3114) were 58.74 ± 4.85 (n = 9) and 79.42 ± 3.85 (n = 7) nmol per gram of tissue, respectively. DOPAC (3,4-dihydroxyphenylacetic acid) levels were 5.38 ± 0.41 in wild type and 5.70 ± 0.62 nmol/g in transgenic, whereas

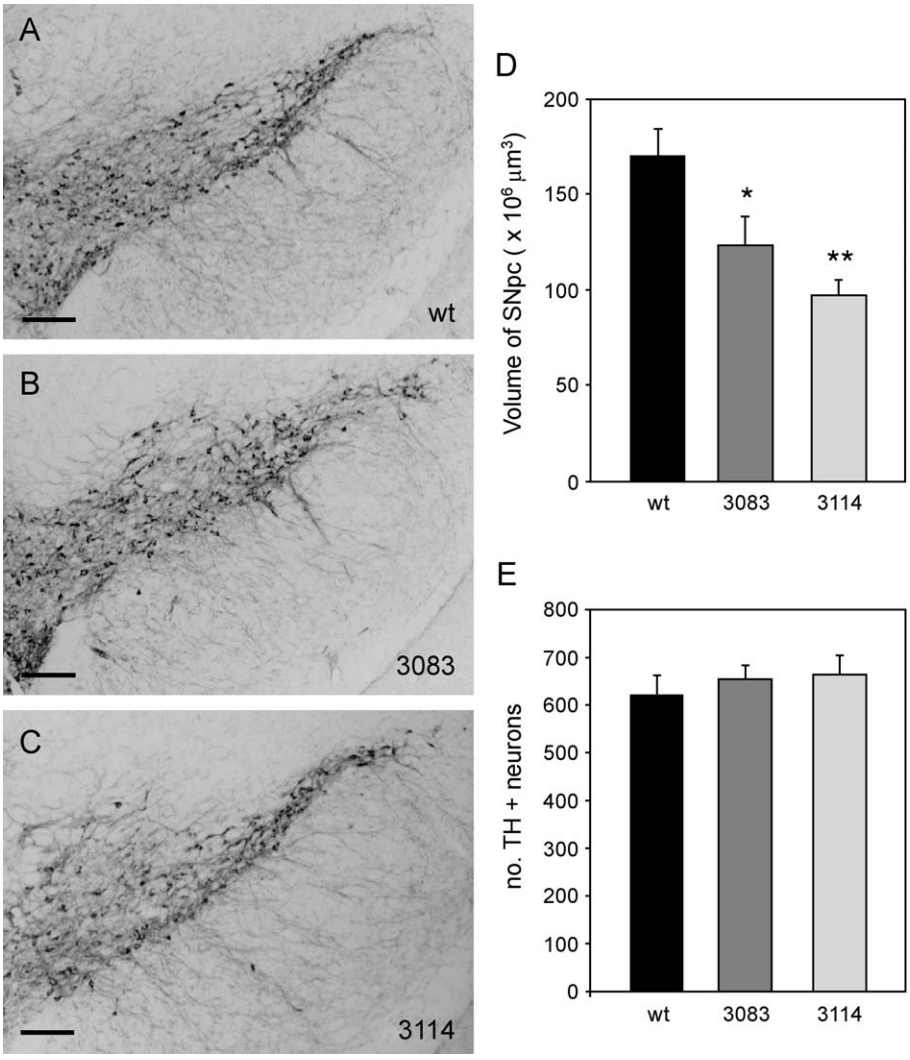

Fig. 3. Reduced volume but normal number of dopaminergic neurons in the substantia nigra of GFAP-EphA5Fc transgenic mice. (A–C) Photomicrographs of TH immunohistochemistry in the substantia nigra pars compacta (SNpc) of wild type (wt), and transgenic lines 3083 and 3114. Scale bars, 200 μm. (D) SNpc volumes (mean ± SEM, four animals per group) in transgenic mice were reduced by 28% (3083 line) and 42% (3114 line). *$P < 0.05$, **$P < 0.001$ vs. wild-type animals (ANOVA, LSD post hoc test). (E) Quantification of the number of TH-positive neurons in the SNpc showed no differences between wild type and transgenic mice. Values represent the mean ± SEM ($n = 4$) of the total number of counted TH-positive somas as described in Experimental methods.

HVA (homovanillic acid) levels were 5.72 ± 0.29 in wild type and 5.77 ± 0.40 nmol/g in transgenic animals. Thus, although a fraction of dopaminergic neurons reached the striatum in transgenic compared to wild-type mice, total dopamine levels in the striatum appeared normal, suggesting a compensation in the innervating neurons.

Normal behavioral responses to cocaine in GFAP-EphA5Fc transgenic mice

The nigrostriatal pathway plays a prominent role in the control of locomotor activity, and several drugs affect these behaviors by directly interacting with molecular and cellular components of dopaminergic neurotransmission. We therefore investigated whether transgene expression had an effect on basal locomotor activity and its regulation by cocaine and amphetamine, two central psychostimulants that are known to mediate motor stimulant effects via increased dopaminergic transmission.

As a control experiment, we first tested basal and drug-induced locomotor activity in the two parental strains of our wild type and GFAP-EphA5Fc transgenic mice, namely, C57BL/6 and CBA. Locomotor activity is a polygenic trait that is known to vary among inbred strains of mice (Kelly et al., 1998). In agreement with previous studies, the two strains differed in basal locomotor activity levels and habituation to the test environment, with the C57BL/6 strain showing consistently higher levels of locomotor activity than CBA (Fig. 5A). Despite these differences, treatment with either cocaine or amphetamine was able to induce similar relative increases in the levels of activity in both strains (Fig. 5A).

Wild type and GFAP-EphA5Fc transgenic mice (3083 line) did not differ in their basal locomotor activity and habituated to the test environment with a similar gradual reduction in rearing, motility and locomotion (Fig. 5B). Low levels of activity were observed after 60 min in the test environment in both transgenic and wild-type animals, indicating comparable habituation. We then tested

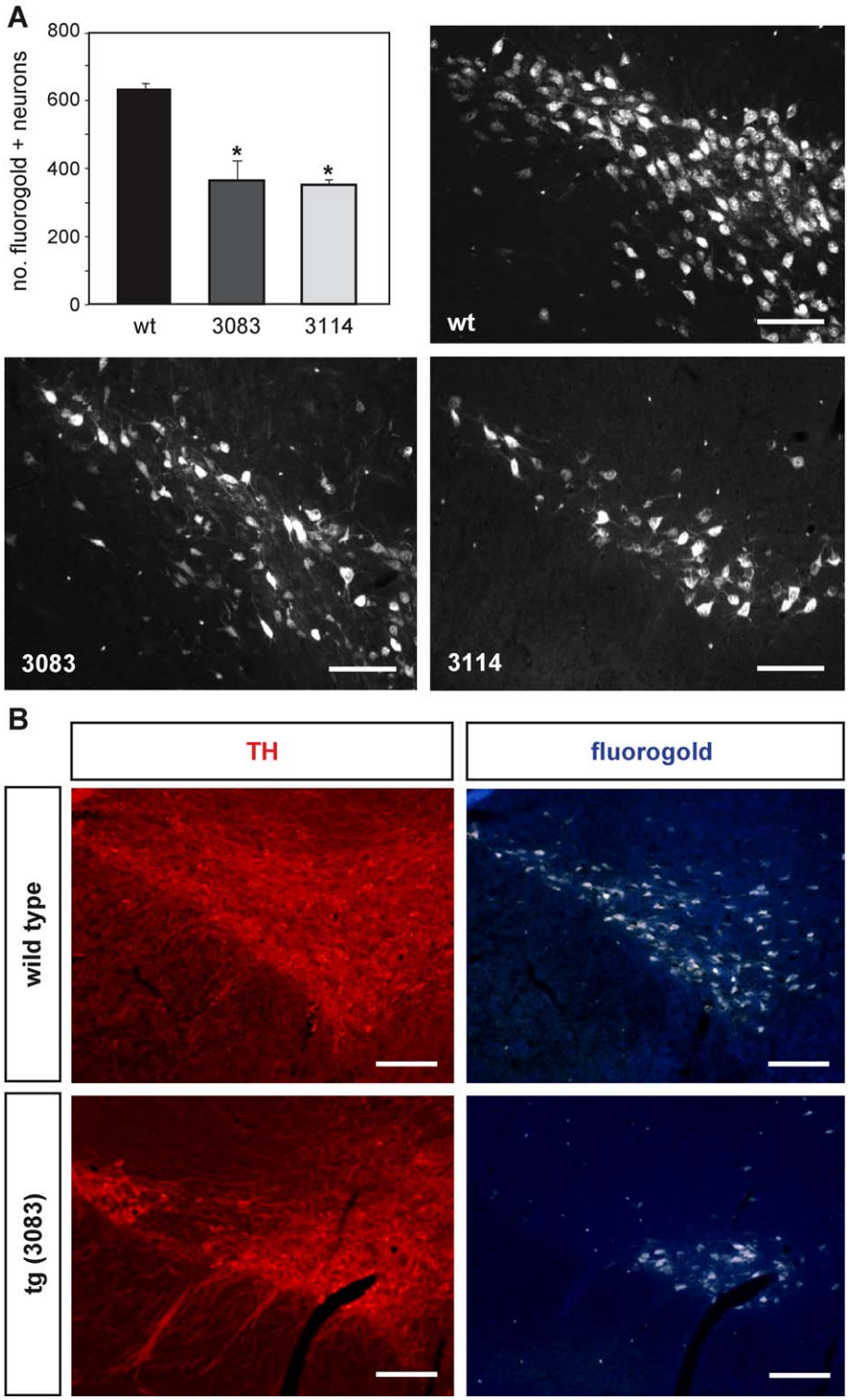

Fig. 4. Reduced dopaminergic innervation of the striatum of GFAP-EphA5Fc transgenic mice. (A) Fluorogold tracer was injected in the striatum, and 2 days later, sections of the substantia nigra were processed for analysis. The histogram shows the quantification of the number of fluorogold-labeled neurons in the substantia nigra ipsilateral to the striatum injected. Values represent the mean ± SEM ($n = 4–6$) of the number of fluorogold-positive cells counted in serial sections through. *$P < 0.0001$ vs. wild type (wt) (ANOVA, LSD post-hoc test). No significant differences were observed between the 3083 and 3114 transgenic lines. Representative photomicrographs of fluorogold transport in the substantia nigra of the three types of animals are also shown. Scale bars, 200 μm. (B) Double labeling with fluorogold and TH immunohistochemistry showing that only a fraction of dopaminergic neurons were able to transport fluorogold from the striatum in transgenic mice (only line 3083 is shown here) compared to wild type. Scale bars, 200 μm.

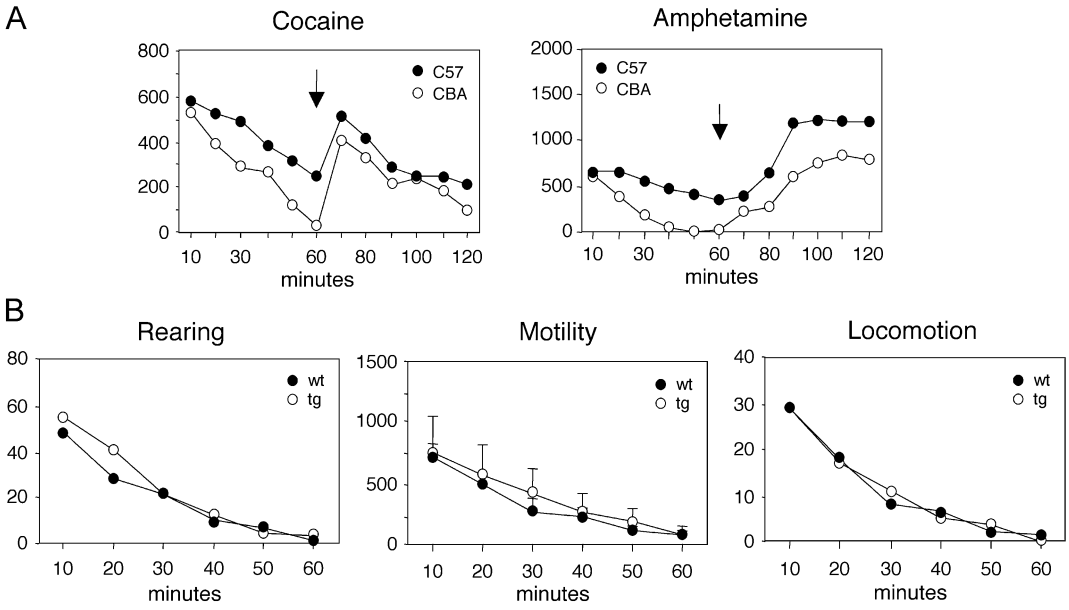

Fig. 5. Behavioral responses in parental lines C57BL/6 and CBA, and basal locomotor activities in wild type and GFAP-EphA5Fc transgenic mice. (A) Motility responses of wild-type C57BL/6 and CBA parental strains to cocaine (5 mg/kg) and amphetamine (1.5 mg/kg) after 60 min of habituation. (B) Wild type (wt) and GFAP-EphA5Fc transgenic mice (3083 line) (tg) did not differ in their basal locomotor activity and habituated to the test environment with a similar gradual reduction in rearing, motility and locomotion. Measurements (in arbitrary units) were taken at 10-min intervals following placement of the animals in the activity monitor cage. Results are expressed as mean ± SEM, $n = 8$. ANOVA confirmed a strong habituation [$P < 0.00001$, $F(5,610) = 228.9$] but no difference between strains nor strain–time interaction ($P = 0.21$ and $P = 0.75$, respectively).

motility responses of wild type and GFAP-EphA5Fc transgenic mice (3083 line) to cocaine and amphetamine. After a period of habituation of 60 min, mice were treated with one of the psychostimulants and their locomotor response recorded for an additional 60 min. Both wild type and GFAP-EphA5Fc transgenic mice responded to cocaine administration with a similar elevated level of motility (Fig. 6). As expected, a greater response was observed at 20 mg/kg than at 5 mg/kg cocaine (Fig. 6). Cocaine-induced

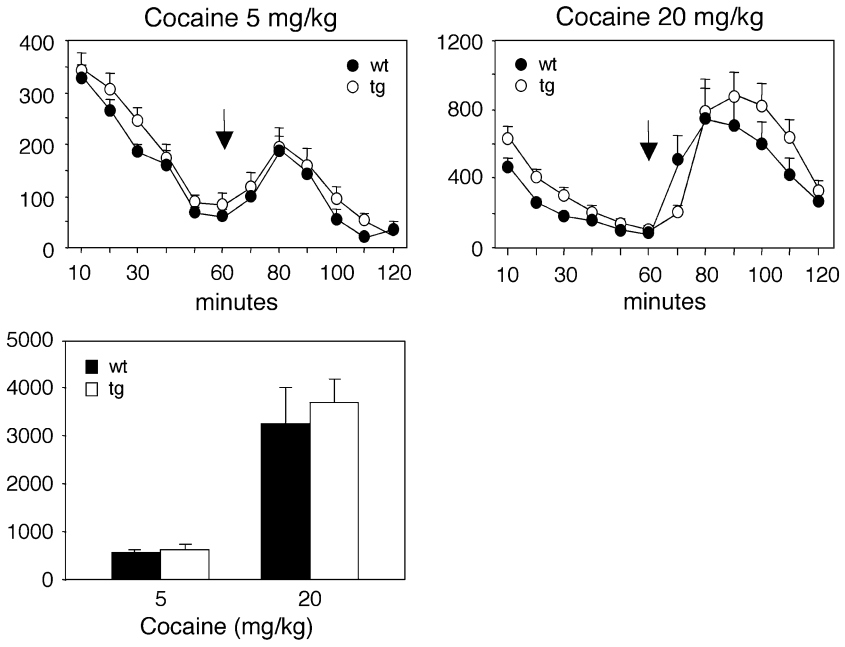

Fig. 6. Normal behavioral responses to cocaine in GFAP-EphA5Fc transgenic mice. Motility responses of wild type and transgenic mice (3083 line) to different doses of cocaine after 60 min of habituation. The drug was administered intraperitoneally after 60 min in the activity monitor cage (arrow). Results are expressed as mean ± SEM, $n = 8$. The histogram below shows a comparison of the compound activities during a 60-min period following drug administration (mean ± SEM). Both strains of mice displayed a dose-related stimulation of motor activity [dose effect: $F(2,25) = 6.3$, $P < 0.05$], but we could not detect an effect of strain ($P = 0.5$) nor strain–dose interaction ($P = 0.61$).

motility reached a peak 30 min after drug administration and declined thereafter. No differences between wild type and transgenic mice could be observed in either the time course or the magnitude of the response to cocaine treatment, measured as the accumulated motility scores during the 60 min following drug administration (Fig. 6). Similar results were obtained for rearing and locomotion (data not shown).

Reduced behavioral responses to amphetamine in GFAP-EphA5Fc transgenic mice

In contrast to the effects observed with cocaine, treatment with amphetamine revealed dramatic differences in the locomotor responses of wild type and GFAP-EphA5Fc transgenic mice. Wild-type animals responded to amphetamine with a typical bell-shaped dose–response curve of accumulated motility scores with maximal stimulation of motor activity at 1.5 and 4.5 mg/kg (Fig. 7A). In contrast, transgenic mice (3083 line) responded only very poorly (4.5 mg/kg) or almost not at all (1.5 mg/ml) to amphetamine administration without any significant dose–response correlation of accumulated motility scores (Fig. 7A). At much higher doses (10 mg/kg), amphetamine treatment produced stereotypy in all animals, which is known to interfere with locomotor activity (Fig. 7A). Similar results were obtained for tests of rearing and locomotion (data not shown). Heterozygous animals carrying only one copy of the GFAP-EphA5Fc transgene presented normal motility responses to amphetamine, comparable to those observed in wild-type mice (Fig. 7B and data not shown). Importantly, animals from the independently derived 3114 transgenic line also failed to adequately respond to amphet-

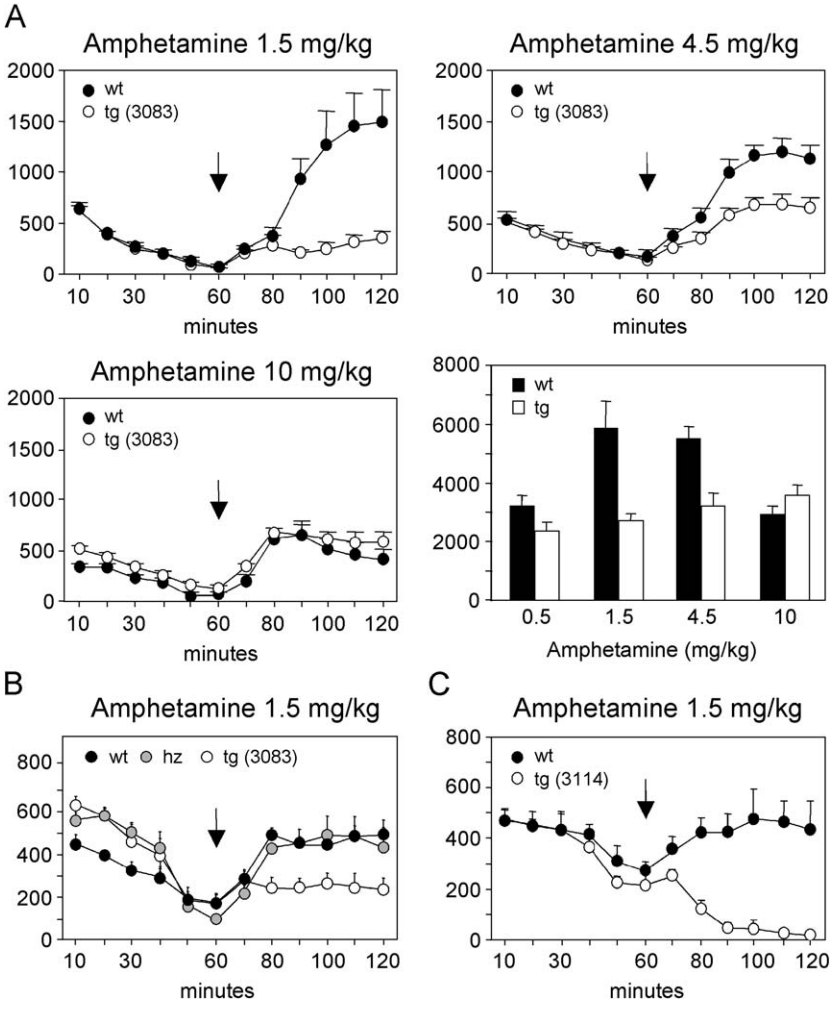

Fig. 7. Reduced behavioral responses to amphetamine in GFAP-EphA5Fc transgenic mice. (A) Motility responses of wild type and transgenic mice (3083 line) to different doses of amphetamine after 60 min of habituation. The drug was administered intraperitoneally after 60 min in the activity monitor cage (arrow). Results are expressed as mean ± SEM, $n = 8$. The histogram below shows a comparison of the compound activities during a 60-min period following drug administration (mean ± SEM). Strong differences between strains were found for the effects of amphetamine by ANOVA. Strain differences: $F(1,122) = 19.5$, $P < 0.0001$; time differences: $F(5,610) = 25.5$, $P < 0.00001$; strain–time interaction: $F(5,610) = 7.9$, $P < 0.00001$). Wild-type mice responded to amphetamine with the a classical "bell-shaped" dose-related stimulation of motor activity (dose effect $P < 0.01$) with highly significant ($P < 0.00001$) stimulation of the motor activity at the doses 1.5 and 4.5 mg/kg. In contrast, transgenic mice showed only a tendency towards increased locomotor activity ($P = 0.08$) at the highest dose tested (10.0 mg/kg). (B) Motility responses of wild type (wt), heterozygous (hz) and homozygous (tg) transgenic mice (3083 line) to amphetamine (1.5 mg/kg) after 60 min of habituation. (C) Motility responses of wild type (wt) and homozygous (tg) transgenic mice of the 3114 line to amphetamine (1.5 mg/kg) after 60 min of habituation.

amine stimulation (Fig. 7C), although they responded normally to cocaine (data not shown).

Discussion

By overexpressing an antagonist of ephrin-A signaling in the central nervous system, we have uncovered a role for this signaling system in the establishment and/or maintenance of the dopaminergic nigrostriatal projection and in the responsiveness to central psychomotor stimulants of dopaminergic neurotransmission. Our results indicate that ephrin-A signaling is an important component of the molecular mechanisms that regulate the development and function of dopaminergic terminals.

Based on correlative patterns of expression, Yue et al. (1999) proposed a role for the EphB1/ephrin-B2 system in the topographic projection of midbrain dopaminergic neurons to the striatum. These observations are reminiscent of complementary expression patterns in other systems were Eph receptors and ephrins are thought to play a role in the organization of topographic projections, such as the retinotectal and hippocamposeptal projections. In these cases, the actions of ephrins on Eph-expressing terminals are believed to be inhibitory, and appear to contribute to the segregation of different projections within a common territory. In this context, the reduction that we observed in the number of substantia nigra dopaminergic neurons reaching the striatum in GFAP-EphA5Fc transgenic mice is unexpected, as it suggests that, unlike their B-class counterparts, class-A ephrins and Eph receptors could play a positive role in the establishment or maintenance of the nigrostriatal projection. Activation of Eph receptors by ephrins has also been implicated in the elimination of mistargeted neurons by the induction of cell death (Yue et al., 1999). However, because normal numbers of TH-positive cells were observed in the substantia nigra of GFAP-EphA5Fc transgenic mice, the reduction in nigrostriatal projections observed in these animals could not be due to cell death of dopaminergic neurons. In light of what is known about the role of Eph signaling in the establishment of axonal projections, it is possible that disruption of EphA/ephrin-A interactions resulted in the mistargeting of a fraction of substantia nigra dopaminergic projections to ectopic sites outside the striatum. A recent report has implicated ephrin-A5 in cell adhesion by its ability to interact with differentially spliced, truncated EphA receptors lacking catalytic activity (Holmberg et al., 2000). Although it is presently unknown whether non-catalytic forms of EphA receptors are expressed in ventral midbrain dopaminergic neurons, the deficits in the nigrostriatal projections of GFAP-EphA5Fc transgenic mice could reflect a role for endogenous EphA/ephrin-A interactions in the establishment or maintenance of adhesion between dopaminergic terminals and their target cells in the striatum.

Our behavioral studies in GFAP-EphA5Fc mice indicate a role for the EphA/ephrin-A signaling in dopaminergic neurotransmission. Transgenic mice had a reduced response to motor-stimulant doses of amphetamine but showed normal responses to cocaine. This differential response could not be attributed to differences in the genetic contributions of parental strains or to the site of transgene integration, but represented specific effects of EphA5Fc overexpression in the CNS of transgenic mice. Despite their differences in the underlying mechanisms, both amphetamine and cocaine are known to induce their behavioral effects by increasing dopamine levels in the synaptic cleft. In line with this, both drugs normally produce a similar stimulatory effect on locomotor behavior. In this context, the differential responses to amphetamine and cocaine that we observed in GFAP-EphA5Fc transgenic mice were highly unexpected and represent, to the best of our knowledge, the first case in which the behavioral responses to these two psychostimulants are not directly correlated.

The normal responsiveness to cocaine of GFAP-EphA5Fc transgenic mice suggests that the changes induced by transgene overexpression may be presynaptic, although postsynaptic mechanisms cannot be entirely excluded at present. The normal levels of basal locomotor activity of transgenic mice suggest that these animals have no impairments in basal dopaminergic neurotransmission. In agreement with this, and although the number of dopaminergic neurons that reached the striatum was lower in transgenic mice, total levels of striatal dopamine were comparable to wild type. In addition, other studies have shown that behavioral deficiencies due to loss of dopaminergic neurons are only seen after losses greater than 85–90% in the substantia nigra (Ungerstedt, 1971). A presynaptic locus for the deficit in the responsiveness to amphetamine is also consistent with the expression of EphA5 receptors by midbrain dopaminergic, but not striatal, neurons. In this regard, it is interesting to note that recent studies have highlighted the possibility that, despite lacking an intracellular domain, ephrin-A molecules may still be able to transmit a signal across the membrane when engaged by EphA receptors (Davy et al., 1999; Knoll and Drescher, 2002). However, we think it is unlikely that the effects of EphA5-Fc overexpression were due to increased signaling by ephrin-A molecules since (i) this requires membrane associated or high-order clustered receptors, and (ii) as explained above, the effects we observe are more readily explained by presynaptic, as opposed to postsynaptic, alterations.

Although the actual mechanistic bases for the actions of cocaine and amphetamine have been a matter of debate, recent work on mutant mice lacking the dopamine transporter (DAT) has begun to shed light on this question (Jones et al., 1998). Although it has been accepted for some time that the DAT is the main molecular target of cocaine, amphetamine appears to have multiple sites of action in addition to the DAT. Cocaine increases extracellular dopamine by inhibiting the reuptake of released dopamine by plasma membrane DAT (Heikkila et al., 1975). The effects of cocaine are therefore mediated by dopamine released from vesicles because of neuronal activity. Amphetamine, on the other hand, is able to enter the cell by lipophilic diffusion and by DAT-mediated transport, where it functions by displacing dopamine from secretory vesicles to the cytoplasm and by promoting reverse transport of cytoplasmic dopamine through the DAT to the extracellular space (Jones et al., 1998). Previous work has shown that the stimulatory effect of lower doses of amphetamine (<2 mg/kg) are primarily dependent upon the cytoplasmic pool of dopamine in dopaminergic terminals, while at higher doses (>5 mg/kg) amphetamine mainly affects the vesicular release of dopamine (Ögren and Ross, 1977; Sabol and Seiden, 1998). Unlike vesicular release, this transport-mediated release of cytoplasmic dopamine is independent of nerve impulses. Thus, both drugs potentiate dopaminergic transmission by increasing extracellular dopamine content albeit by different mechanisms. In light of this, the differential responsiveness of transgenic mice to cocaine and amphetamine may thus involve a change in the balance between the vesicular pool of dopamine, which is mainly affected by cocaine, and the cytosolic pool, which is released by amphetamine. In agreement with this, the dose of amphetamine that showed the greatest differences

between wild type and transgenic mice (i.e. 1.5 mg/kg) is probably mainly affecting the release of cytoplasmic dopamine.

It is at present unclear how disruption of EphA/ephrin-A signaling may affect the balance between vesicular and cytoplasmic pools of dopamine in the terminals of substantia nigra neurons. The central role played by the DAT in the responsiveness to cocaine and amphetamine suggests that this molecule could somehow be involved in the behavioral effects observed in GFAP-EphA5Fc transgenic mice. Regulation of DAT function by presynaptic EphA receptors could also explain the differences we observed between the two drugs, as amphetamine, but not cocaine, requires reverse dopamine transport through the DAT for its effects on dopamine transmission. The possibility that Eph receptors regulate synaptic membrane components has recently received support by the demonstration of a physical and functional interaction between EphB and NMDA glutamate receptors in hippocampal cells (Dalva et al., 2000; Takasu et al., 2002).

In conclusion, we have observed both anatomical and functional abnormalities in the nigrostriatal system following disruption of EphA/ephrin-A signaling in the brain of transgenic mice. Although the discussion presented above favors multiple and largely independent effects of EphA signaling on the anatomy and function of dopaminergic terminals, we cannot at present rule out that the effects we observed on dopaminergic innervation and amphetamine responsiveness may somehow be related. In any event, our analysis indicates that EphA/ephrin-A signaling plays an important role in the normal connectivity and function of midbrain dopaminergic neurons.

Experimental methods

Transgenic mice and genotyping

A 2.2-kb fragment containing the promoter region of the human *GFAP* gene (Brenner et al., 1994) was linked to a fusion of the extracellular domain of rat EphA5 and the Fc fragment of human IgG. Transgenic mice were generated by pronuclear injection at the transgenic core facility of the Karolinska Institute according to standard procedures. Genotyping was performed by slot-blot or PCR analyses of mouse tail DNA. Tail-blots were hybridized with a probe derived from the human Fc fragment of the transgene, and were quantified in a Storm 840 phosphorimager. After normalization, this analysis allowed the identification of mice that were hetero- or homozygous for the transgene. Homozygosity was confirmed in crosses with wild-type mice from which all resulting pups carried the transgene. For PCR analysis, primers were derived from the downstream region of the EphA5 ectodomain and the middle portion of the human Fc fragment. Primer sequences are available upon request.

Brain extracts, synaptic membranes and immunoblotting

The brains of postnatal day 4 or 14 mice were homogenized in 1 ml/100 mg tissue of 0.32 M sucrose, 25 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM sodium orthovanadate and protease inhibitors (Roche) with 4 × 20 strokes in a 7-ml glass homogenizer. Samples were then centrifuged for 10 min at 1000 × *g* and the nuclear pellet was discarded. The supernatant was recentrifuged for 40 min at 14,000 × *g* and the pellet containing synaptic membranes, synaptosomes and mitochondria was resuspended in NP40 lysis buffer (1% NP40, 20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitors). Two milligrams of protein from this fraction was immunoprecipitated with anti-EphA5 antibodies (L15 from Santa Cruz) overnight at 4 °C. For total brain extracts, 1 mg total brain lysate (in NP40 lysis buffer with protease inhibitors) was immunoprecipitated with Gamma Bind Plus Sepharose overnight at 4 °C. Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The product of the EphA5Fc transgene was detected using an alkaline-phosphatase antihuman Fc (Promega) followed by ECF detection system (Amersham). Purified recombinant EphA5-Fc protein was from Promega. Mixed glial cultures were established from cortices of P1 transgenic pups from line 3083 as previously described (O'Malley et al., 1994). Purified Type 1 astrocyte cultures were obtained by passaging mixed glial cultures as described (O'Malley et al., 1994). Conditioned medium (CM) was collected from confluent cultures that were incubated in defined, serum-free medium for 3 days. Media were then concentrated 25 times before immunoblot analysis. Cell monolayers were lysed in lysis buffer containing 1% NP-40 after media collection. Immunoblot analysis was performed as described above.

Immunohistochemistry

Animals were deeply anesthetized in a CO_2 chamber and transcardially perfused with 4% paraformaldehyde solution in 0.1 M sodium phosphate (pH 7.2). The brains were postfixed 2 h in the same solution, cryoprotected in 10% sucrose in phosphate-buffered saline (PBS) for 15 h and frozen in dry-ice cooled isopentane. Coronal sections obtained with a cryostat (14 μm) through the whole substantia nigra (SN) were collected serially and processed for immunohistochemistry. Slices were preincubated for 1 h with PBS containing 10% methanol and 1% H_2O_2. After three washes in PBS, incubated with the monoclonal antibodies directed against tyrosine hydroxylase (TH; 1/4000; DiaSorin, Stillwater, MN) or antihuman Fc (Sigma, St. Louis, MO, USA) in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA) for 16 h at 4 °C. Sections were then washed three times with PBS and incubated with a biotinylated goat anti-mouse antibody (1/200; Pierce, Rockford, IL, USA) for 1–2 h at room temperature in the same buffer as the primary antibody. Thereafter, tissue was incubated with avidin–biotin complex (Pierce) and developed with the SG chromogen (Vector Laboratories, Burlingame, CA, USA).

Fluorogold tracing

To study the axonal transport, we injected a retrograde transport tracer as described previously (Canals et al., 2001). Fluorogold (0.2 μl) (2% solution; Fluorochrome, Denver, CO, USA) was injected into the left striatum at two different coordinates (AP +0.7 and L +1.8 from Bregma, and −2.6 from dura; AP +0.23 and L −2.1 from bregma, and −2.6 from dura). Two days after tracer injection, mice were transcardially perfused with 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2, postfixed for 1–2 h with the same solution and cryoprotected in 10% sucrose in PBS. Thereafter, brains were frozen in dry-ice cooled isopentane and cryostat serial horizontal sections (14 μm) were mounted with mowiol (Calbiochem, San Diego, CA, USA) and visualized under fluorescent microscope. For double-labeling fluorogold immunohistochemistry, sections were processed for immunofluorescence. These sections were postfixed 10 min with 4% paraformaldehyde/

PBS, washed three times with PBS and incubated with primary anti-TH (1:1000) antibody as above. The sections were then washed and incubated with a Rhodamine-conjugated donkey anti-mouse (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature, washed and mounted with mowiol (Calbiochem). In control experiments, we avoid the primary antibody to show its specificity.

Image analysis and morphometry

All morphometry and cell counting were performed in a blind coded fashion. The measurements of the SN pars compacta (SNpc) volume and the number of fluorogold- or TH-positive cells were performed using the AnalySIS program from Soft Imaging System GmbH, Münster, Germany) on a computer attached to an Olympus microscope. For the volume estimations, consecutive sections (an average of 16–18 sections/animal) were visualized on a computer and the perimeter of the SNpc was outlined. TH-positive landmarks within each section were used to delimit the perimeter of the SNpc. The SNpc volume was estimated by multiplying the sum of all sectional areas (μm^2) by the distance between successive sections (70 μm). Fluorogold- and TH-positive neurons through the entire substantia nigra were counted in serial sections every 70 μm. Neurons showing a clear TH-positive cytoplasm surrounding a nonstained nucleus were counted as positive. Cell counts were expressed as the sum of positive cells and were corrected by the Abercrombie (1946) method. Statistical analysis was performed using one-way ANOVA, followed by LSD (least significant difference *t* test) post hoc test.

Measurement of dopamine content in striatum

The supernatants of striatal homogenates were assayed for levels of dopamine, DOPAC and HVA using high-performance liquid chromatography (HPLC) with electrochemical detection as described earlier (Magnusson et al., 1980) with minor modifications (Larsson et al., 1990).

Measurement of locomotor activity

Mice were individually tested in a dimly lit sound controlled room. They were removed from their home cages and placed in the middle of an activity monitor (standard transparent A3 Macrolon® cage with 50 ml of wooden shavings on the floor) and the data collecting system was immediately activated. In experiments with non-habituated animals, the mice were treated with the drug just before placement in the activity box and locomotor activity was recorded during 6 × 10 min intervals. In experiments with habituated animals, the mice were habituated to the test cages for 60 min and thereafter the injection was made and the activity was recorded as above.

Locomotion, motility and rearing behaviors were recorded in eight animals simultaneously by means of a multicage red and infrared-sensitive motion detection system (Ögren et al., 1986). The system is fully computerized and uses beams of red and infrared lights in combination with vertical and horizontal photocell arrays (4 cm distance between cells) to detect movements of animals. Rearing was recorded by counting the number of times an animal stands on its hind legs and interferes with any of the six invisible infrared beams passing horizontally through the cages. The height of these photocells was adapted to the size of the animals. Motility was defined as all movements of a distance of 4 cm or more detected by 48 vertical photocells, and it represents a measurement of general activity. Locomotion was defined by counting the number of times an animal had covered eight horizontal photocells and moved from one side of the test cage to the other side (a distance of at least 32 cm).

The dose-dependent effects of drugs on rearing, motility and locomotion in mice were analyzed by two-way repeated-measures ANOVA, the between group factors being strain [homozygote transgenics (tg) vs. heterozygotes (hz) vs. wild-type mice (wt)] and drug dose and time (6 × 10 min recording) were used as repeated measurement factors. This was followed by a post hoc Newman–Keul's test when appropriate for every time point separately. The whole study was designed as a between-subjects (independent groups) experiment (i.e. each animal was used only once).

Drugs

Amphetamine sulphate (0.5–1.5–4.5–10.0 mg/kg ip, Sigma), Cocaine HCl (5.0–20.0 mg/kg ip, Sigma). All drugs were dissolved in saline.

Acknowledgments

We thank Renping Zhou, Rutgers University, for sharing unpublished results, and Prof. Svante Ross, AstraZeneca, Södertälje, Sweden, for help with HPLC analysis. Financial support was obtained from the Swedish Medical Research Council (K99-33X-10908-06C), the Göran Gustafssons Stiftelse, and the Karolinska Institute (to C.F.I.), and Wenner-Gren Stiftelsen and KI Fonder (to B.-A. S.).

References

Abercrombie, M., 1946. Estimation of nuclear populations from microtome sections. Anat. Rec. 94, 239–247.

Brenner, M., Messing, A., 1996. GFAP transgenic mice. Methods 10, 351–364.

Brenner, M., Kisseberth, W.C., Su, Y., Besnard, F., Messing, A., 1994. GFAP promoter directs astrocyte-specific expression in transgenic mice. J. Neurosci. 14, 1030–1037.

Canals, J., Checa, N., Marco, S., Åkerud, P., Michels, A., Pérez-Navarro, E., Tolosa, E., Arenas, E., Alberch, J., 2001. Expression of brain-derived neurotrophic factor in cortical neurons is regulated by striatal damage. J. Neurosci. 21, 117–124.

Cheng, H.J., Nakamoto, M., Bergemann, A.D., Flanagan, J.G., 1995. Complementary gradients in expression and binding of ELF-1 and mek4 in development of the topographic retinotectal projection map. Cell 82, 371–381.

Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W., Greenberg, M.E., 2000. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell 103, 945–956.

Davy, A., Gale, N.W., Murray, E.W., Klinghoffer, R.A., Soriano, P., Feuerstein, C., Robbins, S.M., 1999. Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. Genes Dev. 13, 3125–3135.

Dodelet, V.C., Pasquale, E.B., 2000. Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. Oncogene 19, 5614–5619.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., Bonhoeffer, F., 1995. In vitro guidance of retinal ganglion cell axons

by RAGS, a 25 kDa tectal protein related to ligands for eph receptor tyrosine kinases. Cell 82, 359–370.

Flanagan, J.G., Vanderhaeghen, P., 1998. The ephrins and eph receptors in neural development. Annu. Rev. Neurosci. 21, 309–345.

Frisen, J., Holmberg, J., Barbacid, M., 1999. Ephrins and their Eph receptors: multitalented directors of embryonic development. EMBO J. 18, 5159–5165.

Gale, N.W., Holland, S.J., Valenzuela, D.M., Flenniken, A., Pan, L., Ryan, T.E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D.G., et al., 1996. Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron 17, 9–19.

Gao, P.P., Zhang, J.H., Yokoyama, M., Racey, B., Dreyfus, C.F., Black, I.B., Zhou, R., 1996. Regulation of topographic projection in the brain-elf-1 in the hippocamposeptal system. Proc. Natl. Acad. Sci. U. S. A. 93, 11161–11166.

Gao, W.Q., Shinsky, N., Armanini, M.P., Moran, P., Zheng, J.L., Mendozaramirez, J.L., Phillips, H.S., Winslow, J.W., Caras, I.W., 1998. Regulation of hippocampal synaptic plasticity by the tyrosine kinase receptor, rek7/epha5, and its ligand, al-1/ephrin-a5. Mol. Cell. Neurosci. 11, 247–259.

Heikkila, R.E., Orlansky, H., Cohen, G., 1975. Studies on the distinction between uptake inhibition and release of (3H)dopamine in rat brain tissue slices. Biochem. Pharmacol. 24, 847–852.

Holder, N., Klein, R., 1999. Eph receptors and ephrins: effectors of morphogenesis. Development 126, 2033–2044.

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M., Pawson, T., 1996. Bidirectional signalling through the eph-family receptor nuk and its transmembrane ligands. Nature 383, 722–725.

Holmberg, J., Frisen, J., 2002. Ephrins are not only unattractive. Trends Neurosci. 25, 239–243.

Holmberg, J., Clarke, D.L., Frisen, J., 2000. Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature 408, 203–206.

Jones, S.R., Gainetdinov, R.R., Wightman, R.M., Caron, M.G., 1998. Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. J. Neurosci. 18, 1979–1986.

Kelly, M.A., Rubinstein, M., Phillips, T.J., Lessov, C.N., Burkhart-Kasch, S., Zhang, G., Bunzow, J.R., Fang, Y., Gerhardt, G.A., Grandy, D.K., Low, M.J., 1998. Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. J. Neurosci. 18, 3470–3479.

Klein, R., 1999. Bidirectional signals establish boundaries. Curr. Biol. 9, R691–R694.

Knoll, B., Drescher, U., 2002. Ephrin-As as receptors in topographic projections. Trends Neurosci. 25, 145–149.

Krull, C.E., Lansford, R., Gale, N.W., Collazo, A., Marcelle, C., Yancopoulos, G.D., Fraser, S.E., Bronnerfraser, M., 1997. Interactions of ephrelated receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. Curr. Biol. 7, 571–580.

Lackmann, M., Oates, A.C., Dottori, M., Smith, F.M., Do, C., Power, M., Kravets, L., Boyd, A.W., 1998. Distinct subdomains of the EphA3 receptor mediate ligand binding and receptor dimerization. J. Biol. Chem. 273, 20228–20237.

Larsson, L.G., Renyi, L., Ross, S.B., Svensson, B., Angeby-Moller, K., 1990. Different effects on the responses of functional pre- and postsynaptic 5-HT1A receptors by repeated treatment of rats with the 5-HT1A receptor agonist 8-OH-DPAT. Neuropharmacology 29, 86–91.

Lemke, G., 1998. Eph receptors and ligands in axon pathway choice, target recognition, and synaptogenesis. Prog. Brain Res. 117, 171–176.

Magnusson, O., Nilsson, L.B., Westerlund, D., 1980. Simultaneous determination of dopamine, DOPAC and homovanillic acid. Direct injection of supernatants from brain tissue homogenates in a liquid chromatography-electrochemical detection system. J. Chromatogr. 221, 237–247.

Maisonpierre, P.C., Barrezueta, N.X., Yancopoulos, G.D., 1993. Ehk-1 and Ehk-2: two novel members of the Eph receptor-like tyrosine kinase family with distinctive structures and neuronal expression. Oncogene 8, 3277–3288.

Ögren, S.O., Ross, S.B., 1977. Substituted amphetamine derivatives: II. Behavioural effects in mice related to monoaminergic neurones. Acta Pharm. Toxicol. (Copenh.) 41, 353–368.

Ögren, S.O., Hall, H., Köhler, C., Magnusson, O., Sjöstrand, S.E., 1986. The selective dopamine D2 receptor antagonist raclopride discriminates between dopamine-mediated motor functions. Psychopharmacology (Berl.) 90, 287–294.

O'Leary, D.D., Wilkinson, D.G., 1999. Eph receptors and ephrins in neural development. Curr. Opin. Neurobiol. 9, 65–73.

O'Malley, E.K., Sieber, B.A., Morrison, R.S., Black, I.B., Dreyfus, C.F., 1994. Nigral type I astrocytes release a soluble factor that increases dopaminergic neuron survival through mechanisms distinct from basic fibroblast growth factor. Brain Res. 647, 83–90.

Sabol, K.E., Seiden, L.S., 1998. Reserpine attenuates D-amphetamine and MDMA-induced transmitter release in vivo: a consideration of dose, core temperature and dopamine synthesis. Brain Res. 806, 69–78.

Takasu, M.A., Dalva, M.B., Zigmond, R.E., Greenberg, M.E., 2002. Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB receptors. Science 295, 491–495.

Ungerstedt, U., 1971. Postsynaptic supersensitivity after 6-hydroxy-dopamine induced degeneration of the nigro-striatal dopamine system. Acta Physiol. Scand., Suppl. 367, 69–93.

Wilkinson, D.G., 2001. Multiple roles of EPH receptors and ephrins in neural development. Nat. Rev., Neurosci. 2, 155–164.

Yue, Y., Widmer, D.A., Halladay, A.K., Cerretti, D.P., Wagner, G.C., Dreyer, J.L., Zhou, R., 1999. Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. J. Neurosci. 19, 2090–2101.

Zhang, J.H., Cerretti, D.P., Yu, T., Flanagan, J.G., Zhou, R.P., 1996. Detection of ligands in regions anatomically connected to neurons expressing the eph receptor bsk-potential roles in neuron-target interaction. J. Neurosci. 16, 7182–7192.