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ORIGINAL RESEARCH ARTICLE

# Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine

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**Netrins are guidance cues that play a fundamental role in organizing the developing brain. The netrin receptor, DCC (deleted in colorectal cancer), is highly expressed by dopaminergic (DA) neurons. DCC may therefore participate in the organization of DA circuitry during development and also influence DA function in the adult. Here we show that adult *dcc* heterozygous mice exhibit a blunted behavioral response to the indirect DA agonist amphetamine and do not develop sensitization to its effects when treated repeatedly. These behavioral alterations are associated with profound changes in DA function. In the medial prefrontal cortex, *dcc* heterozygotes exhibit *increased* tyrosine hydroxylase (TH) protein levels and dramatic *increases* in basal concentrations of DA and DA metabolites. In contrast, in the nucleus accumbens, *dcc* heterozygotes show no changes in either TH or DA levels, but exhibit *decreased* concentrations of DA metabolites, suggesting reduced DA activity. In addition, *dcc* heterozygous mice exhibit a small, but significant reduction in total number of TH-positive neurons in midbrain DA cell body regions. These results demonstrate for the first time that alterations in *dcc* expression lead to selective changes in DA function and, in turn, to differences in DA-related behaviors in adulthood. These findings raise the possibility that changes in *dcc* function early in life are implicated in the development of DA dysregulation observed in certain psychiatric disorders, such as schizophrenia, or following chronic use of drugs of abuse.**
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Repeated exposure to stimulant drugs, such as amphetamine, leads to the development of increased sensitivity to the behavioral-activating effects of these drugs. This phenomenon, known as behavioral sensitization, is long-lasting and is accompanied by enhanced stimulant-induced dopamine (DA) release in the nucleus accumbens (NAcc).[1,2] Individual differences in susceptibility to the effects of stimulants on behavior and on the release of DA have been demonstrated in both humans and adult laboratory animals.[3–8] Although the processes that lead to differences in vulnerability to stimulant drugs remain to be elucidated, evidence indicates that both genetic factors[9] and exposure to environmental insults early in life are involved.[10]

Perinatal insults can result in sensitized behavioral and mesolimbic DA responses of adult rats to an acute injection of amphetamine.[11] Similarly, lesions of forebrain structures made in neonatal rats lead to enhanced behavioral responses to amphetamine in adult animals.[5,12] These findings suggest that anomalies in the development and organization of DA circuitry contribute to subsequent functional and behavioral abnormalities in the adult. However, little is known about the molecular mechanisms that regulate the development of DA systems.

Netrins are a family of secreted proteins that play a fundamental role in the organization of the developing brain by directing growing axons toward appropriate targets.[13,14] Interestingly, the netrin receptor DCC is highly expressed by DA neurons in both the developing and the adult brain[15,16] and may, therefore, contribute to the development and organization of DA circuitry. However, a role for DCC in the

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development of DA function has not been identified. Homozygous *dcc* knockout mice die soon after birth and, although *dcc* heterozygous mice survive to adulthood, no mutant phenotype has been identified in these mice.[17]

We hypothesized that DCC participates in the development and organization of DA circuitry and that abnormal levels of DCC expression may lead to changes in DA function and behavior in the adult. To investigate this, we examined the activity of the mesolimbic DA system of adult *dcc* heterozygous mice by assessing their behavioral response to acute injections of amphetamine. In addition, to determine whether changes in DCC function would result in altered plasticity of DA systems in adulthood, we assessed whether *dcc* heterozygous mice would develop sensitization to the locomotor-activating effects of amphetamine, when repeatedly exposed to this drug. We found that adult *dcc* heterozygotes have a blunted behavioral response to acute amphetamine exposure and do not develop sensitization to its effects when treated repeatedly. Importantly, these behavioral alterations are associated with profound molecular and anatomical changes in mesocortical DA function.

## Materials and methods

*Subjects*

Mice heterozygous for *dcc* were obtained from Robert Weinberg[17] (Harvard University, Cambridge, MA, USA) and bred in the Montreal Neurological Institute animal care facility. Mice were housed on a 12-h light/dark cycle with access to food and water *ad libitum*. Pups were weaned at P25 and housed with same-sex littermates. Three-month-old male DCC heterozygous and control wild-type littermates were used in all the experiments. All procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

*Locomotor activity*

Locomotor activity was quantified using an infrared activity monitoring apparatus modified for use with mice as described.[18] Briefly, activity boxes consisted of three pressed-wood walls, one Plexiglas front wall, a wire screen top, and a stainless-steel rod floor. Four photocells were located around the perimeter of the box. Interruptions of each of the photocell beams were detected and recorded via an electrical interface by a computer located in an adjacent room. The activity boxes were kept in the dark throughout all activity session and a white noise generator (75 dB) was used to mask extraneous noise.

In the initial experiment, to assess possible differences in basal locomotor activity *dcc* heterozygous and wild-type littermates were habituated to activity boxes for 30 min and then were given an intraperitoneal (i.p.) injection of saline and their activity was monitored for 90 min. In the next experiment, the acute effects of amphetamine were studied in three different groups of *dcc* heterozygous and wild-type littermates. Mice were placed in activity boxes for 30 min, then given an i.p. injection of either 1.5, 2.5, or 4 mg/kg D-amphetamine sulfate (Sigma) and their activity was measured for 90 min. Data for each 15 min are expressed as mean ($\pm$SEM) activity counts (number of interruption of photocell beams).

In the third experiment, the sensitization experiment, different *dcc* heterozygous and wild-type littermate mice were treated repeatedly with D-amphetamine sulfate (4 mg/kg, i.p.) or saline, one injection every other day, five times. In this 'pretreatment phase', animals were taken to the activity room and their locomotor activity was measured 30 min before and 90 min after each amphetamine or saline injection. To test for sensitization, one week after the last saline or amphetamine injection, mice were taken to the activity room, placed in activity boxes for 30 min, and then *all* of them, whether saline or amphetamine pretreated, were given a single injection of D-amphetamine sulfate (2 mg/kg, i.p.). This lower dose was used in the test to avoid any masking effects of stereotyped behaviors that might have developed to the higher dose. The locomotor activity induced by this amphetamine challenge was measured for 90 min. Data for each 15 min are expressed as mean ($\pm$SEM) activity counts (number of interruption of photocell beams).

*Western blotting*

Western blotting for DCC (deleted in colorectal cancer), tyrosine hydroxylase (TH), and dopamine-$\beta$-hydroxylase (DBH) was conducted using tissue from 3-month-old *dcc* heterozygous and wild-type mice. Bilateral punches of mPFC, including cingulate cortex area 1 and 2, NAcc, including both core and shell, and striatum (STR) were excised from 1-mm-thick coronal slices. Protein samples (20 $\mu$g) were separated using SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with antibodies against DCC (G97-449; 1 : 1000; Pharmigen, Mississauga, Canada), TH (1 : 5000; Chemicon, Temicula, CA, USA), and DBH (1 : 1000, Chemicon), and then incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, PA, USA). Reactive bands were visualized by chemiluminescence (NEN Life Science Products, MA, USA). Optical density was quantified on scanned images of immunoblots using NIH Image software (National Institutes of Health). Data were analyzed using Student's *t*-tests and are expressed as percent of wild-type group. All statistical analyses were conducted on the raw data.

*Stereology*

Unbiased estimates of the number of midbrain DA neurons were obtained using the optical dissector method of West and Gundersen[19] as described.[20] The

entire rostrocaudal extent of the midbrain was examined in TH-stained (1 : 1000, Chemicon) 50-$\mu$m coronal serial sections of 3-month-old *dcc* heterozygous and wild-type mice. TH cell counts of every third section were conducted at $\times 100$ magnification using a $60 \times 60\,\mu$m counting frame. A 10-$\mu$m dissector was placed 2 $\mu$m below the surface of the section at counting sites located at 150 $\mu$m intervals after a random start. Data were analyzed using repeated measures ANOVA.

*Biochemistry*

Bilateral punches of mPFC, NAcc, and STR were excised from 1-mm-thick coronal slices of brains from 3-month-old DCC heterozygous and wild-type mice. Punches were homogenized and centrifuged at 3000 rpm for 15 min at 4°C. Pellets were suspended in 0.1 M NaCl and analyzed for protein content. The supernatant was removed and assayed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) using HPLC with electrochemical detection.[21] Supernatant was injected into a 15-cm $C_{18}$ column (Higgins Analytical, Inc.). Compounds were detected and quantified with a coulochem III detector (model 5100A; ESA, Inc.). Concentrations were estimated from peak height by comparison with injection of known amounts of pure standards (Sigma). Data were analyzed using repeated measures ANOVA and Student's *t*-tests and are expressed as percent of wild-type group. All statistical analyses were conducted on the raw data.

## Results

*Blunted behavioral response of adult* dcc *heterozygotes to a single injection of amphetamine*

No difference was detected in basal locomotor activity between *dcc* heterozygotes ($n=16$) and wild-type mice ($n=10$). Locomotor activity of adult *dcc* heterozygous mice 30 min before or 90 min after an i.p. injection of saline was similar to that of wild-type type littermates (Figure 1a). In contrast, *dcc* heterozygotes were significantly less sensitive than wild-type littermate controls to the locomotor-activating effects of acute injections of amphetamine at three different doses, 1.5, 2.5, and 4 mg/kg (Figure 1b). At all doses, *dcc* heterozygous mice (1.5 mg/kg, $n=12$; 2.5 mg/kg $n=20$; 4 mg/kg $n=6$) were less active (total activity scores in 90 min) in response to amphetamine than their wild-type littermates (1.5 mg/kg, $n=6$; 2.5 mg/kg $n=15$; 4 mg/kg $n=6$). The magnitude of this difference, however, was small at the 1.5 mg/kg dose. A two (genotype) by four (dose: saline, 1.5, 2.5, 4 mg/kg) ANOVA revealed significant effects of genotype ($F_{(1,83)}=8.3$, $P=0.005$) and dose ($F_{(3,83)}=41.7$, $P=0.0001$). This is the first demonstration of a phenotypic difference between adult *dcc* heterozygous and wild-type mice.

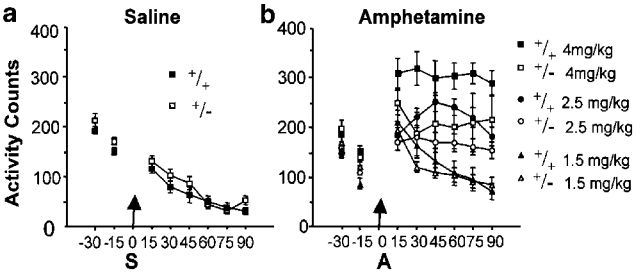


**Figure 1** Behavioral response of adult *dcc* heterozygous mice to acute injections of amphetamine. (a) Locomotor activity observed 30 min before and 90 min after an i.p. injection of saline did not differ between *dcc* heterozygous (+/−) and wild-type (+/+) mice. (b) Following a 30 min habituation period, the effects of acute exposure to D-amphetamine sulfate on locomotor activity were assessed in *dcc* heterozygous and wild-type mice by comparing three different doses: 1.5, 2.5, and 4 mg/kg. At all doses tested, *dcc* heterozygous mice were less active (total activity scores) in response to amphetamine than their wild-type littermates; the magnitude of this difference, however, was small at the lowest amphetamine dose.

*Adult* dcc *heterozygotes do not develop sensitization to the effects of amphetamine following repeated exposure*

To assess whether *dcc* heterozygous mice would develop sensitization to the locomotor-activating effects of amphetamine, we treated new groups of *dcc* heterozygotes and wild-type littermates with five injections of amphetamine (4 mg/kg, i.p.) or saline, one injection every other day. In this 'pretreatment phase', the locomotor activity of both *dcc* heterozygous ($n=6$) and wild-type mice ($n=6$) increased in response to each amphetamine injection when compared to saline injections (wild-type $n=6$; heterozygous $n=4$). However, as was observed in the acute study (Figure 2), amphetamine-induced locomotor activity was significantly reduced in *dcc* heterozygotes in comparison to wild-type mice (data not shown). At 1 week after the last 'pretreatment' injection, a test for sensitization was conducted. In this test, *all* animals, whether pre-exposed to amphetamine or saline, were given an injection of amphetamine (2 mg/kg) and their locomotor activity was measured. As expected, wild-type mice previously exposed to amphetamine showed a robust sensitized response; their locomotor activity in response to the amphetamine challenge was significantly greater than that of mice given amphetamine for the first time in this test (saline-pretreated mice). These data were analyzed with a two (treatment) by six (time) ANOVA (main effect of time $F_{(5,50)}=11.4$, $P=0.0001$; treatment by time interaction $F_{(5,50)}=3$, $P=0.02$). In contrast, *dcc* heterozygous mice that had received amphetamine in the pretreatment phase did not show sensitization; their behavioral response to the amphetamine challenge did not differ from that observed in saline-pretreated mice (Figure 2; main effect of time $F_{(5,40)}=4.2$, $P=0.003$; treatment by time interaction

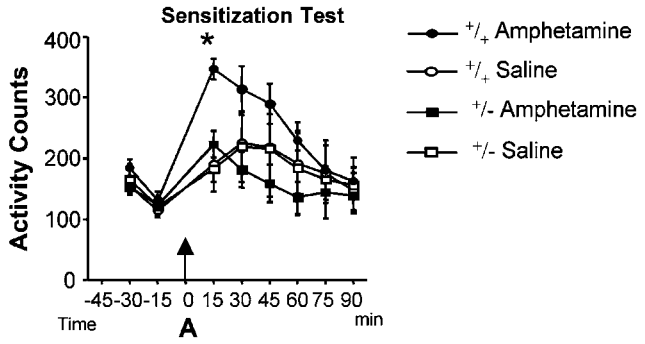


**Figure 2** Behavioral response of adult *dcc* heterozygous mice to repeated amphetamine exposure. Groups of mice previously treated with saline or D-amphetamine sulfate (i.p., 4 mg/kg; once every other day, five times) were tested for sensitization 1 week after the last 'pretreatment' injection. During this sensitization test *all* mice, whether pretreated with amphetamine or saline, received an amphetamine injection (i.p. 2 mg/kg). Wild-type mice showed a sensitized response to amphetamine; wild-type mice pre-exposed to amphetamine showed greater activity than those exposed to amphetamine for the first time. In contrast, *dcc* heterozygous mice previously exposed to amphetamine did not show a sensitized response; their locomotor activity in response to amphetamine challenge was not different from mice pre-exposed to saline. *Wild-type amphetamine pretreated group significantly different from all the other groups.

$F_{(5,40)} = 2$, $P = 0.08$). A one-way ANOVA revealed that 15 min after the amphetamine challenge, the locomotor activity exhibited by wild-type amphetamine-pretreated mice was significantly different from all the other groups ($F_{(3,18)} = 3.8$, $P = 0.02$).

### *Adult* dcc *heterozygotes have fewer midbrain TH-positive neurons, but exhibit increased TH protein expression in the mPFC*

To determine whether the behavioral differences between *dcc* heterozygous and wild-type mice were accompanied by alterations in DA function, we examined the expression of DCC and TH, the rate-limiting enzyme for the synthesis of DA and norepinephrine, in samples of tissue from DA terminal regions of adult *dcc* heterozygous ($n = 4$) and wild-type littermate mice ($n = 4$). Immunoblot analysis of samples of mPFC identified a ~70% decrease in the amount of DCC protein in *dcc* heterozygotes, but a ~50% *increase* in TH (Figure 3a; DCC: Student's *t*-test: $t_6 = 5.4$, $P = 0.05$; TH: $t_6 = 5.7$, $P = 0.05$). As the mPFC receives both DA and noradrenergic innervation, we determined whether the increase in mPFC TH expression corresponded to changes in TH in DA or noradrenergic terminals. Thus, we assessed mPFC expression of DBH, the enzyme that converts DA to norepinephrine. We found that DBH in mPFC of *dcc* heterozygous mice was, if changed at all, decreased, indicating that the increases in mPFC TH reflects changes in DA terminals ($t_6 = 3$, $P = 0.13$). Although

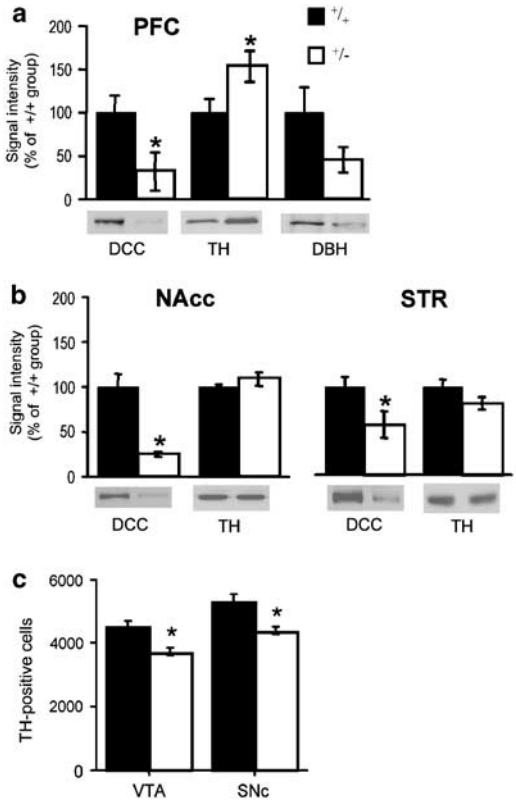


**Figure 3** Altered levels of DCC and TH protein levels in adult *dcc* heterozygous mice. (a) Western blot analysis revealed significantly decreased mPFC DCC expression in heterozygous (+/−) mice in comparison to wild-type (+/+) group and significantly *increased* TH expression. DBH expression was not significantly affected. (b) DCC expression in NAcc and STR was significantly reduced in *dcc* heterozygous mice, but TH was not changed. Representative examples of Western blot analysis are shown below the graphs. (c) Unbiased estimates of TH-positive neurons in VTA and SNc revealed significant decreases in number of DA neurons in *dcc* heterozygous mice.

DCC protein levels were decreased by ~80% in NAcc and by ~40% in STR of *dcc* heterozygotes compared to wild type ($t_6 = 42$, $P = 0.001$; $t_6 = 5.4$, $P = 0.05$), TH expression in these two regions did not differ from that found in brains of wild-type mice (Figure 3b).

Stereological analysis of the number of TH-positive cells in ventral tegmental area (VTA) and substantia nigra compacta (SNc) indicated that adult *dcc* heterozygous mice had ~20% fewer midbrain TH-positive cells than wild-type mice (Figure 3c; genotype effect: $F_{(1,6)} = 36$, $P = 0.001$; $n = 4$ per group).

### *Increased TH levels in prefrontal cortex of* dcc *heterozygous mice are associated with dramatic increases in prefrontal cortex dopamine activity*

Measurement of basal tissue concentrations of DA and its metabolites, DOPAC and HVA, in medial prefrontal cortex (mPFC) of adult *dcc* heterozygous and wild-type mice, identified a ~200% increase in

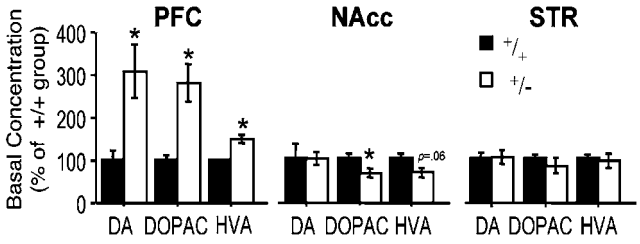


**Figure 4** Alterations in basal concentrations of DA and DA metabolites in adult *dcc* heterozygous mice. HPLC analysis revealed significant increases in mPFC concentrations of DA, DOPAC, and HVA in *dcc* heterozygous mice (+/−) in comparison to wild-type (+/+) group. NAcc DOPAC and HVA concentrations were decreased in *dcc* heterozygous mice. No significant changes were observed in STR.

DA ($P=0.01$), a ~200% increase DOPAC ($P=0.003$), and a ~50% increase in HVA ($P=0.001$) in *dcc* heterozygotes (Figure 4; genotype by metabolite interaction: $F_{(2,16)}=5.4$, $P=0.01$). Remarkably, these increases were accompanied by a *decrease* in basal concentrations of DOPAC ($t_9=6.3$; $P=0.03$) and HVA ($t_9=4.6$; $P=0.06$) in NAcc. DA concentrations in NAcc and STR did not differ between *dcc* heterozygous and wild-type mice ($p>0.05$; $n=5$–6 per group).

## Discussion

The findings reported indicate that adult *dcc* heterozygous mice are significantly less sensitive to the behavioral-activating effects of acute amphetamine than wild-type mice. This is the first identification of a *dcc* haplo-insufficient phenotype in the adult. Locomotor activity before or following an acute injection of saline did not differ between the two groups. Thus, the differential response to amphetamine does not result from alterations in basal activity levels. As the acute effects of amphetamine on locomotor activity are largely mediated by amphetamine-induced DA release in the Nacc,[22–24] it is possible to speculate that the blunted response to amphetamine observed in *dcc* heterozygotes results from decreased mesoaccumbens DA transmission.

Several lines of evidence indicate that DA neurons that project to the NAcc (mesolimbic) have anatomical and functional characteristics that are distinct from those that project to the mPFC (mesocortical).[25–28] Furthermore, mPFC DA activity has been shown to attenuate mesolimbic DA release.[29,30] In the present study, we show that in adult *dcc* heterozygous mice there is a selective increase in basal TH levels in the mPFC, without a corresponding increase in DBH levels. The increase in TH, therefore, reflects changes in DA and not noradrenergic terminals. During postnatal development, the DA system shows a progressive ingrowth of fibers into the mPFC that continues until early adulthood. Furthermore, mPFC DA innervation has the capacity for significant structural plasticity during postnatal life following brain lesions in neonates.[31] Although further studies are needed, one can speculate that increased TH expression in the mPFC of *dcc* heterozygous mice results from increased DA fiber ingrowth in this region. This idea is supported by our finding that increased TH in the mPFC is not associated with an increase in midbrain DA cell number.

Increased TH expression in the mPFC observed in *dcc* heterozygotes suggests greater DA activity within this region that, in turn, may attenuate NAcc DA transmission.[32,33] This attenuation would be predicted to reduce the behavioral response to acute amphetamine. Indeed, when we measured basal tissue concentrations of DA and its metabolites in adult *dcc* and wild-type mice, we found a dramatic increase in DA, DOPAC and HVA concentrations in the mPFC of *dcc* heterozygotes. Although, the basal DA concentration in NAcc of *dcc* heterozygotes was unchanged, concentrations of DOPAC and HVA were reduced, indicating decreased DA metabolism within this region.

Since decreased sensitivity to the acute effects of amphetamine does not in itself prevent the development of sensitized responding when animals are exposed repeatedly to this drug,[34] we investigated the sensitizing effects of amphetamine in adult *dcc* heterozygous and wild-type mice. During the 'pretreatment phase' of the sensitization experiment, locomotor activity of both *dcc* heterozygous and wild-type mice was increased by amphetamine when compared to saline, but, as in the acute study, this response was significantly reduced in *dcc* heterozygotes. However, on the test day, when both saline and amphetamine pretreated animals received amphetamine, *dcc* heterozygous mice failed to show behavioral sensitization. These findings suggest that reduced levels of DCC throughout life protect against the long-lasting consequences of repeated amphetamine exposure.

Although, the mechanisms underlying the lack of sensitization to the effects of amphetamine observed in *dcc* heterozygous mice remain to be elucidated, it is likely that the robust increase in mPFC DA function observed in these mice contributes to this effect. It has been shown that repeated exposure to stimulants results in blunted DA release in mPFC in response to drugs or stress.[35,36] Importantly, decreased mPFC DA response following repeated drug treatment has been demonstrated to contribute to the expression of sensitized DA release in the Nacc.[37,38] Thus, it is likely that enhanced mesocortical DA transmission in adult *dcc* heterozygous mice protects against sensitized function of mesolimbic DA neurons and, in turn, prevents sensitized behavioral responding. Future studies should be aimed at addressing this possibility and at assessing whether other brain regions and other neurotransmitters are implicated.

As mentioned in the introduction, individual differences in sensitivity to the behavioral and DA effects of stimulants have been demonstrated in both

humans and laboratory animals. Interestingly, sensitized function of the mesolimbic DA systems is observed in schizophrenia and appears to contribute to psychotic symptoms. Acute exposure to amphetamine in schizophrenic patients evokes or exacerbates positive symptoms at doses that do not induce psychosis in healthy control subjects.[7,39] Furthermore, imaging studies show that a considerable number of nonmedicated schizophrenic patients exhibit a marked elevation of acute amphetamine-induced striatal DA release in comparison to healthy volunteers; this response correlates significantly with the emergence or worsening of psychotic symptoms (for a review see Laurelle[8]). One might speculate, therefore, that decreased DCC function during development could protect against the development of DA dysfunction observed in schizophrenia and in other psychiatric disorders.[40] In this context, it is important to note that in schizophrenia there is a reduction in the density of DA innervation in the mPFC.[41]

In summary, we find that adult *dcc* heterozygous mice have a blunted behavioral response to acute exposure to the stimulant drug amphetamine and do not develop sensitization to its effects. These behavioral alterations appear to result from molecular and anatomical changes in the mesocortical DA system. Although, adult *dcc* heterozygous mice have a slightly reduced number of DA neurons in VTA and SNc, they exhibit a selective increase in TH protein levels in mPFC accompanied by an increase in mPFC DA and a decrease in DA metabolism in NAcc. Thus, reduced amounts of DCC during development significantly augment mPFC DA function in adulthood. Enhanced DA function in the mPFC could account for the reduced behavioral response to amphetamine and produce a phenotype less vulnerable to the development of sensitized responses of the mesolimbic DA system to stimulant drugs. Taken together, these findings indicate that DCC is involved in the organization and function of DA circuitry and that decreased levels of DCC during development produce alterations opposite to those seen in schizophrenic patients or following chronic use of stimulant drugs.

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