Role of DCC (Deleted in Colorectal Cancer) in the development and function of the mesocorticolimbic dopaminergic system: implications for schizophrenia

Alanna Grant

Integrated Program in Neuroscience
McGill University, Montréal

December, 2011

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Acknowledgements

This Thesis would not exist without the help and support of many people to whom I am extremely grateful. First, and foremost, I would like to thank my supervisor, Dr. Cecilia Flores. I do not have the words to adequately describe the extent of my gratitude or the level of esteem I have for her. All I can say is that a student could not wish for a better supervisor! I would like to thank Cecilia for all her guidance, her enthusiasm, her time and effort (not to mention patience!) in helping me to develop my scientific and writing skills.

I would also like to thank the members of my advisory committee, Dr. Giamal Luheshi and Dr. Ridha Joober for their constructive criticisms and insightful suggestions they provided throughout our numerous advisory meetings.

I consider myself incredibly lucky to have always been surrounded by an amazing group of lab mates. I realize that much of my research would not have been possible without the technical (and, more importantly, moral) support from all of you. I would especially like to acknowledge the exceedingly bright undergraduate students who have been an integral part of data collection: Stephanie Gallant, Dan Hoops, Harrod Ling, Matt Pokinko, and Zoe Speed; the past research technicians who could always be counted on to assist in experiments: Michael Prevost and Carla Himmelman; the post-doctoral fellows from whom I've learned a tremendous amount: Dr. Cassandre Labelle-Dumais and Dr. Colleen Manitt: and my fellow students: Jessie Argento, Meagan Auger, Mark Daubaras, Conrad Eng, and especially, Leora Yetnikoff. All of you are

more to me than lab mates… thank you for all the fun times in the Flores Lab and for the memories that I will cherish forever. I would especially like to thank Colleen and Leora for their proofreading and comments on this thesis and previous manuscripts.

I would also like to take this opportunity to acknowledge the fantastic support staff at the Douglas Mental Health University Institute, especially Marie-Eve Desaulniers and Eve-Marie Charbonneau (for the daily care of our mice) and Marianne Dufour (for all the moral and administrative support).

I am indebted to NSERC, McGill Graduate and Postdoctoral Studies, the Integrated Program in Neuroscience, and the Faculty of Medicine for their generous fellowships that allowed me to complete my studies.

Finally, I am deeply grateful to my best friend and greatest source of support, Rasha Nasra. Thank you for *everything* over the past few years. I consider myself blessed to have someone like you in my corner.

This thesis is dedicated to the memory of my father, Carlton Richard Grant, and to my mother, Brenda Grant, for without their support and encouragement, I would not be where I am today.

Table of contents

Abstract

Disruption to the development of the mesocorticolimbic dopamine (DA) system is thought to contribute to the symptomatology of schizophrenia and related disorders. However, the molecular mechanisms underlying how disruptions to the development of this system result in abnormal DA function in adulthood are not known. The netrin-1 guidance cue and its receptor, DCC (deleted in colorectal cancer), are highly expressed by DA neurons and their target areas throughout pre- and post-natal life. Therefore, these proteins may play an instructive role in the development and organization of the mesocorticolimbic DA system and influence DA function in adulthood. In the present Thesis, the hypothesis, that variations in the level of DCC expression result in functional and organizational changes of mesocorticolimbic DA circuitry, was tested. A series of experiments comparing *dcc* heterozygous and wild-type mice on various indices of DA function was conducted at juvenile, peri-pubertal, and adult ages. It was found that adult mice that develop with reduced levels of DCC display selective reorganization of DA circuitry in the medial prefrontal cortex (mPFC), show enhanced mPFC DA function, and exhibit a behavioural phenotype opposite to that observed in putative neurodevelopmental animal models of schizophrenia. Furthermore, it was also found that none of the phenotypic traits present in adult *dcc* heterozygous mice are evident prior to adolescence, a period during which mPFC DA circuitry undergoes substantial reorganization and functional refinement. These findings suggest that alterations in DCC function may increase

(or decrease) individual susceptibility to the development of schizophrenia. As a first step in addressing this possibility, a case-control association study comparing the genotypic and allelic frequencies of single nucleotide polymorphisms located in the *DCC* gene between schizophrenic patients and healthy control subjects was conducted. Results from this study provide evidence for an association between schizophrenia and the rs2270954 polymorphism located in the 3' untranslated region of the *DCC* gene. Collectively these results implicate DCC *i)* as a critical determinant in the organization and in the function of the mesocorticolimbic DA system in adulthood, *ii)* as having an important, and previously unknown, role in the normal maturation of mPFC DA circuitry during adolescence, and *iii)* as a promising novel candidate gene that may contribute to the genetic basis behind individual differences in susceptibility to schizophrenia.

Résumé

Des anomalies dans le développement des circuits mesocorticolimbiques de la dopamine (DA) sont présumées contribuer à la symptomatologie de la schizophrénie et des désordres reliés à la schizophrénie. Cependant, les mécanismes moléculaires impliqués dans les effets d'évènements qui influencent le développement du système DA et qui mènent au fonctionnement anormal de ce système chez l'adulte ne sont pas connus. La netrin-1, molécule de guidage axonale, et son récepteur DCC (deleted-in-colorectal-cancer), sont fortement exprimés par les neurones DA ainsi que dans leurs régions d'innervation au cours de la vie pré- et post-natale. Par conséquent, ces protéines pourraient jouer un rôle dans le développement et l'organisation du système DA et pourraient influencer le fonctionnement DA chez l'adulte. Dans cette thèse, l'hypothèse que des variations dans le niveau d'expression de DCC auraient comme conséquence de causer des altérations dans l'organisation et le fonctionnement des circuits mesocorticolimbiques de la DA, a été évaluée. Une série d'expériences comparant les souris portant une mutation hétérozygote pour le gène *dcc* et les souris du type sauvages dans divers index de fonctionnement du système DA ont été entreprises à differentes âges soit : juvéniles, péri-puberté, et chez l'adultes. Les résultats ont indiqués que les souris adultes qui développent avec des niveaux réduits de DCC pendant le développement démontrent une réorganisation du circuit DA spécifiquement dans le circuit qui innerve le cortex préfrontal médial (mPFC), démontrent un hyperfonction DA dans le mPFC ainsi qu'un phénotype

comportemental qui est opposé au phénotype observé dans les modèles animaux neurodevelopmentales putatifs de la schizophrénie. En outre, aucun des traits phénotypiques observés chez les souris adultes portant une mutation hétérozygote dans DCC ne sont évident avant l'adolescence, une période pendant laquelle les circuits DA innervant le mPFC subissent une réorganisation extensive et un raffinement fonctionnel. Ces résultats suggèrent que des changements dans le fonctionnement de DCC pourraient augmenter ou diminuer la susceptibilité de l'individu au développement de la schizophrénie. En premier lieu , une étude d'association génétique de cas-témoins comparant les fréquences de génotypes et d'allélomorphes des polymorphismes simples de nucléotide situés dans le gène de DCC entre les patients schizophrènes et les sujets témoins en bonne santé a été entreprise. Les résultats de cette étude indiquent la présence d'une association entre la schizophrénie et le polymorphisme rs2270954 situé dans la région « 3' untranslated » du gène de DCC. Dand ensemble les résultats impliquent DCC i) comme étant un facteur déterminant dans l'organisation du système mesocorticolimbique DA et le fonctionnement de ce système chez l'adulte, ii) comme ayant un rôle important, inconnu auparavant, dans la maturation normale des circuits DA innervant le mPFC pendant l'adolescence, et iii) comme étant un nouveau gène candidat qui pourrait contribuer à la base génétique des différences dans la susceptibilité à la schizophrénie entre individus.

Contribution of authors

Chapter II-Research Manuscript: Grant *et al,* 2007

Concieved and designed experiments: A. Grant, C. Labelle-Dumais, B. Kolb, A. Arvanitogiannis, and C. Flores

Preformed the experiments: A. Grant, D. Hoops, C. Labelle-Dumais, M. Prevost, H. Rajabi

Analysed data: A. Grant, C. Labelle-Dumais, A. Arvanitogiannis, and C. Flores

Wrote the manuscript: A. Grant, C. Labelle-Dumais, A. Arvanitogiannis, J. Stewart, and C. Flores

Provided equipment and reagents: A. Arvanitogiannis, B. Kolb, and J. Stewart

Chapter III-Research Manuscript: Grant *et al,* 2009

Concieved and designed experiments: A. Grant and C. Flores

Preformed the experiments: A. Grant, Z. Speed, C. Labelle-Dumais

Analysed data: A. Grant and Z. Speed

Wrote the manuscript: A. Grant and C. Flores

Chapter IV-Research Manuscript: Grant *et al*, in prep

Concieved and designed experiments: A. Grant, F. Fathalli, R. Joober, and C. Flores

Preformed the experiments: A. Grant and F. Fathalli

Analysed data: A. Grant and F. Fathalli

Writing of the manuscript: A. Grant and C. Flores

Provided equipment and reagents: G. Rouleau and R. Joober

List of figures

List of tables

Overview

It has been decades since it was first proposed, yet the hypothesis that dysregulation of the mesocorticolimbic dopamine (DA) system is central to the symptomatology of schizophrenia remains as one of the most enduring ideas about the disorder. This "dopamine hypothesis" of schizophrenia posits that malfunction of DA in the medial prefrontal cortex contributes to the cognitive symptoms and enhanced DA function in the ventral striatum (i.e. nucleus accumbens) contributes to the psychotic symptoms. Within the past 20 years, there has been a growing body of evidence that supports the idea that schizophrenia is a neurodevelopmental disorder in which genetic and environmental adverse events that occur early in life alters the normal course of brain development (Weinberger 1987). Because of the role for DA in the symptomatology of schizophrenia, it stands to reason that these events may disrupt the normal course of development of the mesocorticolimbic DA system, thereby resulting in the DA-related cognitive and behavioural abnormalities that become evident in adulthood. Indeed, laboratory animals exposed to manipulations modeling these early developmental insults (i.e. birth anoxia, maternal infection, and peri-natal ventral hippocampal lesions) exhibit abnormalities in DA function and DA-mediated behaviours in adulthood (Boksa 2004; Lipska et al. 1993; Ozawa et al. 2006). However, *how* these early events result in such enduring changes in DA function is unknown. One possibility is that they may alter the course of DA system development by affecting the

function of proteins that are involved in the wiring of this system. Thus, the identification of the mechanisms and molecular players involved in the development and connectivity of this neural network is a critical step in understanding how these adverse events are able to exert their long-lasting effects on DA function.

The development of the DA innervation to the medial prefrontal cortex is a very slow process (Benes et al. 2000; Kalsbeek et al. 1988). Furthermore, this projection undergoes extensive functional remodelling and refinement during the peri-adolescent period (Spear 2000). Intriguingly, the symptoms of several psychiatric disorders associated with altered functioning of the mesocortical DA system, including schizophrenia, only become evident during, or following, this period of substantial reorganization. Therefore, the risk factors that are associated with DA-related psychopathology that manifest in adulthood may be affecting or interacting with the mesocortical DA developmental processes that are occurring during the adolescent period. As such, it is not only important to identify the mechanisms involved in prenatal development of the mesocorticolimbic DA system, but also to elucidate those that play a selective role in the adolescent development of the mesocortical DA system. The experiments presented in this Thesis were aimed at beginning to address this issue.

In this Thesis I argue that alterations in the functioning of netrin-1, a guidance cue that is highly expressed by mesocorticolimbic DA neurons and its target areas, may be a mechanism underlying the selective organization of mesocortical DA circuitry that occurs during adolescence. In Chapter I, the Introduction, I describe key differences between the development and function of

mesolimbic and mesocortical DA systems and provide the rationale behind the idea that alterations in netrin-1 signalling may be involved in the wiring of the mesocorticolimbic DA system. Then in Chapter II, I present experimental evidence showing that the function of the mesolimbic and mesocortical DA systems is indeed altered by varying the levels of the netrin-1 receptor, DCC. Specifically, I demonstrate that adult mice that develop with reduced levels of DCC, display selective reorganization of mesocortical DA circuitry, show enhanced mesocortical DA function, and exhibit a behavioural phenotype opposite to that observed in animal models of schizophrenia. In Chapter III, I show that the emergence of these DA and behavioural phenotypes coincides with the maturational events that occur to the medial prefrontal cortex during the adolescent period, pointing to a potential novel role for netrin-1 signalling in the normal maturation of the mesocortical DA system during adolescence. Taken together, the data I present in Chapters II and III indicate that variations in DCC function may, at least in part, underlie individual differences in susceptibility to the development of psychopathology. Finally, in Chapter IV, I provide evidence to support the idea that *DCC* may be a novel candidate gene associated with differential vulnerability to develop schizophrenia.

Chapter I: Introduction

I.1. Mesocorticolimbic DA system

1.1. Anatomical organization: mesolimbic and mesocortical DA projections

The DA neurons of the ventral tegmental area of the midbrain (VTA; nucleus A10) are comprised of two main subpopulations of cells that give rise to two primary projections, the mesolimbic and mesocortical DA projections (see Fig. I-1). One population, the mesolimbic DA neurons, projects to limbic structures, primarily to the nucleus accumbens (NAcc), but also to the septum, olfactory tubercle, hippocampus, amygdala, and habenular nuclei (Fallon and Loughlin 1987b; Phillipson and Pycock 1982)**.** The second group of cells, the mesocortical DA neurons, targets mainly the medial prefrontal cortex (mPFC), but also innervates some areas of the ventral surface of the frontal lobe including the orbitofrontal cortex (Bjorklund and Lindvall 1984; Fallon and Loughlin 1987b). DA innervation is the densest in the deep layers of the mPFC (Layer V-VI) (Descarries et al. 1987). Interestingly, it is from layer V that the mPFC glutamatergic projection to medium spiny neurons of the NAcc arises (Brog et al. 1993; Pinto and Sesack 2000). Importantly, DA synapses are observed on these NAcc-projecting pyramidal neurons (Carr et al. 1999). Please note that throughout the remainder of this Thesis I will use the term "mesolimbic" to refer to the DA projection to the NAcc and the term "mesocortical" to refer to the DA projection to the mPFC.

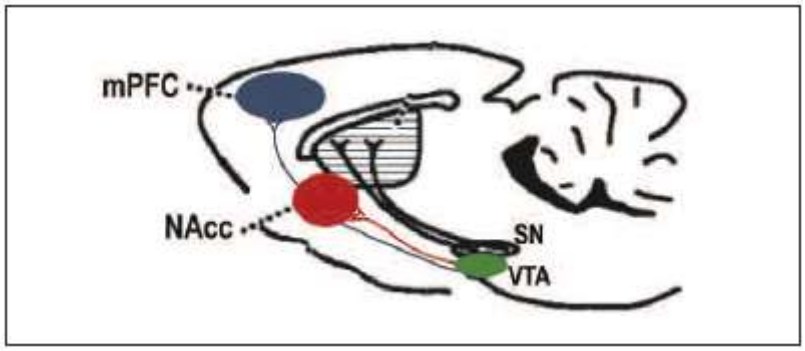


Fig. I-1. Mesocorticolimbic dopamine (DA) system

The cartoon represents the rodent brain shown in sagittal view. The DA projection from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAcc) is shown in red and that to the medial prefrontal cortex (mPFC) is illustrated in blue. The nigrostriatal DA pathway (projection from the substantia nigra [SN] to the dorsal striatum) is also depicted (in black). *Modified from Flores, 2011*.

The mesolimbic and mesocortical projections are anatomically distinct. For example, DA neurons that project to the mPFC, NAcc core, and NAcc medial shell are anatomically segregated from those that project to the lateral shell of the NAcc (Lammel et al. 2008). Furthermore mesolimbic and mesocortical DA neurons receive different glutamatergic inputs; the dense glutamate innervation from the mPFC to the VTA selectively innervates DA neurons that project back to the mPFC, but not those that project to the NAcc (Carr and Sesack 2000b). Mesolimbic DA neurons receive direct excitatory inputs from the laterodorsal tegmentum (Lodge and Grace 2006; Omelchenko and Sesack 2005), pedunculopontine tegmental nuclei (Charara et al. 1996; Floresco et al. 2003; Lokwan et al. 1999; Scarnati et al. 1986), bed nucleus of the stria terminalis (Georges and Aston-Jones 2001; 2002), and lateral habenula (Brinschwitz et al. 2010; Geisler et al. 2007; Omelchenko et al. 2009). The NAcc and mPFC also receive GABAergic afferents from the VTA. Interestingly, there is also selectivity in the glutamatergic inputs onto these VTA GABA projecting neurons (Carr and Sesack 2000a; Van Bockstaele and Pickel 1995). For example glutamatergic afferents from the mPFC innervates GABAergic neurons in the VTA that project to the NAcc, but not those that project to the mPFC (Carr and Sesack 2000b). The functional significance of this selectivity in innervation of these GABA neurons is not yet understood as little is known about the GABAergic mesoaccumbal projection (Carr and Sesack 2000b).

1.2. Functional differences between mesolimbic and mesocortical DA projections

Mesolimbic and mesocortical DA neurons are also functionally distinct from one another. Whereas mesolimbic DA neurons have a dense terminal density and rely on the dopamine transporter for reuptake, mesocortical DA cells have a much lower terminal density and reuptake of DA occurs primarily via the norepinephrine transporter (Garris and Wightman 1994; Lammel et al. 2008; Moron et al. 2002; Sesack et al. 1998; Tanda et al. 1997). Studies in catechol-O-methyltransferase (COMT) knock-out mice have shown that COMT is one of the main mechanisms of degradation of synaptic DA in the mPFC, but not in the NAcc. In comparison to wild-type mice, COMT knock-out mice exhibit selective increases in concentration and elimination time of mPFC DA (Gogos et al. 1998; Yavich et al. 2007).

Mesolimbic and mesocortical DA neurons also exhibit different patterns of firing. In vivo voltammetry experiments in rats have demonstrated that relative to other DA neurons, mesocortical DA neurons have more of a sustained pattern of firing and, therefore, of DA release (Garris and Wightman 1994). This persistent pattern of activity may be explained by the fact that mesocortical DA neurons, but not mesolimbic DA neurons, lack functional somatodendritic D2 autoreceptors (Bannon et al. 1983; Chiodo et al. 1984; Lammel et al. 2008). Furthermore, mesocortical DA neurons, but not mesolimbic DA neurons, express very low mRNA levels of D2 and of GIRK2, the downstream target of D2 autoreceptors (Lammel et al. 2008). Finally, kappa opioid receptor agonists have been shown to

selectively inhibit firing of mesocortical DA neurons, resulting in decreased DA levels in the mPFC and no change in DA concentration in the NAcc (Margolis et al. 2006). These findings clearly demonstrate that mesolimbic and mesocortical DA neurons can be modulated independently of one another. Thus, mesolimbic and mesocortical DA neurons constitute two separate subpopulations of DA neurons that are anatomically, structurally and functionally distinct.

1.3. Inverse functional relationship between mesocortical and mesolimbic DA projections

Since mesolimbic and mesocortical DA neurons receive different input and possess inherent functional characteristics, it is not surprising then that a single manipulation can have divergent effects in these two projections. Although the findings are not completely consistent, there is substantial evidence from pharmacological, stress and lesion experiments in laboratory animals demonstrating that mesolimbic and mesocortical DA activity can be regulated in opposite directions. For example, local injections of the D1 receptor antagonist SCH23390 directly into the NAcc has been shown to decrease the rewarding effects of VTA electrical stimulation, whereas the injection of the same compound into the mPFC increases the rewarding effects (Duvauchelle et al. 1998). In a similar vein, infusion of DA agonists in the NAcc results in enhancement of intracranial self stimulation (ICSS) of the median forebrain bundle (MFB), whereas infusion of DA agonists in the mPFC reduces MFB ICSS (Olds 1990). Additionally, Gratton and colleagues have shown that adult animals

previously exposed to hypoxic conditions at birth (an animal model for obstetric complications), exhibit an increase in DA activity in the NAcc, but blunted release of DA in the right mPFC, in response to repeated stress (Brake et al. 1997; Brake et al. 2000). Importantly, obstetric complication is among the most significant risk factors for developing schizophrenia, with a relative risk estimate of approximately 2.0 (Cannon et al. 2002; Geddes and Lawrie 1995). With this in mind, it is interesting to note that similar to the observation in laboratory animals by Gratton and colleagues, in some individuals with schizophrenia, there is exaggerated striatal DA release in response to amphetamine and evidence for blunted DA activity in the mPFC (Abi-Dargham 2003; Abi-Dargham et al. 2009; Howes et al. 2009; Kegeles et al. 2010; Meyer-Lindenberg et al. 2002).

Many pharmacological and lesion studies suggest that this functional inverse relationship between NAcc and mPFC DA activity observed in adult rodents may stem, at least in part, from the fact that mesocortical DA activity exerts an inhibitory effect on mesolimbic DA functioning (Banks and Gratton 1995; Deutch et al. 1990a; Mitchell and Gratton 1992; Pycock et al. 1980; Rosin et al. 1992; Thompson and Moss 1995b; Ventura et al. 2004; Vezina et al. 1991). For example, reducing DA in the mPFC through 6-hydroxydopamine (6-OHDA) lesions dramatically increases drug-induced DA release in the NAcc (Ventura et al. 2004). On the other hand, activation of mPFC DA receptors decreases stress- and drug-induced NAcc DA release (Banks and Gratton 1995; Doherty and Gratton 1996; Vezina et al. 1991). Furthermore, microinfusions of the DA agonists apomorphine or amphetamine directly into the mPFC results in reduced extracellular concentration of the DA metabolite 3,4- dihydroxypheynlacetic acid

(DOPAC) in striatal regions (Jaskiw et al. 1991; Louilot et al. 1989). Conversely, local administration of DA antagonists sulpiride or flupenthixol in the mPFC is accompanied by an elevation in the concentration of DOPAC in the NAcc (Louilot et al. 1989). It is important to note that although Jaskiw *et al* specify that they measured DA and DA metabolite release in the caudate putamen, the microdiaysis probe they used extended 7.5 mm below dura, and therefore, was most likely sampling both the caudate putamen and the shell of the NAcc (Paxinos and Watson 1998). These fluctuations in extracellular DOPAC concentration in the NAcc induced by altering DA activity in the mPFC are also accompanied by changes in behaviour. For example, increasing DA function in the PFC by local infusion of agonists prevents the behavioural activating effects of systemically administered amphetamine (Broersen et al. 1999). Taken together, these data suggest that the inverse relationship between mesocortical and mesolimbic DA functioning does stem, at least in part, from the inhibitory influence of mPFC DA activity on DA activity in the NAcc.

1.4. Development of mesolimbic and mesocortical DA systems in rodents

1.4.1. Prenatal development (Embryonic day (E)13 to parturition)

In rodents, neurogenesis and differentiation of DA cells in the midbrain begins between embryonic day (E)12-15 (Altman and Bayer 1981; Smidt and Burbach 2007). By E15, the axons of these neurons begin to innervate the ganglionic eminence, the structure that will give rise to striatal structures (Voorn

et al. 1988). Between E15-E17, some of these DA fibres extend beyond the ganglionic eminence and by E19-E20, these fibres reach the cortical sub-plate and begin to innervate what will be the future prefrontal cortex (Kalsbeek et al. 1988).

1.4.2. Post-natal- early life period (Post-natal day (PND)0 – PND21)

NAcc:

At E19, patches of dense DA immunoreactivity are observed in the developing striatum and by PND2, DA fibres are observed in both the dorsal and ventral striatal compartments. From PND4 onwards, DA innervation continues to increase throughout the rostral-caudal axis of the developing NAcc and reaches adult distribution patterns by PND20. During this period, varicosities, sites where DA synthesis, release and reuptake most often occur, become visible and increase in number until PND20, when adult levels are obtained (Benes et al. 1996; Voorn et al. 1988).

mPFC:

At birth, DA-positive fibres are visible in the developing deep layers of the cortex. Although there is a dramatic increase in number of these fibres in the first few days following birth, they still remain relatively sparse. By PND4 the developing infralimbic, prelimbic, and cingulate cortices can be demarcated from other cortical regions based on density of DA fibres. At this period there is also a dramatic change in the morphology of the DA fibres. During embryonic ages and shortly after birth these fibres are thick, but by PND4 they become very thin and contain only a few irregularly spaced varicosities. By PND12, the general

topological pattern of DA innervation to the developing cortex is established, although the density of these fibres and the number of varicosities on the fibres are scarce (Kalsbeek et al. 1988).

1.4.3. Juvenile (post-weaning) period (PND21-PND28)

Unlike the NAcc, that reaches adult innervation patterns by the beginning of the juvenile period, mPFC DA innervation continues throughout this stage of development. During juvenile ages, the density of DA fibres in the mPFC increases substantially (Benes et al. 2000; Kalsbeek et al. 1988). This period is also characterized by a dramatic over-production of D1 and D2 receptors in both the NAcc and mPFC (Andersen et al. 1997; Andersen et al. 2000).

1.4.4. Adolescence period (PND30-PND56)

Definition of "adolescence:"

The terms "adolescence" and "puberty" are often, incorrectly, used interchangeably. Adolescence refers to the developmental period between juvenility to adulthood, whereas puberty refers to the achievement of sexual maturity (Spear 2000). For the purposes of this Thesis I will use the term "adolescence" to refer to the period between PND28-PND56 (Andersen 2003; Spear 2000). The terms "peri-pubertal" and "peri-puberty" refer to the more restricted age span during which C57BL6 mice begin to reach sexual maturation (PND31-PND35) (Gall and Kyle 1968; Gore et al. 1999; Morley and Rodriguez-Sierra 2004; Oakberg 1957).

Developmental events occurring during adolescence:

During adolescence, the rodent mesocortical DA system and its terminal regions undergo substantial reorganization (Spear 2000). For instance, there is considerable pruning of synapses that is accompanied by a decrease in the grey matter volume of the mPFC in rats (van Eden et al. 1990). Furthermore, there is a substantial increase in the density of the DA innervation to this region that occurs throughout adolescence and continues into early adulthood (Benes et al. 2000; Kalsbeek et al. 1988).

In addition, there is also evidence for functional refinement of the mesocortical DA system during the adolescent period. Andersen and colleagues have demonstrated in rats that there is an overproduction of D1 and D2 receptors in the mPFC that begins during the juvenile period and peaks in mid-adolescence (PND40). This overproduction of DA receptors is followed by substantial pruning that begins during late adolescence and continues into early adulthood (PND100) (Andersen et al. 2000; Teicher et al. 1995). Importantly, these changes in DA receptors that occur throughout the juvenile and adolescent periods appear to be independent of pubertal changes in gonadal hormone levels (Andersen et al. 2002).

Many of the developmental events occurring during adolescence described for rodents have also been observed in humans and non-human primates. For example, both the substantial pruning of synapses and the reduction in PFC

cortical volume have been demonstrated to also transpire during human adolescence (Huttenlocher 1979; Mrzljak et al. 1990; Sowell et al. 1999).

1.5. Altered development and functioning of mesocorticolimbic DA systems: implications for schizophrenia

The extensive reorganization of mPFC DA circuitry and changes in its activity that occur during adolescence is intriguing in light of the fact that many symptoms of psychiatric disorders related to the functioning of this system only manifest during (or following) this period (Andersen 2003). For example, overt symptomatology of schizophrenia typically emerges in late adolescence and early adulthood, coinciding with the occurrence of the aforementioned mPFC maturational events. This observation has led to hypotheses that these developmental events either are aberrant in schizophrenia (Feinberg 1982) or they unmask a pre-existing predisposition (Weinberger 1987b). However, it is important to remember that the relationship between the emergence of symptoms and the developmental events occurring to the mPFC DA system is correlational and that causality cannot be inferred. We cannot rule out the alternative possibility that innate alterations in striatal DA pathways results in mPFC DA dysfunction. Indeed, it has recently been demonstrated that mice that develop with increased expression of D2 receptors in striatal regions demonstrate reduced DA functioning in the mPFC as well as deficits in cognitive functioning in adulthood (Kellendonk et al. 2006; Simpson et al. 2010).

1.5.1. Schizophrenia as a neurodevelopmental disorder

There are a number of independent lines of research that suggest that subtle disruptions in normal brain development results in an increased predisposition to develop schizophrenia or related symptoms (Lewis and Lieberman 2000). First, early adverse environmental events such as obstetric complications and maternal infection during the prenatal period occur more frequently in individuals with schizophrenia (Brown et al. 2004; McNeil et al. 2000). Second, minor physical anomalies, such as alterations in head circumference, ocular hyperteliorism, and dermatoglyphics, are thought to be vestigial markers of developmental insults occurring during gestation at a time when craniofacial, limb, and brain development are happening simultaneously (Compton et al. 2011). Incidences of such minor physical anomalies are higher in schizophrenic patients and their first-degree relatives as compared to healthy controls (Boyes et al. 2001; Cantor-Graae et al. 1994; Daly et al. 2008; Green et al. 1989; Gualtieri et al. 1982; O'Callaghan et al. 1991; Rosa et al. 2005). Furthermore, neurological "soft signs" are also common in schizophrenia and are evident prior to the manifestation of psychotic symptoms and in drug-naïve patients (Aksoy-Poyraz et al. 2011; Boks et al. 2004; Gupta et al. 1995; Keshavan et al. 2003; Scheffer 2004; Walker and Lewine 1990). Third, there is evidence for abnormalities in cell migration in the frontal and temporal lobes of schizophrenic patients (Akbarian et al. 1993a; Akbarian et al. 1993b). Finally, the observations of reduced neuropil and cortical thickness also support the role for neurodevelopmental alterations in the aetiology of the disorder (Selemon and Goldman-Rakic 1999; Selemon et al. 1995; 1998). Of relevance for this Thesis,

this seminal work by Goldman-Rakic was conducted on samples of the dorsolateral prefrontal cortex (dlPFC: Brodmann's Areas 9 and 46), the region of the human frontal lobe that is structurally and functionally homologous to the rodent mPFC (Brown and Bowman 2002; Dalley et al. 2004; Ongur and Price 2000; Uylings et al. 2003). These findings of reduced neuropil and cortical thickness may be related to faulty cortical synaptic elimination during adolescence and may explain why the symptoms of the illness emerge around this period (Feinberg 1982). Importantly, the absence of obvious histological evidence of neurodegeneration, such as gliosis or inclusion bodies, suggests a neurodevelopmental, rather than neurodegenerative, nature of the disorder (Rapoport et al. 2005).

1.5.2. Evidence of sensitized mesolimbic DA function in schizophrenia

One of the most enduring hypotheses in neuroscience is the so-called "dopamine hypothesis" of schizophrenia. This hypothesis arose from the discovery of a direct correlation between clinical efficacy of neuroleptic medications and their ability to antagonize DA receptors (Creese et al. 1976; Seeman and Lee 1975). Other pharmacological studies and clinical observations indicate that drugs that increase extracellular DA concentrations in striatal and cortical areas, such as amphetamines, can elicit psychotic symptoms that are indistinguishable from schizophrenia in otherwise healthy individuals (Lieberman et al. 1987; Yui et al. 2000). Furthermore, in a subset of schizophrenic patients, amphetamine can evoke or exacerbate psychotic episodes at doses which are not psychotogenic in healthy controls (Lieberman et al. 1987). In corroboration with

these findings, imaging studies demonstrate that in comparison to healthy controls, many individuals with schizophrenia show enhanced striatal DA release in response to amphetamine and that this exaggerated response correlates with the exacerbation of psychotic symptoms (Abi-Dargham et al. 1998; Abi-Dargham et al. 2009; Breier et al. 1997; Laruelle et al. 1996; Meyer-Lindenberg et al. 2002). Additionally, under baseline conditions, many schizophrenic patients have higher occupancy of the D2 receptor in striatal areas, suggesting excess DA in these regions (Abi-Dargham et al. 2000). The hypersensitivity to amphetamine witnessed in schizophrenic patients is consistent with the notion that there is sensitized mesolimbic DA functioning in schizophrenia. This increased sensitivity of the mesolimbic DA system may also explain the high comorbidity of schizophrenia with substance use disorders, as the rewarding effects of drugs are thought to be mediated by enhanced mesolimbic DA release (Chambers et al. 2001).

1.5.3. Evidence of blunted mesocortical DA function in schizophrenia

It has now become clear that schizophrenia does not simply reflect a hyperdopamingeric state. Other neurotransmitters are likely to be involved; atypical antipsychotic medications such as clozapine have low affinity for DA receptors and yet seem to be as clinically effective as the high-affinity traditional neuroleptics (Jones et al. 2006; Lieberman et al. 2005). Moreover, the level of DA metabolites in the cerebral spinal fluid (CSF) of patients is not consistently elevated, and in fact, is reduced in a subset of patients (Pickar et al. 1990; Widerlov 1988). Drawing on the fact that DA metabolite levels in the non-human

primate PFC is the only brain region that significantly correlates with CSF DA metabolite levels (Elsworth et al. 1987), Davis et al (1991) posited that the reduced concentration of DA metabolites observed in some patients with schizophrenia reflects hypoactivity of the mesocortical DA system. This led to their "revised" dopamine hypothesis of schizophrenia in which they suggested that there is elevated DA activity in striatal regions, but blunted DA function in the PFC. Furthermore, because of the inverse relationship between mesocortical and mesolimbic DA function observed in laboratory animals, Davis et al further postulated that reduced PFC DA function may actually lead to excessive activity in mesolimbic DA neurons (Davis et al. 1991).

There is no *direct* evidence to support reduced DA activity in the PFC of schizophrenic patients. However, there is evidence to suggest that there is decreased DA innervation to the deep layers (layer V/VI) of the dlPFC in some schizophrenic patients (Akil et al. 1999). This area of the PFC is critical for a number of cognitive functions. Individuals with schizophrenia do demonstrate deficits in many cognitive tasks including working memory (Bunney and Bunney 2000). Importantly, working memory has been shown to be dependent on intact DA functioning in the dlPFC of non-human primates and in the mPFC of rodents (Brozoski et al. 1979; Phillips et al. 2004; Sawaguchi and Goldman-Rakic 1991; 1994). Other studies that combined imaging techniques and cognitive tasks provide evidence for decreased activation of the dlPFC in schizophrenic patients relative to control subjects (Bertolino et al. 1999; Meyer-Lindenberg et al. 2002; Weinberger et al. 1986). Furthermore, the degree of decreased activation within the PFC is significantly correlated to the extent of exaggerated subcortical DA

function (Meyer-Lindenberg et al. 2002). Taken together, these data do suggest that there may be reduced DA function in the dlPFC of schizophrenic patients.

1.6. Neurodevelopmental animal models of schizophrenia

In an attempt to better understand the etiology and pathophysiology of schizophrenia, various neurodevelopmental animal models have been developed. Some of these animal models, such as the maternal infection and birth anoxia paradigms, are designed to mimic epidemiological risk factors for schizophrenia, while others, such as the neonatal ventral hippocampal lesion (nVHL) and methylazoymethanol acetate (MAM) administration models, are aimed at understanding the neuropathology of the disorder (Wilson and Terry 2010). Evidence for enhanced mesolimbic DA function has been observed in adult laboratory animal exposed to prenatal immune challenge (Ozawa et al. 2006), anoxic conditions at birth (Brake et al. 1997), lesions of the ventral hippocampus (Lipska et al. 1993), or to prenatal MAM administration (Flagstad et al. 2004). The changes in mesolimbic function seen in these animals are accompanied by schizophrenia-like behavioural abnormalities, such as impairments in sensorimotor gating, working memory, and increased sensitivity to the locomotor-activating effect of stimulant drugs (Aguilar-Valles et al. 2010; Fortier et al. 2004; Lipska et al. 1993; Ozawa et al. 2006). Furthermore, some additional aspects of schizophrenia are reproduced by the nVHL model, including peri-adolescent emergence of the changes in DA function and DA-related behaviours (Lipska et al. 1993), improvement of behavioural abnormalities by neuroleptic

administration (Lipska 2004), alterations in mPFC DA circuitry (Flores et al. 2005b), and enhanced vulnerability to the development of drug addiction (Brady et al. 2008; Tseng et al. 2009).

I.2. Netrin-1

While there is literature that describes the differences in development of mesolimbic and mesocortical DA systems, there is little knowledge regarding the cellular mechanisms that underlie the specification and organization of mesolimbic versus mesocortical DA connectivity. The establishment of neuronal circuitry is accomplished via the actions of guidance cues. One guidance cue, netrin-1, is highly expressed by mesocorticolimbic neurons and its target areas, and therefore, may play a fundamental role in the selective organization of these two systems.

2.1. Structure of netrin protein family

The netrin family of secreted proteins function as guidance cues for migrating cells and neural processes during neural development, and therefore has a pivotal role in the establishment of brain circuitries (Barallobre et al. 2005; Manitt and Kennedy 2002). Netrins are phylogenetically conserved and are structurally related to laminin, an extracellular glycoprotein that is an integral part of the cell associated extracellular matrix. Figure I-2 compares the structures of laminin and netrin protein families (see Fig I-2). The N-terminal region of netrins

contains a domain called VI, which is homologous to the laminin globular domain VI (Fig 1-2). The C-terminal sequence is enriched in basic residues that can act as binding sites for components of the cell surface and for the extracellular matrix (Kappler et al. 2000).

2.2. Netrin as a long-range and short-range guidance cue

The affinity that netrin has for the local extracellular matrix (ECM) and/or plasma membrane, affects how readily netrin can diffuse away from the cells that secrete it (for a comprehensive review see (Barallobre et al. 2005; Kennedy 2000; Manitt and Kennedy 2002). In the case where the affinity is low, netrin can diffuse from its source, establish concentration gradients, and act as a chemotrophic signal over long distances. Conversely, if the affinity for the local ECM and plasma membrane of the netrin-secreting cells is high, netrin will function as a short-range cue, affecting the wiring events that occur following successful axonal pathfinding (Colon-Ramos et al. 2007; Mitchell et al. 1996; Poon et al. 2008; see section 2.4.3 of this Chapter).

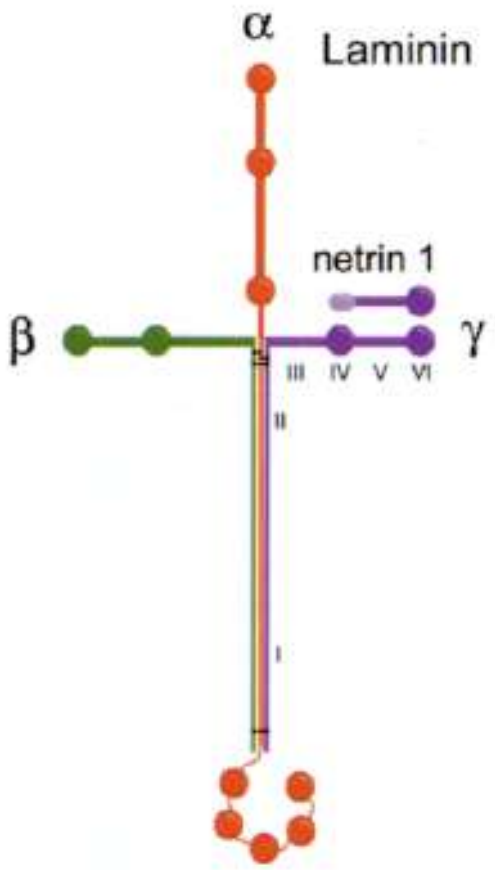


Fig. I-2. Schematic illustrating the structure of laminin and netrin-1

Laminins are trimeric proteins that are a major constituent of the basal lamina. Each laminin polypeptide consists of an α-chain (red), a β-chain (green), and a γ-chain (purple) and is composed of domains I-VI. Netrin-1 contains two regions that share ~ 50% amino acid sequence identity with domains V and VI of laminin γ-chain. *Modified from Manitt & Kennedy, 2002*.

2.3. Netrin-1 as a bifunctional cue

The best-studied member of the netrin family of proteins is the mammalian netrin-1. Netrin-1 is important for both the outgrowth and guidance of commissural axons toward the midline (Kennedy et al. 1994; Serafini et al. 1996). Studies in *netrin-1* deficient mice demonstrate that netrin-1 is critical for midline guidance of forebrain commissures as the corpus callosum, hippocampal commissure, and anterior commissure are all absent in these mice (Kennedy et al. 1994; Serafini et al. 1996). In addition to functioning as an attractive cue for commissural axons toward the midline, results of *in vitro* studies indicate that netrin-1 is also capable of repelling trochlear and craneal motor axons (Colamarino and Tessier-Lavigne 1995; Guthrie and Pini 1995). Netrin-1, therefore, is an example of a bifunctional cue that is capable of attracting some axons while repelling others. How an axon or dendrite responds to a source of netrin depends on the balance of netrin-1 receptors expressed by that process (see next section).

2.4. Netrin-1 receptors

There are two families of netrin-1 receptors, the deleted in colorectal cancer (DCC) and the UNC5 homologue (UNC5H) protein families. Both DCC and UNC5H families of receptors are single-pass transmembrane proteins belonging to the immunoglobulin superfamily of proteins (see Fig I-3). In the presence of netrin-1, DCC and UNC5H recruit Rho GTPases, downstream

proteins that transduce extracellular signals into actin cytoskeletal dynamics (Barallobre et al. 2005). The resultant remodelling of the actin cytoskeleton is the underlying cellular mechanism through which netrin-1 affects the motility of neuronal processes.

It is generally accepted that netrin-1-mediated attraction involves the multimerization of DCC receptors whereas repulsion occurs through the formation of DCC-UNC5H receptor complexes or through UNC5 alone (Hong et al. 1999; Keino-Masu et al. 1996; Keleman and Dickson 2001). It is the ratio of receptors expressed at the cell surface that dictates how an axon or dendrite will respond to a source of netrin-1. For example, Bouchard *et al* showed that increasing the availability of DCC to the plasma membrane in cultured rat embryonic spinal commissural neurons results in increased axon outgrowth and chemoattractive turning to an exogenous source of netrin-1 (Bouchard et al. 2004). Likewise, endocytosis of an UNC5 homologue, UNC5A, converts the responses of axons of cultured hippocampal and cerebellar granule neurons from chemorepulsion to chemoattraction (Bartoe et al. 2006; Williams et al. 2003b). Furthermore, a "switch" in the balance of receptors at the membrane can also affect wiring events that take place after guidance to the target. A recent study demonstrates that guidance of mossy fibres to the CA3 region of the hippocampus is initially established through DCC-mediated netrin-1 signalling. Subsequent increases in neuronal activity, however selectively recruits UNC5A to the membrane, resulting in a conversion from netrin-1 mediated chemoattraction to repulsion and the initiation of sprouting of these axons (Muramatsu et al. 2010).

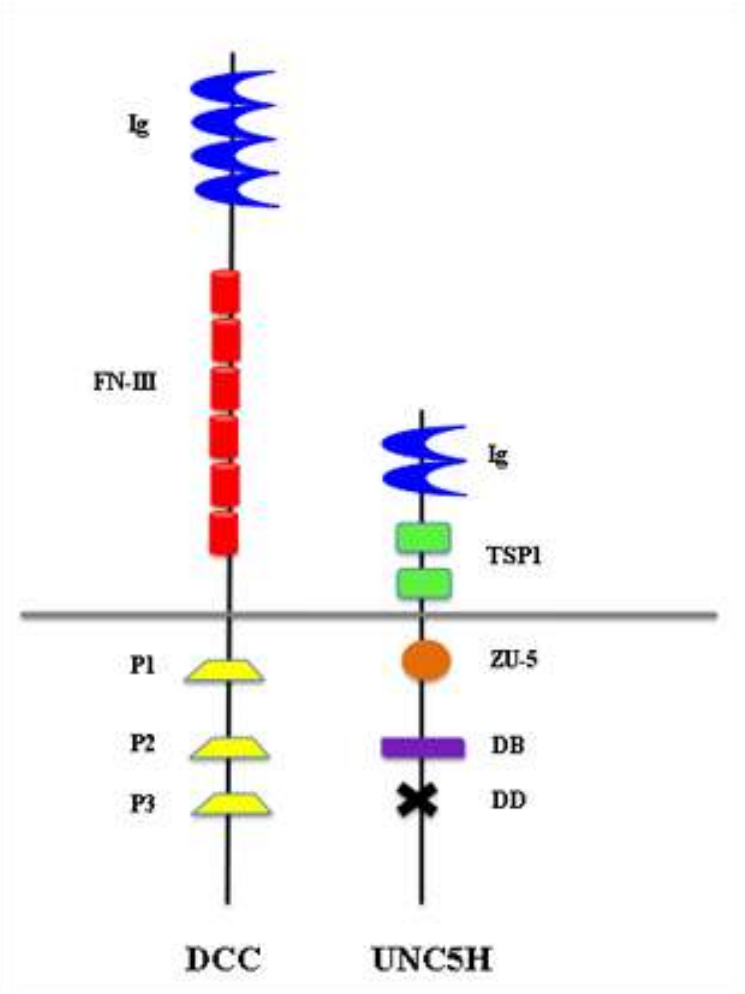


Fig. I-3. Schematic illustrating the structure of netrin-1 receptors

Cartoon illustration of DCC is shown on the left, UNC5H on the right. The horizontal grey line represents the cell membrane.

Abbreviations: Ig, Immunoglobulin-like domain; FN-III, Fibronectin type III domain; P1-P3, three conserved regions found in the cytosolic portion of DCC; TSP, Thrombospondin type I domain; ZU-5, *Zona Occludens-1* domain; DD, death domain; DB, DCC-binding domain. *Adapted from Barallobre et al, 2005*.

Therefore, altering the balance of receptors expressed at the cell surface can affect both the normal pathfinding and connectivity of neuronal processes that express netrin-1 receptors.

2.4.1. DCC family of receptors

In vertebrates, there are two members of the DCC family of receptors, DCC and neogenin. Both DCC and neogenin show sequence similarity with neural cell adhesion molecule (NCAM), a glycoprotein expressed on cell surface of neurons and glia that is important for cell adhesion, neurite outgrowth, target recognition, and synaptic plasticity (Baldwin et al. 1996; Doherty et al. 1995). The extracellular portion of DCC and neogenin contains four extracellular immunoglobulin domains followed by six fibronectin type III repeats. The intracellular domain consists of P1, P2, and P3 regions (Fig I-3). While both DCC and neogenin are expressed in the central nervous system, neogenin mRNA is found mainly in embryonic tissue derived from the mesoderm (Gad et al. 1997). Although, no severe defects in axon guidance have been reported in the *neogenin*-deficient mouse, (Leighton et al. 2001; Srinivasan et al. 2003), recent data do suggest a role for neogenin in neural development (De Vries and Cooper 2008). In contrast, *dcc*-knock-out mice display a phenotype very similar to that of the *netrin-1*-deficient mice, suggesting that DCC is the main netrin-1 guidance receptor (Fazeli et al. 1997; Serafini et al. 1996).

It is important to note that in addition to its fundamental role in netrin-1 mediated guidance, DCC may also be a putative tumour suppressor gene. DCC

was first identified on human chromosome18q in a region that is subject to a loss of heterozygosity (LOH) in over 70% of human colorectal carcinomas (Fearon et al. 1990; Vogelstein et al. 1988). Reduced DCC expression, as well as 18q LOH has been observed in a number of other cancers (Mehlen and Fearon 2004). However there is controversy as to whether DCC is an actual tumour suppressor gene since inactivation of DCC in mouse models has failed to demonstrate increased tumorigenesis (Fazeli et al. 1997).

2.4.2. UNC5H family of receptors

There are four homologues that constitute the vertebrate UNC5H family: UNC5A, UNC5B, UNC5C and UNC5D. The extracellular portion of UNC5H receptors consists of two immunoglobulin domains followed by two type I thrombospondin domains. The cytosolic domain of UNC5H receptors contains three conserved regions: a ZU-5 domain, a DB (DCC-binding) domain, and a DD (death domain) located near the carboxy terminus (Fig I-3). In addition to having an important role in cell migration, (Ackerman et al. 1997), guidance of neuronal processes (Finger et al. 2002; Hong et al. 1999), and cell survival (Llambi et al. 2001), UNC5H, particularly UNC5B, is the critical receptor for netrin-1 mediated angiogenesis (Larrivee et al. 2007).

2.4.3. Role of netrin-1 receptors in synaptic connectivity

Netrin-1 receptors contribute to the wiring events that occur following successful axonal pathfinding. It has been shown that DCC-mediated netrin-1

signalling influences axonal arborization and facilitates synapse formation in both vertebrates (Manitt et al. 2009) and invertebrates (Colon-Ramos et al. 2007; Gitai et al. 2003; Lim et al. 1999; Winberg et al. 1998). Furthermore, through UNC5, netrin-1 signalling is important for establishing polarity between the pre-and post-synaptic compartments by actively excluding presynaptic elements (i.e. synaptic vesicles and active zone proteins) from the dendritic domain (Poon et al. 2008). In doing so, UNC5-mediated netrin-1 signalling affects synaptic patterning and may be important for maintaining the fate of newly forming dendrites (Poon et al. 2008). These results are intriguing in light of the observation that netrin-1 and its receptors continue to be expressed in the adult brain (Manitt et al. 2010; Osborne et al. 2005; Yetnikoff et al. 2007). Thus, the continued expression of these developmental proteins by the adult brain may point to their role in the ongoing plasticity of brain circuitries (Flores 2011).

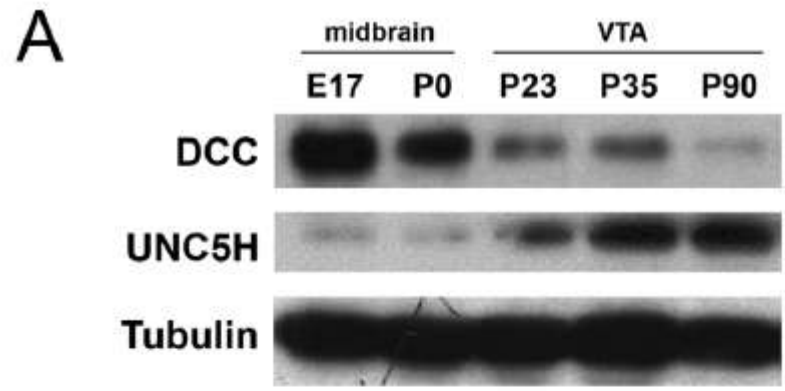
2.5. Expression of DCC and UNC5H proteins by rodent mesocorticolimbic DA neurons

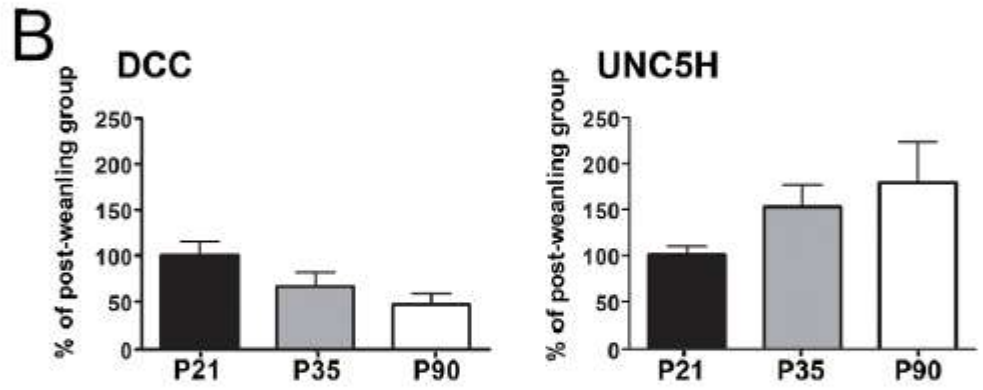
2.5.1. DCC

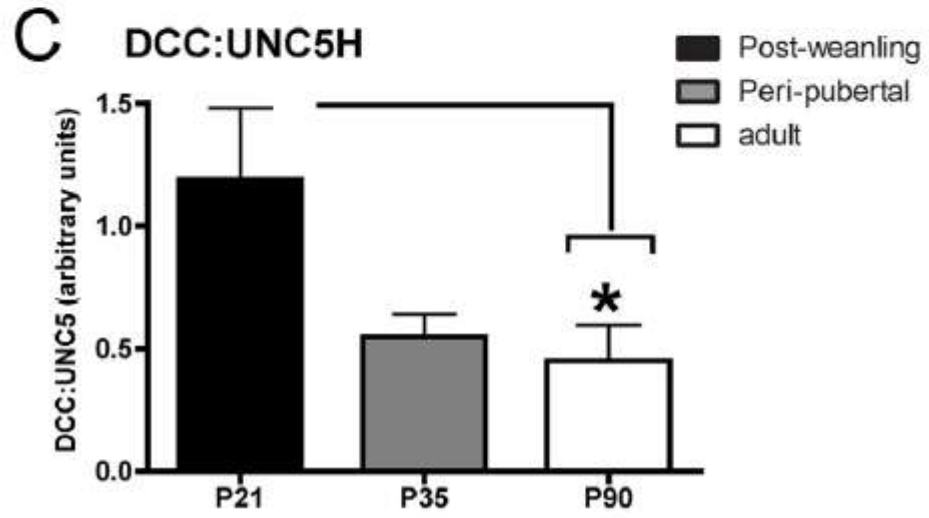
During embryogenesis, expression of *dcc* mRNA is widespread throughout the developing midbrain of wild-type C57BL6 mice from E13 to birth (Gad et al. 1997; Livesey and Hunt 1997). In corroboration with this report, we have shown that DCC protein is highly expressed in the rodent embryonic midbrain (Fig I-4). At birth, DCC immunoreactivity is reduced in comparison to

Fig. I-4. Temporal regulation of DCC and UNC5H expression in the ventral tegmental area of the rodent midbrain

(A) Western blot showing DCC (top), UNC5H (middle) and tubulin (bottom) expression in homogenized tissue lysates from rat midbrain at E7 and P0, as well as lysates of VTA of PND23, PND35, and PND90 rats. (B) Quantitative analysis of Western blots conducted on lysates from VTA of PND21, PND35 and PND90 wild-type BL6 mice. Although the data are expressed as percent of the post-weanling group, all analyses were conducted on raw data and were analysed using oneway ANOVAs. A trend toward a significant effect of age on DCC expression was observed ($F_{2,14}$ = 3.50, p = 0.06); DCC levels decreased progressively between juvenile to adult ages. Conversely, UNC5H expression increased between juvenile and adult periods, although this effect did not reach statistical significance ($F_{2,20}$ = 2.54, p = 0.10). (C) Analysis of data presented in panel B when expressed as the ratio of DCC to UNC5H for each sample (an individual animal). A dramatic shift toward UNC5H predominance occurs between peri-pubertal and adulthood ($F_{2,13}$ = 4.42, p = 0.03). *Reproduced from Manitt et al., 2011*.


A
midbrain
VTA
E17
P0
P23
P35
P90
DCC
UNC5H
Tubulin


B
DCC
% of post-weanling group
250
200
150
100
50
0
P21
P35
P90
UNC5H
% of post-weanling group
250
200
150
100
50
0
P21
P35
P90


C
DCC:UNC5H
Post-weanling
Peri-pubertal
adult
DCC:UNC5 (arbitrary units)
1.5
1.0
0.5
0.0
*
P21
P35
P90

that observed at embryonic ages, but the expression level still remains relatively high. By the post-weaning age, there is a substantial decline in DCC levels. Interestingly, there is a drastic reduction in DCC immunoreactivity that occurs during the pubertal period (Manitt et al. 2010).

2.5.2. UNC5H

Intriguingly, the exact opposite pattern of UNC5H immunoreactivity is observed. During embryonic ages, UNC5H expression is barely detectable in the midbrain. However, by the post-weaning age there is a noticeable increase in UNC5H levels in the VTA. UNC5H expression increases substantially between the post-weaning and peri-pubertal periods (Fig I-4, panel B). Furthermore, when the relative levels of receptor expression within each sample were converted to a DCC:UNC5H ratio, we saw that there was a significant shift toward UNC5H predominance that occurred between puberty and adulthood (Fig I-4 panel C).

It is important to note, that we also conducted double-labelling immunohistochemistry experiments to assess whether mesocorticolimbic DA neurons themselves express DCC and/or UNC5H. Remarkably, while DA neurons express DCC throughout life, expression of UNC5H by these neurons emerges only at the peri-pubertal age (Manitt et al. 2010).

The UNC5H antibody used in the Western blot and immunohistochemistry experiments is reported to recognize UNC5A, UNC5B, UNC5C, and presumably UNC5D (Manitt et al. 2004b; Tong et al. 2001). In order to assess which of the UNC5 homologues are expressed in the VTA, we also preformed RT-PCR

experiments on tissue punches taken from the VTA of adult wild-type mice. Our results indicate that adult mesocorticolimbic DA neurons only express UNC5C and UNC5D homologues (Manitt et al. 2010).

I.3. Rationale

The fact that netrin-1 function is determined by the relative balance of "attractive" and "repulsive" receptors expressed by a particular neuron at a particular time suggests that netrin-1 can organize neuronal connectivity of different populations of neurons in a very selective manner. It is interesting that two functionally distinct pathways with different developmental trajectories (mesolimbic and mesocortical DA systems) arise from a common nucleus (VTA), yet establish very different circuitries. A factor, such as netrin-1, that can play a role in very precise and selective organization of connectivity, could very well account for a scenario such as this.

Whether netrin-1 signalling has a critical role in the normal organization and maturation of the mesocorticolimbic system is the focus of this Thesis. DCC is highly expressed by mesocorticolimbic DA neurons in both the developing and adult brain (see section 2.5.1 of this Chapter and Fig I-4). Therefore, it stands to reason that DCC may participate in the development, organization and ongoing plasticity of mesocorticolimbic DA circuitry. In this Thesis, I assess whether variations in the expression of DCC during development and/or throughout life results in functional and/or organizational changes of the mesocorticolimbic DA circuitry.

There is evidence to suggest that alterations in DCC function throughout life does, in fact, lead to functional reorganization of DA systems. Flores and colleagues have demonstrated that 129Sv/C57BL6 mice heterozygous for the *dcc* gene survive to adulthood and are physically indistinguishable from their wild-type littermates. However, when the mesocorticolimbic DA system was challenged with an acute injection of amphetamine (either 2.5 mg/kg or 4.0 mg/kg, i.p.), adult male *dcc* heterozygous mice showed a blunted locomotor response in comparison to their wild-type counterparts. Furthermore, *dcc* heterozygous, but not wild-type, mice failed to sensitize to the behavioural activating effects of amphetamine following repeated exposure to the drug. These behavioural changes were accompanied by altered DA function of the mesocorticolimbic DA system. High performance liquid chromatography (HPLC) analysis on whole tissue punches excised from the NAcc of drug-naïve *dcc* heterozygous and wild-type mice showed no difference in baseline DA concentration. However, in comparison to wild-type mice, basal DA metabolite levels of *dcc* heterozygous mice were significantly decreased, suggesting reduced mesolimbic DA functioning in these mice. In contrast, HPLC analysis revealed a 200-fold increase in the concentration of basal mPFC DA and DA metabolites in *dcc* heterozygous mice suggestive of increased mesocortical DA functioning relative to wild-type mice. These data demonstrate that altering DCC expression throughout life does lead to changes in DA function in adulthood. Moreover, the results of this initial study imply that decreased function of DCC during development and/or throughout life may protect against the development of DA

and behavioural abnormalities associated with schizophrenia-like symptoms (Flores et al. 2005a).

Here, I expand on these results and demonstrate that reduced expression of DCC *selectively* alters mesocortical DA circuitry in adulthood. Furthermore, I now identify that the critical period for the effects of reduced DCC on the development of this protective phenotype is during the adolescent period, suggesting that DCC may play an important role in the normal mesocortical DA maturation during adolescence. Finally, I demonstrate that a genetic variant in human *DCC* may be associated with individual differences in the vulnerability to develop schizophrenia.

Chapter II: Netrin-1 receptor deficient mice show enhanced mesocortical dopamine transmission and blunted behavioural responses to amphetamine

II.1. Preamble

The results described previously by Flores *et al* (2005) suggest that there is a link between netrin-1 and DA function in adulthood. Briefly, the authors showed that adult *dcc* heterozygous mice exhibit a blunted locomotor response to amphetamine and do not develop sensitization to its locomotor-activating effects when treated repeatedly. These changes in behaviour were accompanied by increases in the expression of tyrosine hydroxylase, (TH; the rate limiting enzyme in catecholamine synthesis) and in basal concentrations of DA and DA metabolites in the mPFC. In contrast, the authors reported that these mice show no changes in the basal levels of TH or DA in the NAcc, but have decreases in the concentrations of DA metabolites, suggesting reduced NAcc DA function. Their results, therefore, raise the interesting possibility that decreased function of the netrin-1 receptor DCC protects against the development of DA and behavioural abnormalities associated with schizophrenia-like symptoms. The first objective of the studies described in this Chapter is to assess this hypothesis and to expand on the behavioural findings of Flores and colleagues. To this end, I conducted a more in-depth characterization of the behavioural phenotype of adult *dcc* heterozygous mice by assessing their performance in other DA-dependent behavioural tests. An additional aim of the studies described herein was to determine whether any behavioural changes observed in these mice were accompanied by alterations in extracellular concentrations of DA in the NAcc and mPFC either at baseline or following a systemic injection of amphetamine. Finally, through the use of Golgi-Cox staining, in this Chapter, I established

whether there are alterations to the neuronal circuitry in the NAcc or mPFC by comparing dendritic spine densities between *dcc* heterozygous and wild-type mice.

II.2. Research Manuscript

Netrin-1 receptor-deficient mice show enhanced mesocortical dopamine transmission and blunted behavioural responses to amphetamine

Alanna Grant[1], Daniel Hoops[1], Cassandre Labelle-Dumais[1], Michael Prévost[1], Heshmat Rajabi[2], Bryan Kolb[3], Jane Stewart[2], Andreas Arvanitogiannis[2], Cecilia Flores[1]

[1]Departments of Psychiatry and of Neurology and Neurosurgery, McGill University, Douglas Hospital Research Centre, 6875 LaSalle Boulevard, Quebec, Canada, H4H 1R3

[2]Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, 7141 Sherbrooke Street West, Montreal, Quebec, Canada, H4B 1R6

[3]Department of Neuroscience, Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Lethbridge, AB, Canada, T1K 3M4

European Journal of Neuroscience, 2007, Vol 26, pp3215-3228.

ABSTRACT

The mesocorticolimbic dopamine (DA) system is implicated in neurodevelopmental psychiatric disorders including schizophrenia, but it is unknown how disruptions in brain development modify this system and increase predisposition to cognitive and behavioural abnormalities in adulthood. Netrins are guidance cues involved in the proper organization of neuronal connectivity during development. We have hypothesized that variations in the function of DCC (deleted in colorectal cancer), a netrin-1 receptor highly expressed by DA neurons, may result in altered development and organization of mesocorticolimbic DA circuitry and influence dopamine function in the adult. To test this hypothesis, we assessed the effects of reduced DCC on several indicators of DA function. Using in vivo microdialysis, we showed that adult mice that develop with reduced DCC display increased basal DA levels in the medial prefrontal cortex and exaggerated DA release in response to the indirect DA agonist amphetamine. In contrast, these mice exhibit normal levels of DA in the nucleus accumbens but significantly blunted amphetamine-induced DA release. Concomitantly, using conditioned place preference, locomotor activity, and prepulse inhibition paradigms, we found that reduced DCC diminishes the rewarding and behavioural-activating effects of amphetamine and protects against amphetamine-induced deficits in sensorimotor gating. Furthermore, we found that adult DCC-deficient mice exhibit altered dendritic spine density in layer V medial prefrontal cortex pyramidal neurons but not in nucleus accumbens medium spiny neurons. These findings demonstrate that reduced DCC during development results in a behavioural phenotype opposite to that observed in developmental models of

schizophrenia and identify DCC as a critical factor in the development of DA function.

INTRODUCTION

A combination of genetic and environmental events occurring during the prenatal or perinatal periods of life appears to lead to abnormalities in dopamine (DA) synaptic organization and neurotransmission in the medial prefrontal cortex (mPFC) as well as sensitized mesolimbic DA function later on in life. Such abnormalities have been linked to psychiatric disorders in humans, including schizophrenia, where under- or malfunction of mPFC DA contributes to cognitive symptoms and sensitized mesolimbic DA function contributes to psychotic symptoms. This suggests that early life events can result in subtle variations in the normal course of DA system development that, in turn, result in differential predisposition to cognitive and behavioural abnormalities in the adult (Lewis and Gonzalez-Burgos 2000; Weinberger 1987a). How such early events might result in enduring changes in DA function is unknown. One possibility is through the reorganization of DA circuitry by altering the function of proteins involved in DA wiring. Netrin-1, a member of the mammalian netrin protein family, is a guidance cue that, by attracting or repelling growing axons, directs them toward their appropriate targets (Manitt and Kennedy 2002). Here we report a study in which we investigated whether abnormal levels of the netrin-1 receptor DCC (deleted in colorectal cancer) during brain development can influence DA function and DA-related behaviours in the adult.

There are two reasons why we thought this influence might occur. First, there is evidence showing that DCC receptors are highly expressed in the mesocorticolimbic system and DA neurons in both the developing and adult brain (Gad et al. 1997; Livesey and Hunt 1997; Osborne et al. 2005; Shu et al. 2000;

Volenec et al. 1998). DCC may, therefore, participate in the development, organization, and maintenance of DA circuitry. Second, in a previous study we showed that adult *dcc*-heterozygous mice (*dcc*-homozygotes die at birth) exhibit sizeable increases in baseline tissue DA levels in the mPFC. Interestingly, these mice showed blunted amphetamine (AMPH)-induced locomotor activity, known to be mediated by drug-induced striatal DA release, and did not develop sensitization to this effect when treated repeatedly (Flores et al. 2005). This may well relate to the inhibitory effect that mesocortical DA activity exerts on mesolimbic DA activity (Grace 1991; Le Moal and Simon 1991). But whether the increased tissue DA levels in mPFC are associated with enhanced extracellular concentrations of DA within this region and are responsible for decreased mesolimbic DA activity remains to be examined.

Nevertheless, these studies suggest a link between netrin-1 and DA function and raise the interesting possibility that decreased function of the netrin-1 receptor DCC protects against the development of DA and behavioural abnormalities associated with schizophrenia-like symptoms. To determine whether this is the case, we tested for differences between adult *dcc*-heterozygous and wild-type mice in a number of AMPH-induced behavioural effects that reflect the 'state' of functioning of the mesocorticolimbic system and that are known to be altered in schizophrenia and related disorders. To determine whether these behavioural changes are indeed accompanied by changes in DA responsiveness, we measured AMPH-induced DA release in the mPFC and nucleus accumbens (NAcc) by in vivo microdialysis in freely moving animals.

MATERIALS AND METHODS

Animals

Adult male and female *dcc*-heterozygous (+/-) mice, originally obtained from Dr. S. Ackerman (The Jackson Laboratory) and maintained in the BL6 background at our animal colony, were used in all experiments. Mice were kept on a 12h light-dark cycle with ad libitum access to food and water. All behavioural testing was conducted during the light phase of the cycle. Pups were weaned at PND25 and housed in cages with same-sex littermates. Different cohorts of +/- and wild-type (+/+) mice were used for each experiment, counterbalancing for genotype, chamber assignment, and treatment. All experiments were preformed in accordance with the guidelines of the Canadian Council of Animal Care, the Animal Committee of the Douglas Hospital/McGill University and the Concordia University Animal Research Ethics Committee.

Drugs

d-Amphetamine sulphate salt (AMPH, Sigma) was dissolved in 0.9% saline and was injected intraperitoneally (i.p.).

Locomotor Activity

Locomotor activity was quantified with an automated infrared activity monitoring apparatus modified for use with mice (AccuScan Instruments, Columbus, Ohio). The animals were placed in a Plexiglas activity box consisting of 8 equidistantly spaced infrared light beams, oriented in both the X- and Y-axes.

The AccuScan software detects beams broken by the animal and determines the location of the animal within the activity box. Data are then converted by the system and was expressed as distance travelled (cm).

Data collection occurred over 3 days: on day 1, mice were habituated to the boxes for 15 minutes; on day 2, mice were habituated to the boxes again for 15 minutes then given a saline injection and returned to the boxes for an additional 30 minutes; on day 3, following the 15 minute habituation period, mice were given an injection of AMPH (females 2.2. mg/kg or males 2.5 mg/kg; i.p.) and continued to be monitored for 90 minutes. These doses were based on our previous study on the locomotor effects of AMPH on adult male mice (Flores et al. 2005) and were adjusted for females so as to produce equivalent drug brain concentrations (Becker et al. 1982). At moderate and higher dose, AMPH can induce stereotyped behaviours that can suppress locomotor activity in rodents (Randrup and Munkvad, 1967). Therefore to ensure that any group difference in locomotor activity observed was not due to genotypic difference in stereotypy, we also assessed stereotypy and expressed these data as stereotypy counts. Stereotypy counts were measured as the number of breakings of the same photocell beam or set of beams repeatedly, as defined by the AccuScan system.

Prepulse inhibition of acoustic startle response (PPI-ASR)

Apparatus: PPI was assessed using startle chambers (SR-LAB, San Diego Instruments, San Diego) containing a clear Plexiglas cylinder that housed the animal during the testing session. Background white noise of 70 dB was delivered throughout the testing session to mask extraneous noise. The startle response for

each trial was calculated as the mean of 65 readings taken at 1-ms intervals from stimulus onset. Prior to each testing session, the chambers were calibrated to ensure equivalent sensitivity to vibration and sound levels (C weighting).

Procedure: In all experiments, testing occurred over 4 days; on day 1 baseline measures of startle response and PPI were obtained. On days 2 and 3 male mice were left undisturbed in their home cages. On day 4, AMPH effects on PPI were tested. Each PPI session consisted of 12 startle, 6 prepulse, and 6 no stimulus (null) trials. Each session began with a 5-min acclimation of background noise followed by trials arranged in a pseudorandom order to prevent consecutive presentations of the same trial type. Startle trials consisted of presentation of a 40 ms-120 dB pulse. In the prepulse trials, the startle pulse was preceded (100 ms) by a 20 ms prepulse of varying intensity (5, 10, and 15 dB above background). The degree of PPI was calculated as a percentage for each prepulse intensity: %PPI=100%[1 - (mean prepulse trial - mean null)/(mean startle-mean null)].

Two doses of AMPH were tested, 3.2 and 6.4 mg/kg ('low' and 'high', respectively) in two different cohorts of $dcc^{+/-}$ and $dcc^{+/+}$ mice. These doses were chosen on the basis of a previous study showing AMPH-induced PPI impairment in adult male BL6 mice (Tsai et al. 2004). The baseline measures of startle response and PPI obtained in the 'low' and 'high' experiment were combined.

Conditioned place preference (CPP)

An automated 3-compartment apparatus modified and adapted for use in mice was used with infrared photobeam detectors (MED Associates Inc., ENV-013, Vermont, USA) interfaced to a MED control system with MED-PC software.

The two conditioning side compartments had distinctive visual and tactile cues that were balanced such that no side preference was exhibited before conditioning. Testing lasted for 5 days and consisted of 3 phases: preconditioning, conditioning and postconditioning test. On day 1 (preconditioning), female mice were allowed to move freely throughout all three compartments for 30 min and the time spent in each compartment was monitored. For the next 3 conditioning days, mice were exposed to twice-daily conditioning sessions. They were randomly assigned to receive AMPH pairings with one of the side compartments and saline pairings with the other compartment in a counterbalanced fashion. In the morning of each of the three conditioning days, they received saline and were confined to one compartment for 30 min. In the afternoon, they received AMPH (2.2 or 4.4 mg/kg, i.p.) in the other side compartment. These doses were based on a previous report showing CPP in adult male BL6 mice (Budygin et al. 2004) and were adjusted for females. The postconditioning test was conducted on the fifth day when mice in a drug-free state, were allowed to move freely between compartments for 30 min, and the amount of time spent in each was recorded. Importantly, this test was conducted between the time periods used previously for the morning and afternoon conditioning sessions.

In vivo microdiaylsis

Cannula implantation: Mice were anaesthetized using sodium pentobarbital (75 mg/kg, i.p.) and then given atropine sulphate (0.25mg/kg; s.c.) to reduced bronchial secretions. Animals were mounted in a stereotaxic apparatus and a 21-gauge guide cannula was implanted into the nucleus accumbens (AP=

+1.8mm, ML= +0.8mm, DV= -4.5mm from the skull; (Paxinos and Franklin 2001) or into the mPFC (AP= +1.8mm, ML= +0.6mm, DV= -1.0mm from the skull; (Paxinos and Franklin 2001). The cannula was held in place using Geristore adhesive (Den-Mat Corp., Santa Maria, California). Mice were allowed to recuperate in their home cages undisturbed for at least 5 days prior to microdialysis experiments.

Microdialysis: The tip of the microdialysis probes consisted of a semipermeable dialysis membrane (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA) with a molecular cut-off weight of 13 000 kDA. The length of the exposed tip of the microdialysis probes was 1 mm for the NAcc and 2 mm for the mPFC. Dialysate was collected from probes outlet tubing (silica) into a 0.2-ml microcentrifuge tube. The night before the experiment, male mice were placed in a lidless cage to allow for habituation to the testing environment. At this time probes were connected to the pump. On the following morning, probes were inserted into the intracranial cannula and then were flushed with artificial cerebral spinal fluid (150mM Cl^-, 145 mM Na^+, 2.7mM K^+, 2 mM Na_2HPO_4, 1.22 mM Ca^{2+}, 1.0 mM Mg^{2+}, 0.2 mM ascorbate, pH=7.4± 0.1) at a rate of 0.05ml/hr (i.e. 0.83 µl per min) for 3 hours prior to collection of samples. Four baseline 20 µl-samples were collected every 20 minutes. Mice then received an injection of AMPH (2.5 mg/kg) and 5 additional samples were collected every 20 minutes. This dose was based on our locomotor studies in adult male mice (Flores et al. 2005a). Throughout the habituation period and during the testing procedure animals were freely moving and had ad libitum access to food and water.

High Performance Liquid Chromatography: The concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanilic acid (HVA) in the collected dialysate were measured by electrochemical detection as previously described (Flores et al. 2005). Data for DA, DOPAC and HVA were analysed through an EZChrom Chromatography Data System (Scientific Software, Inc., San Ramon, CA).

Histology: Animals were given an overdose of sodium pentobarbital (in excess of 75 mg/kg, i.p.) and were perfused intracardially with 0.9% saline to flush the body of blood. Next, brain tissue was fixed through perfusion of 10% formalin solution (v/v, anachemia, Montreal, QC). Brains were then removed and flash frozen in 2-methylbutane chilled with dry ice to approximately -50^{o}C. Frozen brains were coronally-sectioned at 20 µm using a cryostat, mounted onto slides and Nissl-stained. Probe placement was verified by assessment of Nissl stained sections. Only data from animals with correct probe placement and with minimal tissue damage were used in the study.

Western blotting

Expression of DCC and UNC5H, the other netrin-1 receptor family, in the NAcc, mPFC, and ventral tegmental area (VTA) of brains from male experimentally-naïve +/- and +/+ mice was measured through western blotting as described previously (Flores et al. 2005). Briefly, bilateral punches of mPFC, including cingulate cortex area 1 and 2, NAcc, including both core and shell, and VTA were excised from 1mm-thick coronal sections. Sampling areas of mPFC and NAcc were taken starting from sections corresponding to plate 15 and of the

VTA corresponding to plate 55 of the Paxinos & Franklin mouse atlas (Paxinos and Franklin 2001). The tissue punches were suspended in TNE buffer (10 mM Tris-HCl, 1% NP40, 150 mM NaCl, 2 mM Na_3VO_4, 10 mM NaF, 1x protease inhibitor cocktail) and protein concentration of the samples were quantified through Bicinchoninic Acid (BCA) protein assays. Protein samples (25 µg) were resolved using SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The membrane was incubated with antibodies against DCC (1:1000, mouse monoclonal, Pharmigen, Cat# 554223, Mississsauga, Canada), UNC5 (1:7500, kindly provided by Dr. Tony Pawson, University of Toronto), and tubulin (1:4000, mouse monoclonal, Sigma). Bands were detected by chemiluminescence (Perkin Elmer, Waltham, MA, USA) and analysed using Kodak Imaging system software (2000, New Haven, CT, USA).

Immunofluorescence

Mice were anesthetized with an overdose of sodium pentobarbital (>75 mg/kg i.p.) and were perfused intracardially with 50 ml of 0.9% saline followed by 80 ml of fixative solution (4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer). Mouse brains were dissected from the skull and post-fixed in the same fixative for 45 minutes at 4°C. Brains were then cryoprotected in sucrose (30% in phosphate buffered saline) overnight at 4°C. The following morning, tissue was rapidly frozen by immersion in 2-methylbutane (Fisher Scientific, Hampton, NH) chilled with dry ice. Frozen brains were immediately sectioned using a Leica SM2000-R sliding microtome.

Free floating brain sections (40 μm) were processed for dual-labelling immunofluorescence. Briefly, sections were collected and rinsed in phosphate buffered saline and incubated in blocking solution (2% bovine serum albumin, 0.2% Tween-20 in phosphate buffered saline) for 1 hour at room temperature. Sections were incubated overnight at 4°C with combinations of primary antibodies diluted in blocking solution. The primary antibodies against DCC and UNC5H used in this procedure were the same as the ones described in the Western blotting section. The two combinations of primary antibodies were as follows: 1) monoclonal anti-DCC (1:500) and polyclonal anti-tyrosine hydroxylase (TH; 1:500, raised in rabbit, Chemicon, Temecula, CA, USA, Cat# AB152) and 2) polyclonal pan-UNC5 (1:5000) and monoclonal anti-TH (1:300, Chemicon, Cat# MAB318). The sections were then washed several times in blocking solution and incubated with Alexa 488 and Alexa 546-conjugated secondary antibodies raised in goat (1:500, Molecular Probes, Eugene, OR, USA) for 45 minutes at room temperature. Immunofluorescence was visualized using a Leica DM4000B microscope and images were captured with a Microfire camera and PictureFrame software (Microbrightfield, VT, USA).

Golgi-Cox staining

Procedure: Adult male mice were given an overdose of sodium pentobarbital (>75 mg/kg i.p.) and perfused transcardially with 0.9% saline. The brains were immersed in 20 ml of Golgi-Cox solution and stored (in the dark) in the Golgi-Cox fixative for 14 days before being transferred to a solution of 30%

sucrose for 7 days. The tissue was cut into 200 µm-thick sections using a Vibratome™ and developed using a method described by Gibb and Kolb (Gibb and Kolb 1998).

Anatomical analysis: Because of the changes in DA function observed in +/- mice, dendritic spine density was analysed in neurons within the NAcc and mPFC that receive robust DA innervation, i.e. the basilar dendrites of layer V mPFC pyramidal neurons and NAcc medium spiny neurons (Fallon and Loughlin 1987). In addition, to find out if changes in mPFC pyramidal neurons are specific to regions highly innervated by DA cells, we assessed dendritic spine density in layer III. Measurements were taken from the prelimbic subregion of the mPFC. We were also interested in analyzing changes in layer III because, in postmortem brains of schizophrenic patients, there is a reduction in dendritic spine density in layer III pyramidal neurons of the dorsolateral prefrontal cortex (Glantz and Lewis 2000).

A Leica model DM4000 microscope equipped with a Ludl XYZ motorized stage was used to identify cells, trace dendritic segments, and quantify dendritic spines. Relevant regions were first identified at low magnification (250 X). Only dendritic trees of a cell that was intact; well impregnated and not obscured by blood vessels, astrocytes or heavy clusters of dendrites from other cells were included in the analyses. Five cells from each hemisphere were analysed. NEUROLUCIDA® software was used to quantify spine density of selected dendrites as determined by the number of visible spine per 10 µm-length of dendrite (at 5000 X magnification). For medium spiny neurons, a one third-order (or greater) terminal tip was identified and the total number of visible spines

along the length of the dendritic segment (at least 20-µm long) was counted. One dendritic segment was analysed per neuron. Spines were always counted from the last branch point to the terminal tip of the dendrite. For cortical neurons, spines were counted on one third-order tip. No attempt was made to correct for the fact that some spines are obscured from view, so the measure of spine density necessarily underestimates total spine density. Anatomical analysis was conducted blind to treatment condition.

Statistical Analyses

All the results of the statistical tests used are indicated in detail in the figure legends.

Locomotor Activity: Differences in total distance travelled scores were analysed using two-way repeated measures ANOVAs with genotype and time (min) as between- and within-group variables, respectively. Student's *t* tests for independent samples were used to analyze differences in stereotypy counts between groups.

Prepulse inhibition: Differences in baseline magnitude of startle response was analysed using Student's *t* test for independent samples. Differences in baseline PPI scores were analysed using two-way repeated measures ANOVAs with genotype and prepulse intensity as between- and within-group variables, respectively. Data obtained from the AMPH experiments were analysed using three-way repeated measures ANOVAs with genotype and treatment as between group variables and prepulse intensity as the within group variable. *Post-hoc*

analyses of significant interactions were made using ANOVA tests for simple effects.

CPP: Place preference scores (i.e. difference in time spent in the AMPH- vs. the saline-paired compartment before and after conditioning) were analysed using planned paired *t* tests with the significance level adjusted for multiple comparisons using the Holm-Bonferroni sequentially rejective procedure (Holm 1974).

Microdialysis: Data obtained before and after the AMPH challenge, were analysed using two-way repeated measures ANOVAs, with genotype and time (min) as between- and within-group variables, respectively. *Post-hoc* analyses of significant interactions were made using ANOVA tests for simple effects.

Western blotting: Raw data (optical density of scanned immunoblots) was normalized to tubulin to correct for any loading errors and analyses were conducted on these normalized data. Differences in protein expression between *dcc* $^{+/-}$ and *dcc*$^{+/+}$ mice were analysed using Student's *t* tests for independent samples.

Spine density: Differences in dendritic spine density in Golgi-Cox stained NAcc medium spiny and mPFC pyramidal neurons of adult *dcc* $^{+/-}$ and *dcc*$^{+/+}$mice were analysed using Student's *t* tests for independent samples.

RESULTS

Blunted behavioural activation by AMPH in DCC-deficient male and female mice

We determined whether the phenotype previously reported for *dcc* heterozygous mice is dependent on a specific genetic background and/or sex. We previously reported that adult *dcc* heterozygous (+/-) male mice of a 129Sv/BL6 cross (Fazeli et al. 1997) show a blunted locomotor response to a single AMPH injection of 1.5, 2.5, or 4 mg/kg (Flores et al. 2005). Here, however, we conducted all the experiments in a pure BL6 strain (Burgess et al. 2006). As previously found, there was no effect of genotype on basal locomotor activity or following a single i.p. injection of saline (Fig II-1a, b). However, when male mice were given a single AMPH injection, +/- mice were significantly less responsive in comparison to their +/+ littermate controls (Fig II-1c).

We then assessed locomotor activity in adult cycling female +/- and +/+ mice and obtained results identical to the data obtained in males: there was no genotype effect on baseline locomotor activity (Fig II-1a) or following an injection of saline (Fig II-1b). However, a blunted locomotor response to AMPH was observed in +/- mice in comparison to +/+ controls (Fig II-1c). Consistent with previous work (Morse et al. 1995), the locomotor activity observed in male and female BL6 mice was very similar.

Finally, both male and female +/- mice, in comparison to +/+, demonstrated significantly fewer stereotypy counts in the AMPH test (Fig II-1d),

ruling out the possibility that the genotype effect was caused by drug-induced stereotypy. Together, these results indicate that the *dcc* phenotypic response to AMPH does not depend on genetic strain or gender. See Figure II-1 legend for statistical analysis.

Reduced dcc protects against AMPH-induced sensorimotor gating deficits

Sensorimotor gating, a fundamental form of information processing dependent on proper mesocorticolimbic DA function, is impaired in schizophrenia. It has been shown that single or repeated exposure to stimulant drugs, such as AMPH, produces significant deficits in sensorimotor gating in laboratory animals (Tenn et al. 2005). This effect appears to be mediated by AMPH-induced DA release in the NAcc (Swerdlow et al. 1990). For this reason, performance on tests of sensorimotor gating is widely used to assess deficits in animal models of schizophrenia.

Sensorimotor efficacy can be measured by prepulse inhibition (PPI), the phenomenon by which a mild stimulus (prepulse) suppresses another strong startle-eliciting stimulus. Here, we assessed differences in PPI between adult +/- and +/+ mice both at baseline and following a single AMPH injection. No differences in response to startle alone (Fig II-2a), baseline PPI (Fig II-2b), or habituation to the testing session (data not shown) were observed between +/- and +/+ mice. In the low dose AMPH experiment, although +/+ mice appeared to exhibit higher PPI scores than +/- mice, statistical analysis revealed no effect of genotype and no interaction between genotype and treatment (see Fig II-2c for details of statistical analysis). However, there was a significant effect of AMPH,

but only in the +/+ mice. Remarkably, even after doubling the AMPH dose (6.4 mg/kg), +/- mice remained insensitive to AMPH-induced PPI impairments (Fig II-2c). The low baseline percentage of PPI and following AMPH injection is typical for BL6 mice, which exhibit lower PPI and are more sensitive to the behavioural effects of AMPH than other strains (Varty et al. 2001).

These findings are consistent with the idea that the *dcc* heterozygous phenotype only becomes evident upon AMPH challenge. Furthermore, they suggest that changes in the balance of netrin-1 receptors renders individuals more or less vulnerable to attention deficits induced by stimulant drugs. See Figure II-2 legend for statistical analysis.

Reduced DCC diminishes AMPH-induced reward

Sensitized striatal DA function in schizophrenia has been suggested as a possible explanation for the high comorbidity between schizophrenia and drug abuse (Chambers et al. 2001). Moreover, increased NAcc DA release is known to play a critical role in mediating the rewarding effects of AMPH (Di Chiara et al. 2004). We, therefore, tested whether *dcc* heterozygous mice would be less sensitive to the rewarding effects of AMPH using conditioned place preference (CPP).

In the CPP paradigm, animals learn to associate the effects of a drug with a particular environment. Therefore, preference for the drug-paired environment later on can be considered as an index of the rewarding properties of the drug. Sensitivity to the rewarding effects of AMPH were determined in a place preference test conducted 1 day after 3 consecutive days of conditioning trials, in

which AMPH and saline were paired to particular compartments. As previously shown (Budygin et al. 2004), in a postconditioning test, +/+ mice spent a significantly greater amount of time in the compartment previously paired with 2.2 mg/kg of AMPH than in the one paired with saline. This preference, however, was not observed in +/- mice. When mice were tested with a higher AMPH dose (4.4 mg/kg), a rightward shift in the dose-effect response was observed in the +/- group; both groups exhibited significant preference for the AMPH-associated compartment (Fig II-3). These results indicate that +/- mice have a diminished sensitivity to the incentive properties of AMPH because they fail to show preference for an environment previously paired with an AMPH dose that does induce CPP in wild-type controls. These findings raise the interesting possibility that subtle alterations in the balance of netrin-1 receptors, render individuals more or less prone to drug abuse. See Figure II-3 legend for statistical analysis.

Impaired AMPH-induced DA release in NAcc in dcc heterozygotes

AMPH-induced locomotion, CPP and PPI deficits have been shown to be dependent on AMPH-induced DA release in NAcc in rodents (Sellings and Clarke 2003; Swerdlow et al. 1990). Thus, we predicted that the behavioural phenotype observed in the adult +/- mice would be associated with altered AMPH-induced DA release in this region. Here we assessed NAcc DA function through *in vivo* microdialysis experiments on freely moving mice during baseline conditions and following a single injection of AMPH (Fig II-4). Importantly, since no behavioural differences were found between genotypes following a single

injection of saline, microdialysis experiments were only conducted in AMPH-treated animals.

Baseline levels of extracellular DA did not differ between +/- and +/+ mice. This finding is consistent with the lack of genotype effect observed in baseline locomotor activity and PPI. However, there were considerably lower baseline extracellular concentrations of DOPAC and HVA in +/- mice, indicating decreased DA activity in this region. These findings concur with the results of our previous study where high performance liquid chromatography analysis was conducted on whole tissue punches taken from the NAcc of +/+ and +/- mice (Flores et al. 2005).

Importantly, however, and in contrast to the lack of differences in baseline extracellular DA, there was a large effect of genotype on DA release induced by an AMPH challenge. While AMPH produced a large increase in extracellular DA concentrations in the NAcc of +/+ mice, this effect was less than half in +/- mice. It is important to point out that the slight reduction in DOPAC concentrations in +/- mice by the AMPH challenge most likely reflects a floor effect as the baseline levels were already very low in these mice. See Figure II-4 legend for statistical analysis.

DCC deficiency results in enhanced mPFC DA activity

DA activity in the NAcc is highly regulated by DA neurotransmission in the mPFC. In fact, mPFC DA activity has been shown to attenuate mesolimbic DA release (Deutch et al. 1990; Sesack and Pickel 1992; Ventura et al. 2004). Thus, we hypothesized that the blunted DA response to AMPH observed in the

NAcc of +/- mice results from greater DA activity in the mPFC. To this end, we measured DA and DA metabolites in the mPFC of adult +/- and +/+ mice using in vivo microdialysis. In agreement with our hypothesis, +/- mice showed, at the beginning of the sample collection period, significantly higher baseline extracellular levels of mPFC DA and DOPAC, indicating hyperactivity of the mesocortical DA system (Fig II-5). Although HVA concentrations were also elevated in mPFC of +/- mice, this effect was not statistically significant. These findings are in accordance with our previous results on whole tissue punches (Flores et al. 2005).

There were large differences in the DA response to AMPH (2.5 mg/kg) in the mPFC between +/- and +/+ mice. It can be seen in Figure II-5 that the increase in DA in the mPFC of +/- mice was significantly greater than in the +/+ mice. These results show that mPFC DA function is significantly enhanced in +/- mice and suggest that the hyperactivation in this region by AMPH may account for the blunted AMPH-induced DA release in the NAcc and the behavioural effects observed.

The evidence suggests that, in addition to DA, mPFC norepinephrine (NE) also plays a role in the effects of AMPH on locomotion, PPI and reward (Blanc et al. 1994; Swerdlow et al. 2006; Ventura et al. 2003). We, therefore, measured extracellular mPFC NE concentrations before and after the AMPH challenge (see Fig II-9 in supplementary data section). No differences, however, were observed between +/- and +/+ mice at baseline (+/+ mice = 5.4 ± 1.1 pg/10 µl; +/- mice = 5.2 ± 1.2 pg/10 µl) or after the AMPH challenge (+/+ mice = 5.8 ± 0.8 pg/10 µl; +/- mice = 5.9 ± 0.7 pg/10 µl). See Figure II-5 legend for statistical analysis.

Reduced dcc is not associated with compensatory changes in the expression of UNC5

Netrin-1 is a bifunctional cue that can attract or repel growing axons depending on the balance between 'attractive' and 'repulsive' receptors these axons express. It is generally accepted that while attraction is mediated by DCC receptors, repulsion occurs through DCC/UNC5 homologue receptor complexes (Manitt and Kennedy 2002). To determine whether decreased expression of DCC would be associated with compensatory changes in the expression of UNC5, we conducted western blot analysis on tissue punches excised from the ventral tegmental area (VTA), mPFC, and NAcc of adult +/- and +/+ mice. We used a pan-UNC5 antiserum, which recognizes UNC5H1, UNC5H2, and UNC5H3 homologues (Manitt et al. 2004). As expected, significant decreases in DCC protein levels were found in brains of +/- mice as previously shown (Flores et al. 2005). However, no differences in UNC5 protein expression were observed between the two genotypes (Fig II-6). These results indicate that *dcc* haplo-insufficiency indeed alters the balance between DCC and UNC5 protein expression. See Figure II-6 legend for statistical analysis.

Both DCC and UNC5 receptors are expressed by VTA DA neurons

Our results suggest that altered regulation of netrin-1 receptor expression leads to functional reorganization of midbrain DA systems. It remains to be addressed, however, whether midbrain DA neurons do indeed express both attractive and repulsive receptors. Using double-labelling immunofluroescence, we assessed whether VTA DA neurons express netrin-1 receptors in adult +/- and

+/+ mice. We found that both DCC and UNC5 proteins are expressed in VTA neurons of +/- and +/+ mice and that, importantly, both proteins are expressed in TH-positive cells (Fig II-7). In addition, several double-labelled DCC/UNC5 neurons were observed in the VTA, strongly suggesting that both types of receptors are expressed by single DA neurons. Finally, DCC and UNC5 immunolabelling was observed in TH-positive terminals within the mPFC and NAcc (data not shown). These results although in agreement with those reported previously on DCC expression in the adult mouse (Osborne et al. 2005) are, to our knowledge, the first to demonstrate UNC5 expression in adult midbrain DA neurons.

dcc haplo-insufficiency leads to changes in neuronal structure in the mPFC

Reduced DCC leads to substantial changes in mesocorticolimbic DA function and DA-dependent behaviours in the adult. However, the processes underlying this effect remain to be determined. As a first step toward addressing this question, we assessed whether reduced DCC results in enduring reorganization of mesocorticolimbic DA circuitry. Because the vast majority of synaptic inputs onto neurons are on dendritic spines, we analysed differences in dendritic spine density in NAcc and mPFC regions that receive robust DA innervation (Fallon and Loughlin, 1987) in Golgi-Cox processed brains of adult +/- and +/+ mice. In NAcc medium spiny neurons, no difference in dendritic spine density was observed between +/- and +/+ mice (Fig II-8a). Remarkably, however, in layer V mPFC neurons, +/- mice show a large and significant reduction (~ 40%) in dendritic spine density, in comparison to the +/+ group (Fig

II-8b). This reduction appeared to be specific to pyramidal neurons localized in a cortical region highly innervated by DA neurons because no differences between groups were observed in layer III pyramidal cells (Fig II-8c). Our findings, therefore, are consistent with the idea that alterations in levels of netrin-1 receptors during development may lead to reorganization of synaptic connectivity of mesocortical DA system. See Figure II-8 legend for statistical analysis.

DISCUSSION

In this study we show that adult male and female mice that develop with decreased levels of the netrin-1 receptor, DCC, a developmental protein involved in directing growing axons toward appropriate targets, have elevated basal as well as AMPH-induced extracellular DA concentrations in the mPFC, but decreased basal and AMPH-induced DA activity in the NAcc. Correspondingly, these *dcc* heterozygous mice exhibit blunted locomotor activation in response to a single injection of AMPH, are resistant to AMPH-induced deficits in sensorimotor gating, and are less sensitive to the rewarding properties of AMPH. Together, these findings show that *dcc* haplo-insufficiency results in a phenotype which is opposite to that observed in developmental animal models of schizophrenia (Boksa 2004; Chen et al. 2006; Harrison and Weinberger 2005; Ross et al. 2006).

Numerous pharmacological, stress and lesion experiments show that DA function in the NAcc and mPFC can be regulated differently and that, in fact, DA function in the mPFC has an inhibitory control over DA activity in the NAcc. For instance, selective lesions of the DA input to the mPFC have been shown to result in increased DA release in the NAcc via disinhibition of cortical glutamatergic efferents (Deutch et al. 1990; Sesack and Pickel 1992; Ventura et al. 2004). Conversely, stimulation of DA receptors in the mPFC decreases DA activity in the NAcc (Banks and Gratton 1995; Doherty and Gratton 1996; Thompson and Moss 1995; Vezina et al. 1991). Thus, the reduced NAcc DA activity observed in *dcc* heterozygous mice at baseline and, importantly, following the AMPH challenge, is likely to result from hyperactivity of DA in the mPFC.

Ventura *et al.* (Ventura et al. 2004) showed that BL6 mice exhibit a smaller increase in extracellular concentrations of mPFC DA following an AMPH challenge in comparison to DBA/2J mice. They also showed that this smaller increase accounts for the greater AMPH-induced DA release in the NAcc observed in the BL6 background; selective mPFC DA depletion in DBA/2J mice abolished differences in DA release in NAcc between the two strains. Similar to their observations, we find a small response to AMPH in wild-type BL6 mice used in this study. Interestingly, *dcc* haplo-insufficiency reverses this poor mPFC response to AMPH, resulting, in turn, in significantly diminished AMPH-induced DA release in the NAcc. Behaviourally, these DCC-deficient mice show blunted AMPH-induced locomotion and reward (as measured in the CPP paradigm) and are protected against AMPH-induced deficits in sensorimotor gating. This pattern of results is consistent with the fact that many of the behavioural effects of AMPH are dependent on AMPH-induced DA release in the NAcc (Sellings and Clarke 2003; Swerdlow et al. 1990).

A complete understanding of the mechanisms underlying the effects of *dcc* haplo-insufficiency on baseline as well as on AMPH-induced alterations in extracellular concentrations of DA and DA metabolites in the NAcc and mPFC is yet to be determined. Changes in the expression and/or activity of proteins involved in the regulation of DA synthesis, metabolism, and clearance are likely to be involved. However, the findings from the present and our previous study (Flores et al. 2005), suggest that the increases in DA activity in the mPFC could originate from anatomical alterations in this region, namely, increased DA fibers ingrowth. In fact, as we showed in our previous study, there is an increase in basal

levels of TH expression in the mPFC of adult *dcc* heterozygous mice without a corresponding increase in dopamine-beta-hydroxylase expression. This increase, which was not observed in the NAcc or dorsal striatum, suggests axonal sprouting of DA terminals in the mPFC. This interpretation is further justified by the fact that DCC-deficient mice have a small reduction (~20%) in TH-positive neurons in the VTA and substantia nigra (Flores et al. 2005).

It is important to note that in addition to DA, NE function in the mPFC has also been shown to be a critical regulator of the effects of AMPH on DA release in the NAcc and on behaviour (Blanc et al. 1994; Swerdlow et al. 2006; Ventura et al. 2003). However, consistent with the lack of changes in dopamine-beta-hydroxylase expression (Flores et al. 2005), our present results from the microdialyisis studies revealed no differences in extracellular NE concentrations at baseline or after an AMPH challenge in the mPFC. This lack of difference suggests that the higher level of DA in the mPFC, in itself, may account for the adult phenotype described here. Nonetheless, changes in the function of other neurotransmitters known to directly or indirectly influence NAcc DA function, such as glutamate (Grace et al. 2007), cannot be ruled out. Furthermore, aberrations in neuronal circuits within brain regions that innervate the mesocorticolimbic DA system, such as the hippocampus, have been shown in the developing brain of *dcc* homozygotes (Barallobre et al. 2000). Although it is not known whether these differences are exhibited by *dcc* heterozygous mice and, importantly, whether they are present in the adult, they could contribute to the observed phenotype. Future studies to address these issues are warranted.

Ultimately the question to be addressed is how does reduced DCC during development produce differential effects on DA transmission and function in the mPFC and the NAcc. As mentioned before, it is generally accepted that while attraction is mediated by DCC receptors, repulsion occurs through DCC/UNC5 homologue receptor complexes (Bouchard et al. 2004; Hong et al. 1999; Williams et al. 2003). Thus, subtle changes in the expression of one these receptors during development should lead to alterations in neuronal connectivity between very specific groups of neurons. Although the mPFC and NAcc components of the mesocorticolimbic DA system originate in the VTA, they have distinctive anatomical and functional characteristics (Carr and Sesack 2000; Knable and Weinberger 1997; Le Moal and Simon 1991; Tam and Roth 1997). It is likely, therefore, that variations in DCC alter the organization of one component of this system, which in turn induces functional changes in the other component. Importantly, our results from the western blot analysis show no 'compensatory' decrease in UNC5 protein expression suggesting that *dcc* haplo-insufficiency does favour UNC5 function.

On the basis of our findings, we hypothesize that reduced DCC leads to selective reorganization of mPFC DA circuitry, which in turn affects DA function in the NAcc. If this is the case, mPFC neurons that receive robust DA innervation would be likely to exhibit alterations in synaptic connectivity (Fallon and Loughlin 1987). Because the vast majority of synaptic inputs onto neurons are on dendritic spines (Harris and Kater 1994), we assessed dendritic spine density in neurons within layer V of the mPFC, which receives rich DA innervation, as well as in the NAcc. A large and significant reduction in dendritic spine density was

observed in layer V mPFC pyramidal neurons of DCC-deficient mice. In contrast, there was no difference between genotypes in dendritic spine density in NAcc medium spiny neurons. Indeed, the reduced dendritic spine density observed in the mPFC was specific to pyramidal neurons localized to a region highly innervated by DA neurons; spine density of layer III pyramidal cells was not altered in *dcc* heterozygous mice. Intriguingly, the decreases in dendritic spine density found in postmortem brain of schizophrenic patients are observed in pyramidal neurons of layer III (Glantz and Lewis 2000).

Several studies have shown that changes in dendritic spine density determined using Golgi-Cox staining are accompanied by corresponding changes in the number of synapses per neurons assessed with electron microscopy (Kolb et al. 1998; Woolley et al. 1990; Woolley and McEwen 1992). For this reason, the alterations in dendritic spine density detected in Golgi-Cox stained material in the present study can be considered evidence for changes in patterns of synaptic connectivity (Harris and Kater 1994). Furthermore, because the number of dendritic spines is inversely associated with synaptic activity (Kirov and Harris 1999), the reduction in dendritic spine density in layer V mPFC is likely to be a compensatory response to increased DA function in this region. This is consistent with evidence showing that DA neurons in mPFC form synapses onto GABA interneurons (Sesack et al. 1995), which, in turn, regulate the activity of pyramidal cells. Our interpretation that reorganization of mesocortical DA circuitry is the critical determinant of the phenotype observed in the adult *dcc* heterozygotes is also consistent with the fact (i) that the ingrowth of DA terminals into the mPFC is a slow process, which continues until early adulthood and,

therefore, has an increased vulnerability for significant structural plasticity (Benes et al. 2000); (ii) that adult *dcc* heterozygous mice have increased basal TH protein expression in mPFC, but not in NAcc or dorsal striatum (Flores et al. 2005); and (iii) that higher levels of DA in the mPFC protect against AMPH-induced DA release in the NAcc and associated behavioural effects, such as deficits in sensorimotor gating. Within this context, it is important to note that the direct projection of mPFC to the medium spiny neurons in the NAcc arises from layer V pyramidal neurons (Brog et al. 1993; Carr et al. 1999).

To conclude, increasing evidence indicates that subtle disruptions to the normal course of brain development result in increased predisposition to schizophrenia and related disorders later on in life (Lewis and Gonzalez-Burgos 2000; Weinberger 1987a). Here we show that variations in levels of the netrin-1 receptor DCC during development tip the balance between over- and under-mPFC DA influence on NAcc DA function in the adult. When the balance is in favour of over-mPFC influence, the resulting phenotype appears to be protected against the development of behavioural abnormalities, such as those associated with schizophrenia-like symptoms (Seamans and Yang 2004). These findings add credence to our hypothesis that altered netrin-1 receptor function may be a common mechanism by which the diverse perinatal factors could exert their specific enduring effects on brain function and behaviour, rendering individuals more or less vulnerable to psychopathology. Furthermore, much speculation has being given to the processes and molecular players involved in the specification and establishment of mesocorticolimbic DA connectivity (Smidt and Burbach

2007). Our findings suggest that DCC is a critical determinant in the organization of the wiring of this system.

FIGURES

Fig. II-1 Baseline and AMPH-induced locomotion in male and female *dcc*+/- and +/+ mice

Adult male (n=8) and female *dcc* +/- (n=9) mice are less sensitive to the locomotor-enhancing effects of AMPH than +/+ littermates (n=11,10). Data points represents total distance (cm) travelled (mean ± SEM). Injection time point is indicated as an arrow. (**a**) No difference between groups was observed during the habituation period (day 1). (**b**) On day 2, no difference between +/- and +/+ mice was found following an injection of 0.9% saline, 15 min after habituation. (**c**) Day 3: The locomotor response to a single injection of AMPH (males 2.5 mg/kg, females 2.2 mg/kg, i.p.) was blunted in +/- mice. Repeated measures ANOVA revealed a significant main effect of genotype (males: $F_{(1,17)} = 7.7$; $p = .01$; females: $F_{(1,17)} = 12.2$; $p = .002$). (**d**) Stereotypy counts following AMPH administration (males: $t_{(17)} = 2.5$; $^*p = .03$; females: $t_{(17)} = 4.2$; $^*p = .0005$).

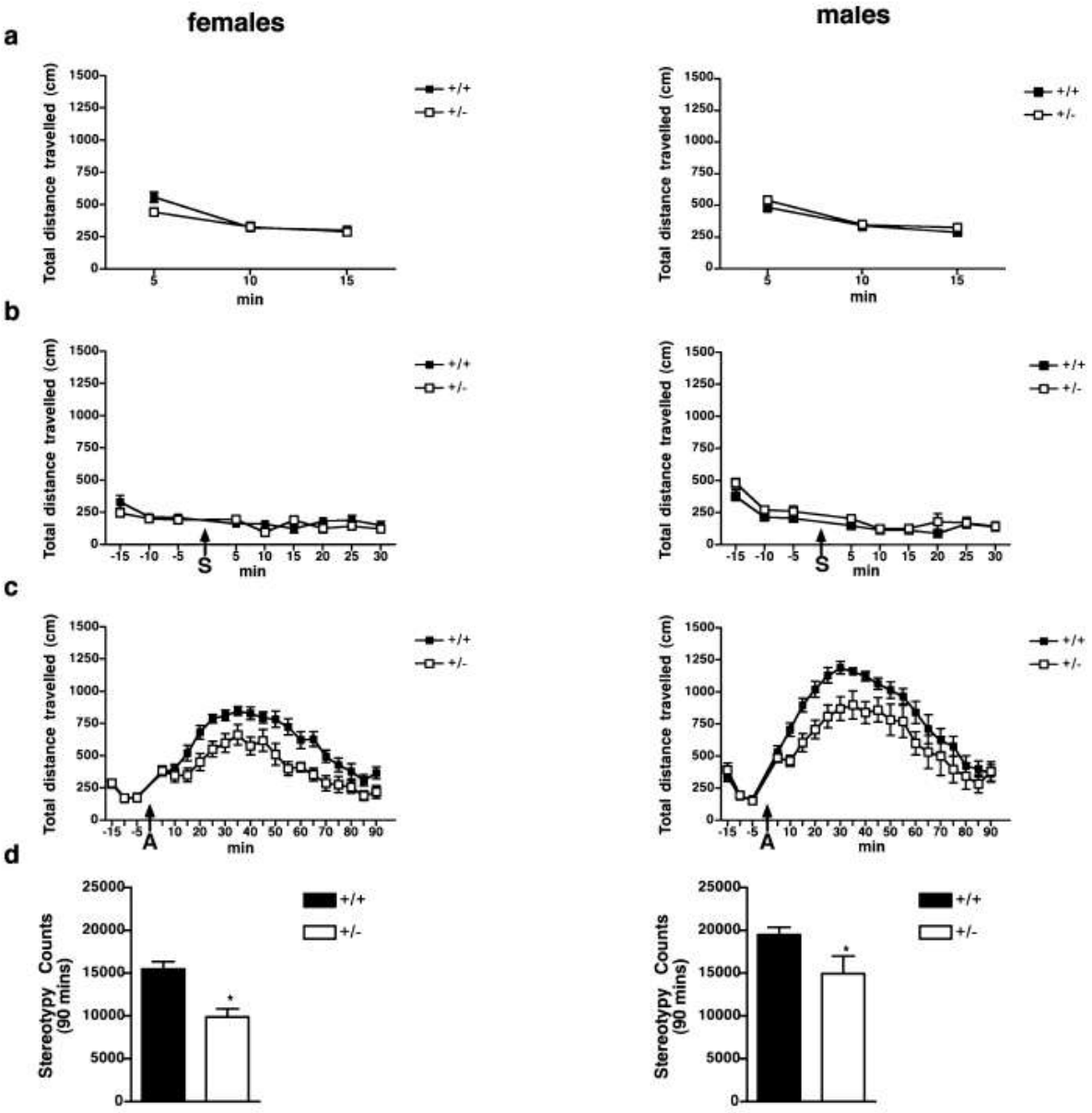

females
males
a
b
c
d
Total distance travelled (cm)
min
+/+
+/-
S
A
Stereotypy Counts (90 mins)
*

Fig. II-2 Baseline and AMPH-induced deficits in prepulse inhibition in *dcc* +/- and +/+ mice

Adult *dcc* +/- mice are protected against AMPH-induced deficits in PPI. (**a,b**) +/- (n = 28) and +/+ (n = 27) mice showed no difference in magnitude of startle response at baseline ($t_{(53)}$ = 1.69, p = 0.1). Repeated measures ANOVA revealed no significant effect of genotype ($F_{(1, 54)}$ = 0.27, p=0.60), and no significant interaction ($F_{(2, 108)}$ = 0.38, p=0.68). **(c)** For the low AMPH dose experiment, a three-way ANOVA with genotype and treatment as between variables and prepulse intensity as the within factor revealed no significant effect of genotype ($F_{(1,27)}$ = 2.76, p=0.1) and no significant interaction ($F_{(1,54)}$ = 1.31, p=0.26), but there was a main effect of treatment ($F_{(1,27)}$ = 4.92, p=0.03). * Student's *t* test for independent samples revealed a significant reduction in PPI in +/+ mice treated with AMPH ($t_{(9)}$ = 2.6; p = 0.03). **(d)** For the high AMPH dose experiment, a three-way ANOVA with genotype and treatment as between variables and prepulse intensity as the within factor revealed a significant interaction between genotype and treatment ($F_{(1,19)}$ = 5.55, p=0.03). A *post-hoc* ANOVA test for simple effects indicated a significant effect of AMPH in +/+ mice ($F_{(1, 19)}$ = 8.5, p=0.009), but not in +/- mice. No differences between genotypes was observed in saline-treated animals ($F_{(1,19)}$ = 0.54, p= 0.47). Low dose experiment: saline +/+, n = 6; +/-, n = 9; AMPH: +/+, n = 8; +/-, n = 8. High dose experiment: saline +/+, n = 6; +/-, n = 6; AMPH: +/+, n = 5; +/-, n = 6. AMPH: d-amphetamine sulphate; PPI: prepulse inhibition.

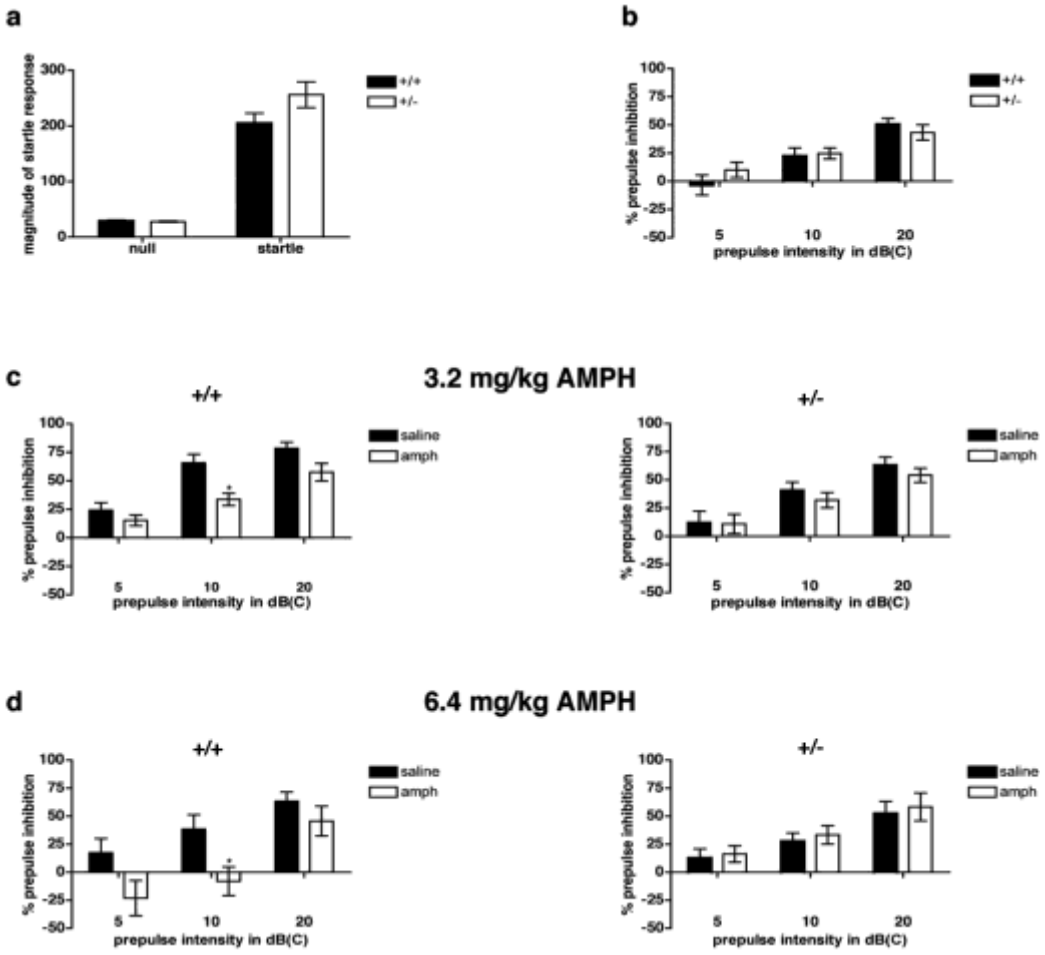
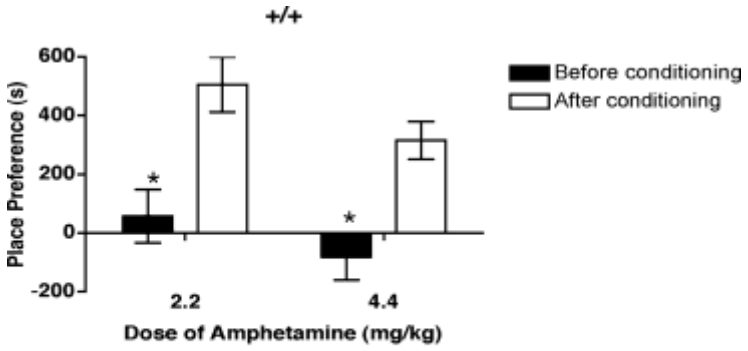

a
magnitude of startle response
300
200
100
0
null
startle
+/+
+/-
b
% prepulse inhibition
100
75
50
25
0
-25
-50
5
10
20
prepulse intensity in dB(C)
+/+
+/-
c
3.2 mg/kg AMPH
+/+
+/-
saline
amph
d
6.4 mg/kg AMPH

Fig. II-3 Preference for the compartment associated with AMPH in adult *dcc* +/- and +/+ mice (n = 10 per group)

Place preference is expressed as the difference in time spent in the AMPH- vs. the saline-paired compartments before and after conditioning. Planned paired *t* tests, with the significance level adjusted for multiple comparisons using the Holm-Bonferroni sequentially rejective procedure (Holm 1974), revealed that although a lower dose of AMPH (2.2 mg/kg) established a place preference for the drug-paired compartment in +/+ mice ($t_{(9)} = 2.884$, $p = 0.018$; adjusted $\alpha = 0.025$), it failed to do so in +/- mice ($t_{(9)} = 1.312$, $p = 0.222$). When mice were given 4.4 mg/kg of AMPH, however, both +/+ ($t_{(9)} = 3.805$, $p = 0.004$; adjusted $\alpha = 0.013$) and +/- ($t_{(9)} = 3.327$, $p = 0.009$; adjusted $\alpha = 0.017$) spent more time in AMPH-associated compartments after conditioning than they did before.


+/+
600
400
200
0
-200
Place Preference (s)
*
*
2.2
4.4
Dose of Amphetamine (mg/kg)
Before conditioning
After conditioning

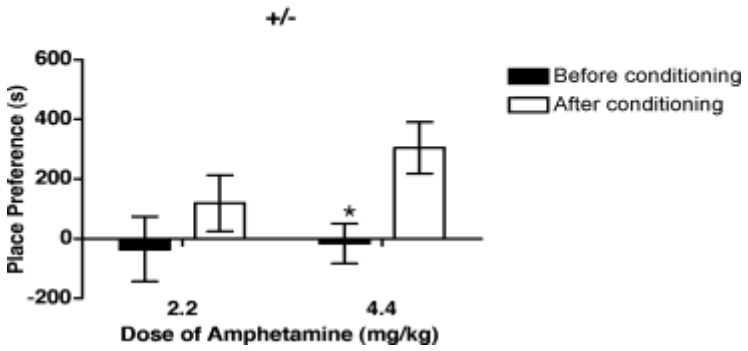

+/-
600
400
200
0
-200
Place Preference (s)
*
2.2
4.4
Dose of Amphetamine (mg/kg)
Before conditioning
After conditioning

Fig. II-4 Extracellular concentrations of DA in the NAcc of adult *dcc* +/- mice during baseline and following an AMPH injection

Dialysates were collected through microdialysis probes and analysed for DA, DOPAC, and HVA (mean ± SEM). Samples were collected during baseline and following an injection of AMPH (2.5 mg/kg, i.p. indicated by an arrow). (**a**) Data obtained before and after the AMPH challenge, were analysed using two-way repeated measures ANOVAs, with genotype and time (min) as variables. *DA*: While there was no difference in basal levels of DA between +/- and +/+ mice (main effect of genotype: $F_{(1,14)} = 1.3$, $p = 0.28$; genotype by time interaction: $F_{(3,42)} = 1$, $p = 0.4$), a significant main effect of genotype in AMPH-induced DA release was revealed ($F_{(1,15)} = 6.04$; $p = 0.02$) and significant interaction ($F_{(4,60)} = 4.01$; $p = 0.006$). *DA metabolites:* Extracellular concentrations of DOPAC at baseline and following AMPH challenge were reduced in +/- as compared to +/+ mice (baseline: main effect of genotype, $F_{(1,15)} = 6.5$, $p = 0.02$; interaction, $F_{(3,45)} = 1.7$, $p = 0.16$; AMPH: main effect of genotype, $F_{(1,15)} = 4.8$, $p = 0.04$, interaction, $F_{(4,60)} = 3.6$, $p = 0.01$). Extracellular concentrations of HVA at baseline and following AMPH challenge were reduced in +/- as compared to +/+ mice, but these effect did not reach statistical significance (baseline: main effect of genotype, $F_{(1,16)} = 3.7$; $p = 0.07$; AMPH: main effect of genotype, $F_{(1,15)} = 2.2$; $p = 0.15$; with no significant interactions). (**b**) Placement of tip of the probes was verified using Nissl staining; only data from animals with correct probe placement were used in the analysis. n = 7-10 per group.

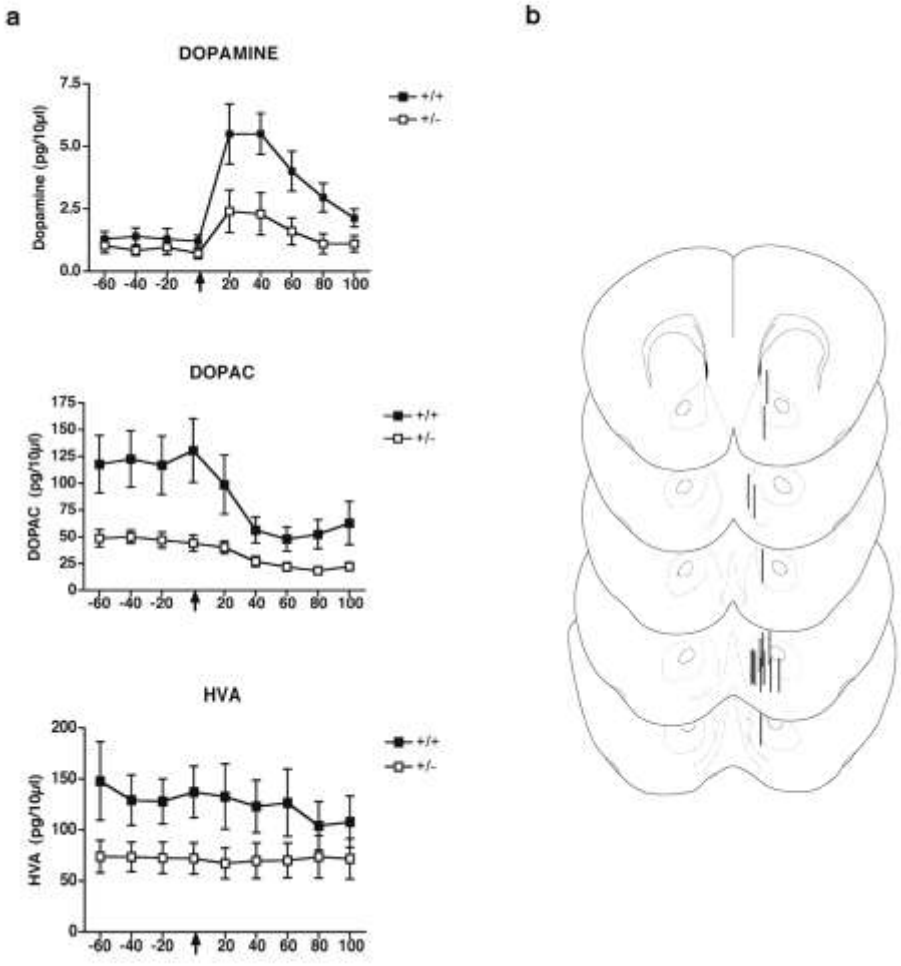
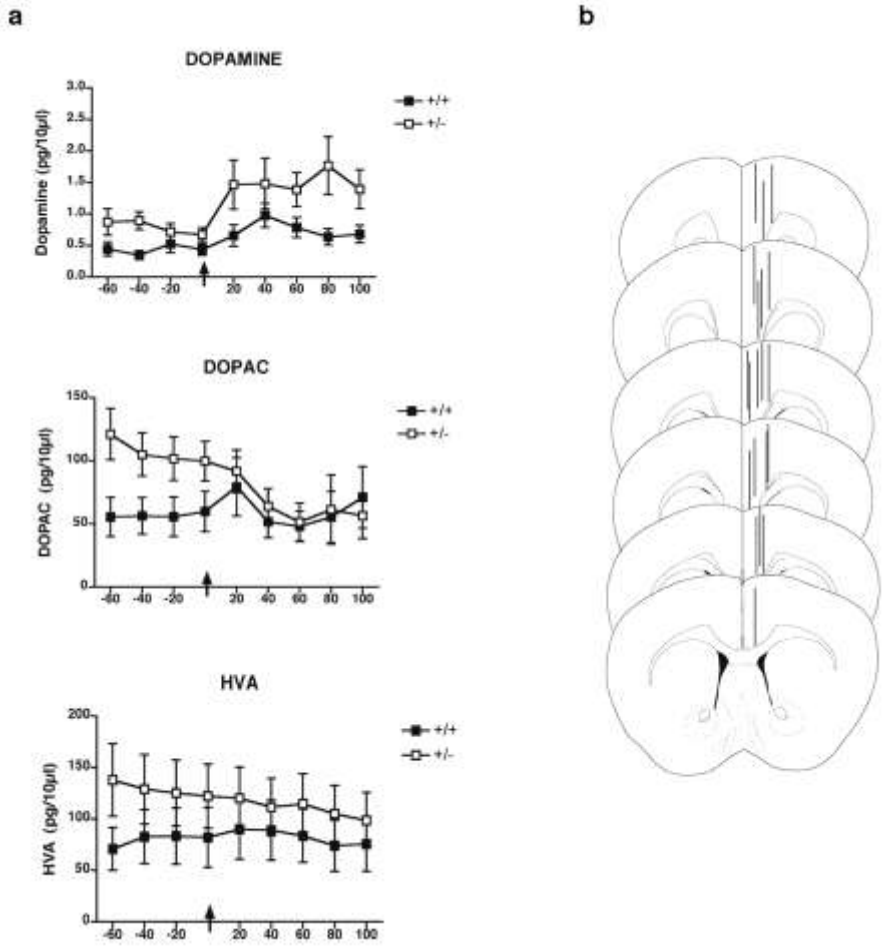

a
DOPAMINE
Dopamine (pg/10µl)
7.5
5.0
2.5
0.0
-60 -40 -20 20 40 60 80 100
+/+
+/-
DOPAC
DOPAC (pg/10µl)
175
150
125
100
75
50
25
0
-60 -40 -20 20 40 60 80 100
+/+
+/-
HVA
HVA (pg/10µl)
200
150
100
50
0
-60 -40 -20 20 40 60 80 100
+/+
+/-
b

Fig. II-5 Extracellular concentrations of DA in the mPFC of adult *dcc* +/- mice during baseline and following an AMPH injection

Dialysates were collected through microdialysis probes and analysed for extracellular levels of DA, DOPAC, and HVA (mean ± SEM). Samples were collected during baseline and following an injection of AMPH (2.5 mg/kg, i.p. indicated by an arrow). (**a**) Data obtained before and after the AMPH challenge, were analysed using two-way repeated measures ANOVAs, with genotype and time (min) as variables. *DA:* For baseline DA, there was a significant main effect of genotype ($F_{(1,17)} = 4.1$; $p = 0.05$) and a significant genotype by time interaction ($F_{(3,51)} = 2.7$; $p = 0.05$). In addition, a significant main effect of genotype in AMPH-induced DA release was revealed ($F_{(1,16)} = 4.9$; $p = 0.04$) with no significant interaction ($F_{(4,64)} = 0.9$; $p = 0.47$). *DA metabolites*: Baseline concentrations of DOPAC were significantly higher in +/- as compared to +/+ mice (main effect of genotype: $F_{(1,14)} = 4.8$; $p = 0.04$, interaction: $F_{(3,42)} = 2.5$; $p = 0.07$). Although baseline HVA concentrations were reduced in +/- mice, this effect was not statistically significant ($F_{(1,17)} = 1.3$; $p = 0.26$; interaction: $F_{(3,51)} = 2.3$; $p = 0.08$). (**b**) The placement of exposed tips of microdialysis probes was verified using Nissl staining and is indicated on the coronal plates taken from Paxinos & Franklin, 2001. Probe placements of +/- and +/+ groups were evenly distributed across the dorsal-ventral and rostral-caudal axes. Only data from animals with correct probe placement were used in the analysis. n = 7-10 per group.


a
DOPAMINE
Dopamine (pg/10µl)
+/+
+/-
DOPAC
DOPAC (pg/10µl)
HVA
HVA (pg/10µl)
b

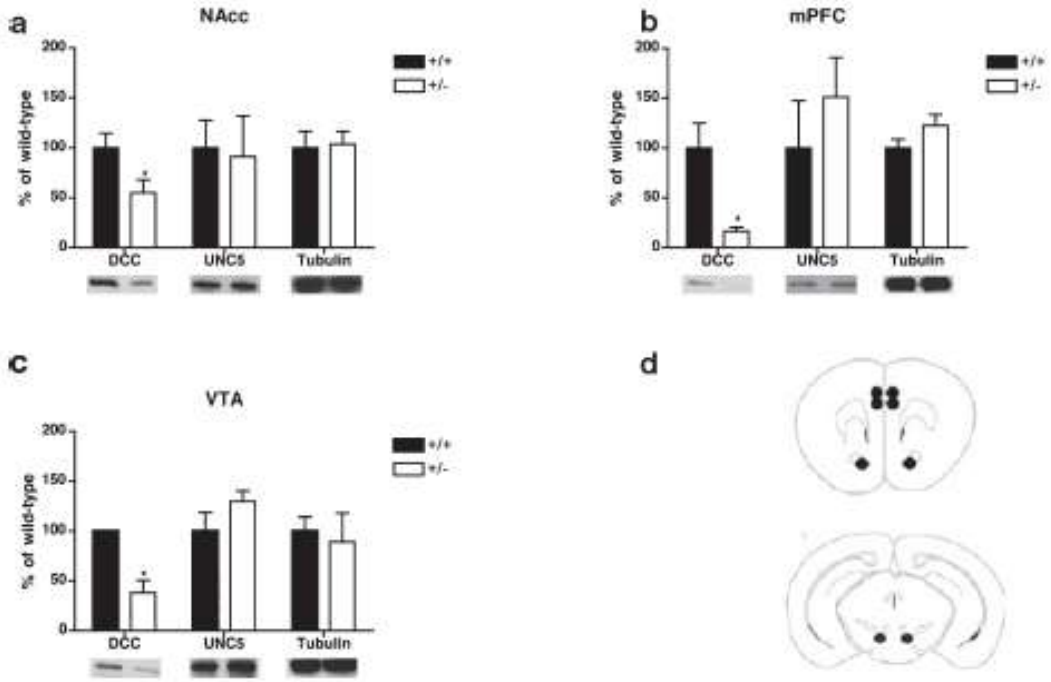


Fig. II-6 Expression of netrin-1 receptors in adult +/- and +/+ mice at baseline conditions

(a, b, c) Optical density data were converted to percent of +/+ group. Student's *t*-tests for independent samples revealed significantly lowered expression of DCC in +/- mice (n = 4 to 8 per group) in the NAcc ($t_{(7)} = 2.5$; $*p = .05$), mPFC ($t_{(15)} = 3.6$; $p = .003$) and VTA ($t_{(6)} = 3.7$; $p = .04$) No differences between groups were found in UNC5 or tubulin expression. Representative examples of western blots for +/- and +/+ mice are shown below the graphs. (**d**) Representative locations of bilateral tissue punches of the mPFC, NAcc (top) and VTA (bottom) used for western blot analysis.

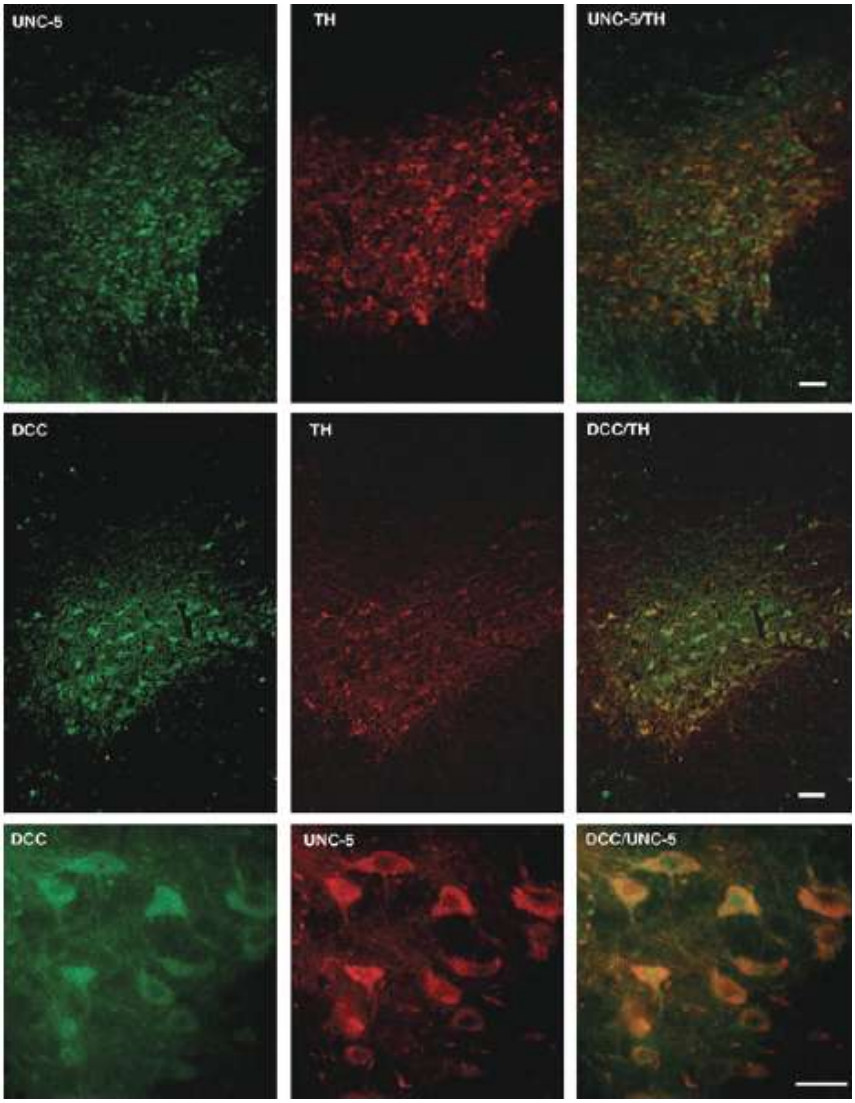


Fig. II-7 Localization of netrin-1 receptors in the VTA of adult +/+ mice Digitized images of coronal VTA sections from +/+ mice under baseline conditions. Both DCC and UNC5 immunopositive cells were visualized in the VTA. Most DCC- and UNC5 positive cells were also TH immunoreactive. The same was observed in brains of adult +/- mice. In addition, a double-labelling experiment revealed many VTA neurons coexpressing DCC and UNC5. Scale bars = 200 µm**.**

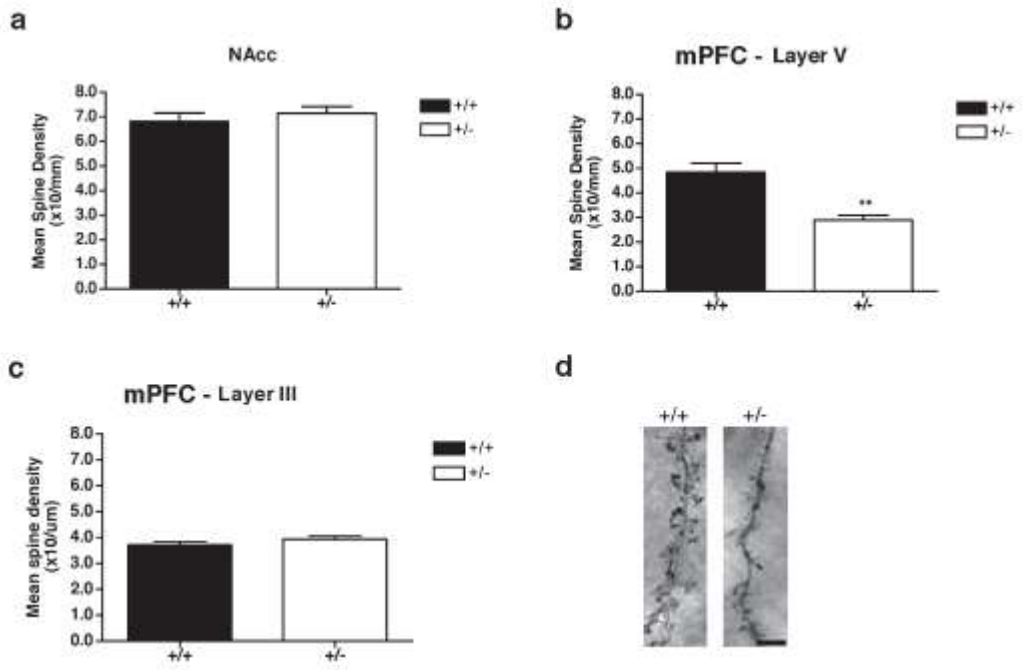


Fig. II-8 Dendritic spine density in Golgi-Cox stained NAcc medium spiny neurons and mPFC pyramidal neurons of adult *dcc* +/+ and +/- mice (n = 4-6 per group)

Data points represent group means (±SEM). (**a**) No difference was observed in NAcc medium spiny neurons between groups. (**b**) A significant ~40% reduction in dendritic spine density was observed in layer V mPFC pyramidal neurons of *dcc* +/- mice as compared to +/+ littermates (Student's *t*-tests for independent samples: $t_{(10)}$= 6.477, p = 0.00007). (**c**) No difference between groups was observed in layer III mPFC pyramidal neurons. (**d**) Photomicrograph illustrating representative Golgi-Cox-impregnated dendrites of mPFC pyramidal neurons in the basilar field of layer V of *dcc* +/+ (left) and +/- (right) mice. Scale bar = 5 μm.

ACKNOWLEDGEMENTS

We thank S. Ackerman (The Jackson Laboratory) for the original *dcc* heterozygous breeders, T. Pawson (University of Toronto) for the pan-UNC5 antiserum, G. Luheshi for critical reading of the manuscript and C. Himmelman, X. Wen and Z. Speed for their excellent technical assistance. This work was funded by the Canadian Institute for Health Research (C.F., J.S), the Natural Science and Engineering Research Council of Canada (C.F., A.A., B.K., A.G.), the Canada Research Chairs Program (A.A.), and the Fonds de la Recherche en Santé du Québec (C.F.).

REFERENCES

Banks, K.E. & Gratton, A. (1995) Possible involvement of medial prefrontal cortex in amphetamine-induced sensitization of mesolimbic dopamine function. *Eur. J. Pharmacol.*, **282**, 157-167.

Barallobre, M.J., Del Rio, J.A., Alcantara, S., Borrell, V., Aguado, F., Ruiz, M., Carmona, M.A., Martin, M., Fabre, M., Yuste, R., Tessier-Lavigne, M. & Soriano, E. (2000) Aberrant development of hippocampal circuits and altered neural activity in netrin 1-deficient mice. *Development*, **127**, 4797-4810.

Becker, J.B., Robinson, T.E. & Lorenz, K.A. (1982) Sex differences and estrous cycle variations in amphetamine-elicited rotational behaviour. *Eur. J. Pharmacol.*, **80**, 65-72.

Benes, F.M., Taylor, J.B. & Cunningham, M.C. (2000) Convergence and plasticity of monoaminergic systems in the medial prefrontal cortex during the postnatal period: implications for the development of psychopathology. *Cereb. Cortex*, **10**, 1014-1027.

Blanc, G., Trovero, F., Vezina, P., Herve, D., Godeheu, A.M., Glowinski, J. & Tassin, J.P. (1994) Blockade of prefronto-cortical alpha 1-adrenergic receptors prevents locomotor hyperactivity induced by subcortical D-amphetamine injection. *Eur. J. Neurosci.*, **6**, 293-298.

Boksa, P. (2004) Animal models of obstetric complications in relation to schizophrenia. *Brain Res. Brain Res. Rev.*, **45**, 1-17.

Bouchard, J.F., Moore, S.W., Tritsch, N.X., Roux, P.P., Shekarabi, M., Barker, P.A. & Kennedy, T.E. (2004) Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: A novel mechanism regulating commissural axon extension. *J. Neurosci.*, **24**, 3040-3050.

Brog, J.S., Salyapongse, A., Deutch, A.Y. & Zahm, D.S. (1993) The patterns of afferent innervation of the core and shell in the "accumbens" part of the rat ventral striatum: immunohistochemical detection of retrogradely transported fluoro-gold. *J. Comp. Neurol.*, **338**, 255-278.

Budygin, E.A., Brodie, M.S., Sotnikova, T.D., Mateo, Y., John, C.E., Cyr, M., Gainetdinov, R.R. & Jones, S.R. (2004) Dissociation of rewarding and dopamine transporter-mediated properties of amphetamine. *Proc. Natl. Acad. Sci. U. S. A.*, **101**, 7781-7786.

Burgess, R.W., Jucius, T.J. & Ackerman, S.L. (2006) Motor axon guidance of the mammalian trochlear and phrenic nerves: dependence on the netrin receptor Unc5c and modifier loci. *J. Neurosci.*, **26**, 5756-5766.

Carr, D.B., O'Donnell, P., Card, J.P. & Sesack, S.R. (1999) Dopamine terminals in the rat prefrontal cortex synapse on pyramidal cells that project to the nucleus accumbens. *J. Neurosci.*, **19**, 11049-11060.

Carr, D.B. & Sesack, S.R. (2000) Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J. Neurosci.*, **15**, 3864-3873.

Chambers, R.A., Krystal, J.H. & Self, D.W. (2001) A neurobiological basis for substance abuse comorbidity in schizophrenia. *Biol. Psychiatry*, **50**, 71-83.

Chen, J., Lipska, B.K. & Weinberger, D.R. (2006) Genetic mouse models of schizophrenia: from hypothesis-based to susceptibility gene-based models. *Biol. Psychiatry*, **59**, 1180-1188.

Deutch, A.Y., Clark, W.A. & Roth, R.H. (1990) Prefrontal cortical dopamine depletion enhances the responsiveness of mesolimbic dopamine neurons to stress. *Brain Res.*, **521**, 311-315.

Di Chiara, G., Bassareo, V., Fenu, S., De Luca, M.A., Spina, L., Cadoni, C., Acquas, E., Carboni, E., Valentini, V. & Lecca, D. (2004) Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology*, **47 Suppl 1**, 227-241.

Doherty, M.D. & Gratton, A. (1996) Medial prefrontal cortical D1 receptor modulation of the meso-accumbens dopamine response to stress: an electrochemical study in freely-behaving rats. *Brain Res.*, **715**, 86-97.

Fallon, J. & Loughlin, S. (1987) Monoamine innervation of cerebral cortex and a theory of the role of monoamines in cerebral cortex and basal ganglia. In Jones, E., Peters, A. (eds.) *Cerebral Cortex*. Plenum, New York, pp. 41-127.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu M, Rayburn, H., Simons J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M. & Weinberg, R.A. (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature*, **386,** 796-804.

Flores, C., Manitt, C., Rodaros, D., Thompson, K.M., Rajabi, H., Luk, K.C., Tritsch, N.X., Sadikot, A.F., Stewart, J. & Kennedy, T.E. (2005) Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Mol. Psychiatry*, **10**, 606-612.

Gad, J.M., Keeling, S.L., Wilks, A.F., Tan, S.S. & Cooper, H.M. (1997) The expression patterns of guidance receptors, DCC and Neogenin, are spatially and temporally distinct throughout mouse embryogenesis. *Dev. Biol.*, **192**, 258-273.

Gibb, R. & Kolb, B. (1998) A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J. Neurosci. Methods*, **79**, 1-4.

Glantz, L.A. & Lewis, D.A. (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch. Gen. Psychiatry*, **57**, 65-73.

Grace, A.A. (1991) Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: a hypothesis for the etiology of schizophrenia. *Neuroscience*, **41**, 1-24.

Grace, A.A., Floresco, S.B., Goto, Y. & Lodge, D.J. (2007) Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci.*, **30**, 220-227.

Harris, K.M. & Kater, S.B. (1994) Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu. Rev. Neurosci.*, **17**, 341-371.

Harrison, P.J. & Weinberger, D.R. (2005) Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol. Psychiatry*, **10**, 40-68

Holm, S. (1979) A simple sequentially rejective multiple test procedure. *Scand. J. Stat.*, **6**, 65-70.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M. & Stein, E. (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell*, **97**, 927-941.

Kirov, S.A. & Harris, K.M. (1999) Dendrites are more spiny on mature hippocampal neurons when synapses are inactivated. *Nat. Neurosci.*, **2**, 878-883.

Knable, M.B. & Weinberger, D.R. (1997) Dopamine, the prefrontal cortex and schizophrenia. *J. Psychopharmacol.*, **11**, 123-131.

Kolb, B., Forgie, M., Gibb, R., Gorny, G. & Rowntree, S. (1998) Age, experience and the changing brain. *Neurosci. Biobehav. Rev.*, **22**, 143-159.

Le Moal, M. & Simon, H. (1991) Mesocorticolimbic dopaminergic network: functional and regulatory roles. *Physiol. Rev.*, **71**, 155-234.

Lewis, D.A. & Gonzalez-Burgos, G. (2000) Intrinsic excitatory connections in the prefrontal cortex and the pathophysiology of schizophrenia. *Brain Res. Bull.*, **52**, 309-317.

Lin, L., Rao, Y. & Isacson, O. (2005) Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons. *Mol. Cell. Neurosci.*, **28**, 547-555.

Livesey, F.J. & Hunt, S.P. (1997) Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. *Mol. and Cell. Neurosci.*, **8**, 417-429.

Manitt, C. & Kennedy, T.E. (2002) Where the rubber meets the road: netrin expression and function in developing and adult nervous systems. *Prog. Brain Res.*, **137**, 425-442.

Manitt, C., Thompson, K.M. & Kennedy, T.E. (2004) Developmental shift in expression of netrin receptors in the rat spinal cord: predominance of UNC5 homologues in adulthood. *J. Neurosci. Res.*, **77**, 690-700.

Morse, A.C., Erwin, V.G. & Jones, B.C. (1995) Behavioral responses to low doses of cocaine are affected by genetics and experimental history. *Physiol. Behav.*, **58**, 891-897.

Osborne, P.B., Halliday, G.M., Cooper, H.M. & Keast, J.R. (2005) Localization of immunoreactivity for deleted in colorectal cancer (DCC), the receptor for the guidance factor netrin-1, in ventral tier dopamine projection pathways in adult rodents. *Neuroscience*, **131**, 671-681.

Paxinos, G. & Franklin, K.B.J. (2001) *The Mouse Brain in Stereotaxic Coordinates*. Academic Press, San Diego.

Ross, C.A., Margolis, R.L., Reading, S.A., Pletnikov, M. & Coyle, J.T. (2006) Neurobiology of schizophrenia. *Neuron*, **52**, 139-153.

Seamans, J.K. & Yang, C.R. (2004) The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Prog. Neurobiol.*, **74**, 1-58.

Sellings, L.H. & Clarke, P.B. (2003) Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. *J. Neurosci.*, **23**, 6295-6303.

Sesack, S.R. & Pickel, V.M. (1992) Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. *J. Comp. Neurol.*, **320**, 145-160.

Sesack, S.R., Snyder, C.L. & Lewis, D.A. (1995) Axon terminals immunolabeled for dopamine or tyrosine hydroxylase synapse on GABA-immunoreactive dendrites in rat and monkey cortex. *J. Comp. Neurol.*, **363**, 264-280.

Shu, T., Valentino, K.M., Seaman, C., Cooper, H.M. & Richards, L.J. (2000) Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. *J. Comp. Neurol.*, **416**, 201-212.

Smidt, M.P. & Burbach, J.P. (2007) How to make a mesodiencephalic dopaminergic neuron. *Nat. Rev. Neurosci.*, **8**, 21-32.

Swerdlow, N.R., Bongiovanni, M.J., Tochen, L. & Shoemaker, J.M. (2006) Separable noradrenergic and dopaminergic regulation of prepulse inhibition in rats: implications for predictive validity and Tourette Syndrome. *Psychopharmacology (Berl.)*, **186**, 246-254.

Swerdlow, N.R., Mansbach, R.S., Geyer, M.A., Pulvirenti, L., Koob, G.F. & Braff, D.L. (1990) Amphetamine disruption of prepulse inhibition of acoustic startle is reversed by depletion of mesolimbic dopamine. *Psychopharmacology (Berl.)*, **100**, 413-416.

Tam, S.Y. & Roth, R.H. (1997) Mesoprefrontal dopaminergic neurons: can tyrosine availability influence their functions? *Biochem. Pharmacol.*, **53**, 441-453.

Tenn, C.C., Kapur, S. & Fletcher, P.J. (2005) Sensitization to amphetamine, but not phencyclidine, disrupts prepulse inhibition and latent inhibition. *Psychopharmacology (Berl.)*, **180**, 366-376.

Thompson, T.L. & Moss, R.L. (1995) In vivo stimulated dopamine release in the nucleus accumbens: modulation by the prefrontal cortex. *Brain Res.*, **686**, 93-98.

Tsai, G., Ralph-Williams, R.J., Martina, M., Bergeron, R., Berger-Sweeney, J., Dunham, K.S., Jiang, Z., Caine, S.B. & Coyle, J.T. (2004) Gene knockout of glycine transporter 1: characterization of the behavioral phenotype. *Proc. Natl. Acad. Sci. U. S. A.*, **101**, 8485-8490.

Varty, G.B., Walters, N., Cohen-Williams, M. & Carey, G.J. (2001) Comparison of apomorphine, amphetamine and dizocilpine disruptions of prepulse inhibition in inbred and outbred mice strains. *Eur. J. Pharmacol.*, **424**, 27-36.

Ventura, R., Alcaro, A., Cabib, S., Conversi, D., Mandolesi, L. & Puglisi-Allegra, S. (2004) Dopamine in the medial prefrontal cortex controls genotype-dependent effects of amphetamine on mesoaccumbens dopamine release and locomotion. *Neuropsychopharmacology*, **29**, 72-80.

Ventura, R., Cabib, S., Alcaro, A., Orsini, C. & Puglisi-Allegra, S. (2003) Norepinephrine in the prefrontal cortex is critical for amphetamine-

induced reward and mesoaccumbens dopamine release. *J. Neurosci.*, **23**, 1879-1885.

Vezina, P., Blanc, G., Glowinski, J. & Tassin, J.P. (1991) Opposed Behavioural Outputs of Increased Dopamine Transmission in Prefrontocortical and Subcortical Areas: A Role for the Cortical D-1 Dopamine Receptor. *Eur. J. Neurosci.*, **3**, 1001-1007.

Volenec, A., Zetterstrom, T.S. & Flanigan, T.P. (1998) 6-OHDA denervation substantially decreases DCC mRNA levels in rat substantia nigra compacta. *Neuroreport*, **9**, 3553-3556.

Weinberger, D.R. (1987) Implications of normal brain development for the pathogenesis of schizophrenia. *Arch. Gen. Psychiatry*, **44**, 660-669.

Williams, M.E., Strickland, P., Watanabe, K. & Hinck, L. (2003) UNC5H1 induces apoptosis via its juxtamembrane region through an interaction with NRAGE. *J. Biol. Chem.*, **278**, 17483-17490.

Woolley, C.S., Gould, E., Frankfurt, M. & McEwen, B.S. (1990) Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *J. Neurosci.*, **10**, 4035-4039.

Woolley, C.S. & McEwen, B.S. (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *J. Neurosci.*, **12**, 2549-2554.

II.3. Supplementary data and discussion

It is possible that the phenotype observed in *dcc* +/- may result from alterations in NE functioning in the mPFC. However, results from my microdialysis experiment, imply that this is not likely; there are no differences between +/- and +/+ mice in baseline levels of NE or following AMPH injection (see Fig II-9). Nonetheless, I cannot completely rule that this system may contribute to the observed phenotype.

Possible involvement of other neurotransmitter systems in the *dcc* heterozygous phenotype

In situ hybridization experiments demonstrate that DCC mRNA is ubiquitously expressed throughout the rodent CNS during early embryogenesis and does not become restricted to any particular neuronal type or brain region until around E18. At this time, DCC expression becomes limited to the olfactory bulb, hippocampus, midbrain DA nuclei, red nucleus, and cerebellum (Gad et al. 1997). Since DCC is widely expressed throughout the embryonic brain, it is possible that reduced gene dosage of DCC in +/- mice may affect the development of other neurotransmitter systems. Alterations in the development other neural networks may therefore contribute to the +/- phenotype reported in this Chapter.

As a first step to address this possibility, the experiments described in this Chapter should be repeated in mice in which *dcc* is selectively "knocked" out in DA neurons. Our laboratory has begun crossing mice containing loxP sites flanking Exon 23 of the *dcc* gene with mice that contain a transgene expressing

Cre recombinase under the dopamine transporter promoter. With the breeding scheme we use, we are able to produce mice that have just one floxed *dcc* allele, and thus have reduced DCC expression in only a subset of DA neurons. Remarkably, preliminary AMPH-locomotor studies conducted in these mice demonstrate that simply "knocking-down" *dcc* selectively in DA neurons is sufficient to reproduce the blunted locomotor response to AMPH. The initial studies in these conditional *dcc* "knock-down" mice strongly imply that alterations in DA systems are the major determinant of the observed +/- phenotype and that the extent to which other neurotransmitter systems contribute to the phenotype is quite small.

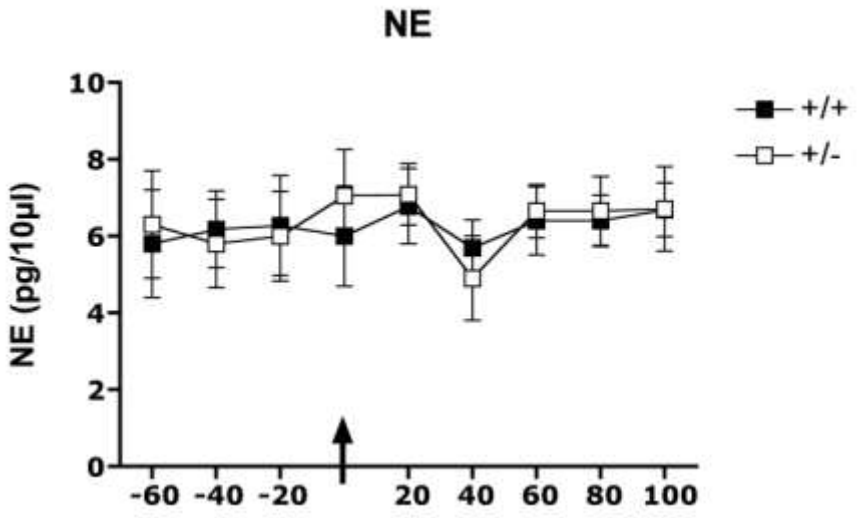


Fig. II-9 Extracellular concentrations of NE in the mPFC of adult *dcc* +/- mice during baseline and following an AMPH injection

Dialysates were collected through microdialysis probes and analysed for NE (mean ± SEM). Samples were collected during baseline and following an injection of AMPH (2.5 mg/kg, i.p. indicated by an arrow). Data obtained before and after the AMPH challenge, were analysed using two-way repeated measures ANOVAs, with genotype and time (min) as variables. Results of these statistical tests are provided in the Results section of the manuscript.

n = 8-10 per group

Chapter III: Postpubertal emergence of a dopamine phenotype in *netrin-1* receptor-deficient mice

III.1. Preamble

The findings presented in Chapter II demonstrate that reduced expression of DCC during development and/or throughout life results in a behavioural phenotype opposite to that observed in neurodevelopmental animal models of schizophrenia, and identify DCC as a critical factor in the development of mesocorticolimbic DA function. Furthermore, the data described in the previous chapter suggest that the DA and behavioural phenotype observed in adult *dcc* heterozygous mice is a result of selective changes in the organization and function of mPFC DA system. However, as these mice develop with reduced levels of DCC throughout life, the precise developmental period during which the reduction in DCC expression results in mPFC reorganization and subsequent manifestation of the "protective" phenotype cannot be determined from these data.

It is well established that adolescence is a critical period for the development and maturation of mPFC DA functioning and for the establishment of individual differences in the vulnerability to develop DA-related psychopathologies. Therefore, one possibility is that adolescence is also the critical period for the alterations in the mPFC DA circuitry and the development of the "protective" phenotype displayed by adult *dcc* heterozygous mice. The primary objective of this Chapter was to determine if the behavioural and DA phenotypes exhibited by adult *dcc* heterozygous mice are present prior to maturation of the mPFC DA circuitry. To this end, I examined whether *dcc* heterozygous post-weanling or peri-pubertal mice show *i*) blunted behavioural

response to amphetamine; *ii*) elevated baseline concentrations of DA and DA metabolites in the mPFC; *iii*) increased mPFC TH protein expression; and *iv*) reduced number of midbrain dopamine neurons.

It is important to clarify the usage of the terms "post-weanling" and "post-weaning." In the following manuscript, juvenile animals that are between the ages of PND20-PND22 are called "post-weanling." The term used to define this age is "post-weaning."

III.2. Research Manuscript

Post-pubertal emergence of a dopamine phenotype in netrin-1 receptor deficient mice

Alanna Grant[1], Zoe Speed[1], Cassandre Labelle-Dumais[1], Cecilia Flores[1]

[1]Departments of Psychiatry and of Neurology and Neurosurgery, McGill University, Douglas Hospital Research Centre, 6875 LaSalle Boulevard, Quebec, Canada, H4H 1R3

European Journal of Neuroscience, 2009, Vol 30, pp1318-1328

ABSTRACT

During the pubertal period the mesocortical dopamine (DA) system undergoes substantial reorganization of neuronal connectivity and functional refinement. Netrins are guidance cues involved in the organization of neuronal circuitry. We have previously shown that adult mice that develop with reduced levels of the netrin-1 receptor, deleted in colorectal cancer (DCC), display selective reorganization of mesocortical DA circuitry, show enhanced mesocortical DA function, and exhibit a behavioural phenotype opposite to that observed in animal models of schizophrenia. Here we assess whether the *dcc* behavioural and DA phenotypes are present prior to the maturation of the mesocortical DA system by comparing *dcc* heterozygous and wild-type mice at the post-weaning and peri-pubertal periods on various indices of DA function. At both the post-weaning and peri-pubertal ages, but unlike in adulthood, *dcc* heterozygous and wild-type mice show no differences in the number of midbrain DA neurones or in tyrosine hydroxylase protein levels in the medial prefrontal cortex. Furthermore, the elevated baseline concentration of mesocortical DA and DA metabolites observed in adult *dcc* heterozygous mice, is not present in either post-weanling or peri-pubertal mice. Interestingly, post-weanling, but not peri-pubertal, *dcc* heterozygous mice show greater baseline concentrations of DA metabolites in the nucleus accumbens, opposite to what was observed in adulthood. Finally, neither post-weanling nor peri-pubertal *dcc* heterozygous mice demonstrate the blunted amphetamine-induced locomotor response observed in adulthood. Thus these findings show that the "protective" *dcc*-phenotype has a post-pubertal emergence

and indicate that DCC may play a role in the normal maturation of the mesocorticolimbic DA system.

INTRODUCTION

Adolescence is a critical stage in the development of the mesocorticolimbic dopamine (DA) system, particularly the medial prefrontal cortex (mPFC) DA projection, which continues to develop until early adulthood. Symptoms of several psychiatric disorders associated with compromised mesocorticolimbic DA function, such as schizophrenia, become evident during or shortly after adolescence. This delay in the emergence of psychopathology-like symptoms, which is also observed in putative developmental animal models, coincides with maturational changes in the DA system occurring during this time period (Adriani and Laviola 2004; Andersen 2003; Andersen et al. 2000; Benes et al. 2000; Benes et al. 1996; Boksa 2004; Crews et al. 2007; Feinberg 1982; Knable and Weinberger 1997; Lewis 1997; Lipska et al. 1993; Lipska and Weinberger 2000; Spear 2000; Teicher et al. 1995). Therefore, adolescence may be a critical period for the establishment of individual differences in the vulnerability to develop altered DA function in adulthood.

Netrin-1 is guidance cue that can either attract or repel growing neurites during development. Generally, attraction is mediated through the deleted in colorectal cancer (DCC) receptor, whereas repulsion is signalled through UNC5 homologue (UNC5H) receptor alone, or UNC5H-DCC receptor complexes (Barallobre et al. 2005; Hong et al. 1999; Keino-Masu et al. 1996; Keleman and Dickson 2001; Manitt and Kennedy 2002). Mesocorticolimbic DA neurons express high levels of DCC from embryonic life to adulthood in the rodent brain (Lin et al. 2005; Grant et al. 2007; Labelle-Dumais and Flores 2008; Osborne et al. 2005; Yetnikoff et al. 2007), but expression of UNC5H receptors by DA

neurons only emerges at the peri-pubertal age (Labelle-Dumais and Flores 2008). Thus, variations in netrin-1 receptor levels at key developmental stages may play an instructive role in the organization of the DA system.

We have shown that altered DCC expression in the adult brain is associated with a functional reorganization of the mesocorticolimbic DA system (Flores et al. 2005; Grant et al. 2007). Briefly, adult *dcc* heterozygous mice (*dcc* homozygotes die shortly after birth), in comparison to wild-type mice, have large increases in baseline as well as amphetamine-induced DA release in the mPFC, but significantly blunted amphetamine-induced DA release in the nucleus accumbens (NAcc). Correspondingly, adult *dcc* heterozygotes exhibit diminished amphetamine-induced locomotion and reward, and are resistant to amphetamine-induced deficits in sensorimotor gating (Flores et al. 2005; Grant et al. 2007).

The behavioural and DA phenotype observed in adult *dcc* heterozygous mice appear to result from *selective* alterations in the organization of mPFC DA circuitry. First, despite having elevated tyrosine hydroxylase (TH) expression in mPFC, but not in NAcc, adult *dcc* heterozygous mice show a ~20% reduction in the total number of DA neurons in the ventral tegmental area, suggesting enhanced DA axonal branching in mPFC (Flores et al. 2005). Second, adult *dcc* heterozygous mice exhibit altered dendritic spine density in layer V mPFC pyramidal cells, but not in NAcc medium spiny neurons (Grant et al. 2007). These findings are significant in light of the fact that DA neurons projecting to the mPFC are anatomically and functionally different from those that project to the NAcc. Moreover, mPFC DA activity has been shown to inhibit DA release in the NAcc, suggesting that the blunted behavioural and NAcc DA response to

amphetamine observed in adult *dcc* heterozygotes may be a consequence of the exaggerated DA activity in mPFC (Banks and Gratton 1995; Bunney and Aghajanian 1976; Carr and Sesack 2000; Deutch et al. 1990; Doherty and Gratton 1996; Gogos et al. 1998; Knable and Weinberger 1997; Lammel et al. 2008; Le Moal and Simon 1991; Margolis et al. 2006; Margolis et al. 2008; Moron et al. 2002; Piazza et al. 1991; Sesack et al. 1998; Tam and Roth 1997; Thompson and Moss 1995; Ventura et al. 2004; Vezina et al. 1994).

If indeed the 'protective' phenotype observed in adult *dcc* heterozygous mice results from selective alterations in mPFC DA circuitry, then this phenotype should only emerge upon maturation of this system. Here we assessed whether the *dcc* behavioural and DA phenotypes observed in adulthood are evident prior to mPFC DA maturation. To this end we examined differences in behavioural responses to amphetamine and in several measures of DA function between *dcc* heterozygous and wild-type mice at post-weaning and peri-pubertal periods.

MATERIALS AND METHODS

Animals

Male and/or female dcc heterozygous (+/-) mice, originally obtained from Dr. S. Ackerman (The Jackson Laboratory) and maintained in the BL6 background at our animal colony, were used in all experiments. Unless otherwise specified, experiments were conducted with male post-weanling (PND21 ± 1) and peri-pubertal (PND33 ± 2) dcc +/- and wild-type (+/+) mice. The peri-pubertal age was chosen based on the time of first spermatozoa production in male BL6 mice and on age of vaginal opening in female BL6 mice (Gore et al. 1999; Morley and Rodriguez-Sierra 2004; Oakberg 1957).

Mice were kept on a 12h light-dark cycle with *ad libitum* access to water and food. Pups were weaned at PND21 (±1) and housed with same-sex littermates. Different cohorts of *dcc* +/- and +/+ mice, from a minimum of five litters, were used in each experiment. All experiments were preformed in accordance with the guidelines of the Canadian Council of Animal Care, and the Animal Committee of the Douglas Mental Health University Institute/McGill University. All efforts were made to minimize the suffering and the number of animals used.

Drugs

On the day of the experiment, *d*-amphetamine sulphate salt (AMPH, Sigma) was dissolved in 0.9% saline and was injected i.p.

Locomotor Activity

All behavioural testing occurred during the light phase. Locomotor experiments were conducted, as described previously (Grant et al. 2007). Briefly, locomotor activity was quantified with an infrared activity monitoring apparatus modified for use with mice (AccuScan Instruments, Columbus, Ohio) and was expressed as distance travelled (in cm). Stereotypy counts were measured as the number of breakings of the same photocell beam or set of beams repeatedly, as defined by the AccuScan system.

Data collection occurred over three consecutive days. On each day the mice were placed in the boxes for 15 minutes to habituate to the testing environment. On the first day, following the 15-minute habituation period, mice were removed from the boxes and returned to the animal facility. On the second day, mice were given a saline injection following the 15-minute habituation period and returned to the activity boxes for an additional 30 minutes. On the final day, mice were given an injection of AMPH following the 15-minute habituation period and were monitored for an additional 3 hours. Both males and females mice were tested with the same dose of AMPH used in our previous studies conducted in adult mice (females: 2.2 mg/kg; males: 2.5 mg/kg) (Flores et al. 2005; Grant et al. 2007). In addition, a dose-dependent experiment was conducted in the female post-weanling group by testing a different cohort with 3.5 mg/kg of AMPH. This higher dose was previously tested in adult mice and produced different effects in *dcc* +/- and +/+ (Flores et al. 2005). Adjustments were made in the AMPH doses to produce equivalent drug brain concentrations in male and female mice (Becker et al. 1982). All doses tested are within the low to moderate

range of doses commonly used in studies conducted in post-weanling and peri-pubertal mice (Adriani and Laviola 2000; Cirulli and Laviola 2000; Niculescu et al. 2005).

High Performance Liquid Chromatography

Concentrations of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured by electrochemical detection as previously described (Flores et al. 2005). Briefly, experimentally-naïve male *dcc* +/- and +/+ post-weanling and peri-pubertal male mice were decapitated and their brains were dissected from the skull cavities and immersed in 2-methylbutane (Fisher Scientific, Hampton, NH, USA) chilled with dry ice and were stored at -80^{o}C. It is important to note that because our goal is to measure concentrations of DA, DOPAC and HVA at baseline, we took special care to minimize stress during this procedure. Bilateral tissue punches of the mPFC, NAcc and dorsal striatum (STR) from 600 µm thick coronal slices were resuspended in 100 µl 0.1 M phosphate buffer, pH 7.0 and filtered using 0.45 µm syringe filters. A 10 µl volume of this filtrate was loaded onto a 15 cm C-18 reverse-phase column via manual injection ports (20 µl loop). Dual-channel coulometric III detectors (model 5100A; ESA, Inc., Bedford, MA, USA) were used to measure the reduction and oxidation currents for DA and DA metabolites (one channel was used for DA, the other for DA metabolites). Concentrations were obtained by comparing peak heights for each compound against peak heights of previously injected standards containing known concentrations of DA, DOPAC and HVA.

The peaks corresponding to DA, DOPAC and HVA were quantified and analysed using EZChrom Data Chromatography Data System (Scientific Software, Inc., San Ramon, CA, USA).

Western blotting

All primary antibodies used are commercially available and the specificity of these antibodies have been previously characterized by immunoblotting and/or immunostaining experiments. The DCC antibody was raised in mouse against a truncated recombinant protein containing the intracellular domain of human DCC and recognizes a single band of ~185 kDa on Western blot of lysate from IMR-32 cells (manufacturer's technical information). The tyrosine hydroxylase antibody was raised in rabbit against denatured tyrosine hydroxylase (TH) protein from rat pheochromocytoma and labels a single band of ~62 kDa on Western blot of lysate from PC12 cells (manufacturer's and technical information). The α-tubulin antibody was produced in mouse against sarkosyl-resistant filaments from sea urchin sperm axonemes and recognizes an epitope located at the C-terminal domain of the α-tubulin isoform. On Western blots, this antibody labels a single band of ~50 kDa on lysate from FS11 cells (manufacturer's technical information).

Western blotting for tyrosine hydroxylase (TH, the rate limiting enzyme of catecholamine biosynthesis) was conducted in tissue samples of mPFC, NAcc and STR of brains from experimentally-naïve post-weanling and peri-pubertal male *dcc* +/- and +/+ mice as described previously (Flores et al. 2005; Grant et al.

2007). Briefly, bilateral punches of mPFC, including cingulate cortex area 1 and 2, NAcc, including both core and shell, and STR were excised from approximately 600 µm-thick coronal sections. Sampling areas were taken starting from sections corresponding to plate 15 of the Paxinos & Franklin mouse atlas (Paxinos and Franklin 2001). Protein samples (25 µg) were resolved using SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The membrane was incubated with antibodies against DCC (1:1000, mouse monoclonal), TH (1:5000, rabbit polyclonal; Chemicon International, Temecula, CA, USA; catalogue number AB152), and α-tubulin (1:4000, mouse monoclonal; Sigma; catalogue number T6074). Bands were detected by chemiluminescence (Perkin Elmer, Waltham, MA, USA) and analysed using Kodak Imaging system software (Kodak Imaging 2000, New Haven, CT, USA).

Neuroanatomy

Tissue preparation: Post-weanling and peri-pubertal male *dcc* +/- and +/+ mice were anesthetized with an overdose of sodium pentobarbital (>75 mg/kg, i.p.) and perfused intracardially with 0.9% saline followed by fixative solution (4% paraformaldehyde in PBS). Brains were removed and post-fixed in the same solution for 45 minutes at 4°C. Fixed brains were then cryoprotected in 30% sucrose-PBS overnight at 4°C. On the following day, brains were flash frozen in 2-methylbutane at -45°C, and were stored at -80°C until needed. Brains were cut on a coronal plane through the midbrain region spanning the VTA and substantia

nigra (SN) with a section thickness of 40 µm using a Leica SM2000-R sliding microtome. Sections were used immediately or kept free-floating in cryoprotectant for subsequent TH immunohistochemistry.

Immunohistochemistry: Midbrain DA neurons within the VTA and SN *pars* compacta were identified by TH immunohistochemistry. A 1:2 series of free-floating coronal sections, starting from sections corresponding to plate 55 of the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin 2001), were washed in PBS and incubated in 0.3% hydrogen peroxide, 0.3% heat-inactivated goat serum in PBS for 30 minutes. Sections were then blocked for 2hr at room temperature in blocking solution (2% BSA, 2% goat serum, 0.3% Triton-X), and incubated with rabbit anti-TH antibody (1:500, Chemicon, Temecula, CA, USA), for 1hr at room temperature and then for 36 hr at 4 °C. Following several rinses with PBS, sections were incubated with a biotinylated secondary antibody (goat anti-rabbit IgG; 1:250, Vector Lab, Cat# BA-1000, Burlingame, CA, USA) for 2hr at room temperature, rinsed in PBS, and incubated with Vectastain Elite ABC reagent (Vector Lab, Burlingame, CA, USA) for 2hr. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB, Sigma). Sections were mounted onto gelatin-coated slides, dried overnight and coverslipped using Permount (Fisher Scientific) the following day.

Stereological analysis: Sections were examined under light microscopy with a Leica DM4000 microscope equipped with a Ludl XYZ motorized stage and a digital camera connected to a computer. The number of DA neurons was estimated using an unbiased fractionator sampling design (Finkelstein et al. 2000;

Flores et al. 2005; Gundersen et al. 1988; Parish et al. 2001; West and Gundersen 1990) using the Optical Fractionator probe of the StereoInvestigator® software (MicroBrightField (MBF) Bioscience, Williston, VT, USA). In each section sampled, VTA and SN *pars* compacta DA neurons were counted using the cell body of TH-immunoreactive neurons as the counting unit. Immunolabelled cells were only counted if the top surface of the cell came into focus within the established counting frame and within the dissector height. For unbiased stereological estimates of VTA and SN *pars* compacta DA neuron numbers, TH-immunoreactive cells were counted on 40 µm-thick serial sections, each 80 µm apart (the section sampling fraction being 1/2 = 0.5), at regular predetermined intervals (x = 150 µm, y = 150 µm). Cell counts were made using a grid program, StereoInvestigator (MBF), through which a systematic sampling of the VTA and SN *pars* compacta was established from a random starting point. An unbiased 60 µm X 60 µm counting frame was employed on brain sections viewed under a 40X objective (numerical aperture: 0.75). The area sampling fraction, obtained by dividing the area of the counting frame by the area of the distance between sampling regions, i.e. x and y intervals, was 3600 / (150x 150) = 0.16. The average mounted thickness of sections was 13 µm, and we used 1µm guard zones to include 84% of the height of the section for sampling. The total number of TH-immunoreactive neurons was calculated by multiplying the number of TH-immunoreactive neurons counted by the reciprocals of the fraction of the brain region sampled and the section-thickness sampled. The precision of the cell number estimates was evaluated by calculating the coefficient of error and

coefficient of variance; values < 0.1 were accepted (Parish et al. 2001; West and Gundersen 1990).

Statistical Analyses

The goal of the study was to determine whether differences between *dcc* +/- and +/+ mice observed in adulthood would be evident at younger ages. Thus, data from experiments conducted with post-weanling and peri-pubertal animals were analysed separately.

Locomotor Activity: Differences in total distance travelled scores were analysed using two-way repeated measures ANOVAs with genotype and time (min) as between- and within-group variables, respectively. Student's *t* tests for independent samples were used to analyze differences in stereotypy counts between groups.

High Performance Liquid Chromatography: All analyses were conducted on raw data (pg/µl/mg protein). Data were analysed using two-way repeated measures ANOVAs with genotype and brain region as the between- and within-group variables, respectively. *Post-hoc* analyses of significant interactions were decomposed using ANOVA tests for simple effects. Additionally, metabolite/DA ratios for the NAcc and STR were analysed using two-way repeated measures ANOVAs to provide an index of DA activity.

Western blotting: Raw data (optical density of scanned immunoblots) was normalized to tubulin to correct for any loading errors and analyses were conducted on these normalized data. Differences in protein expression between *dcc* +/- and +/+ mice were analysed using two way repeated measures ANOVAs

with genotype and brain region as the between- and within-group variables, respectively.

Stereology: The number of TH-immunoreactive cells were analysed using a two-way repeated measures ANOVA with genotype as the between-subjects variable and brain region sampled (VTA or SN *pars* compacta) as the repeated measure.

RESULTS

dcc +/- mice do not exhibit blunted behavioural response to AMPH before puberty

We showed previously that adult male and female *dcc* heterozygous (+/-) mice exhibit blunted locomotor responses to single injections of AMPH with no changes in baseline activity (Flores et al. 2005; Grant et al. 2007). Here we assessed locomotor activity in post-weanling and peri-pubertal male and female *dcc* +/- and wild-type (+/+) mice at baseline and following an AMPH challenge. As previously reported in adult mice, no effect of genotype was detected on baseline locomotor activity (Figs III-1a & III-2a) or following a single injection of saline (Figs III-1b & III-2b) at either age or gender. Repeated-measures ANOVA revealed that both post-weanling and peri-pubertal male *dcc* +/- and +/+ mice exhibited similar AMPH-induced locomotion (Fig III-1c), contrary to what we observed in adult animals (post-weaning: main effect of genotype: $F_{(1, 14)} = 0.01$, $p = 0.91$; genotype by time interaction: $F_{(17, 238)} = 0.67$, $p = 0.83$. Peri-pubertal: main effect of genotype: $F_{(1, 13)} = 0.05$, $p = 0.82$; genotype by time interaction: $F_{(17, 221)} = 0.18$, $p = 0.99$). Importantly, Student's t-tests revealed no group difference in AMPH-induced stereotypy counts in either post-weanling or peri-pubertal mice thereby ruling out the possibility of genotype-dependent differences in sensitivity to the stereotypy effects of AMPH (post-weaning: $t_{(14)} = 0.14$, $p = 0.89$. Peri-pubertal: $t_{(13)} = 0.73$, $p = 0.48$) (Fig III-1d).

In contrast, post-weanling female *dcc* +/- mice, when treated with 2.2 mg/kg of AMPH, showed enhanced AMPH-induced locomotion in comparison to

+/+ controls (main effect of genotype: $F_{(1, 7)} = 6.92$, $p = 0.03$; genotype by time interaction: $F_{(17, 119)} = 1.67$, $p = 0.06$) (Fig III-2c). This is *opposite* to what was observed in adult females when treated with the same dose (insert). In addition, a Student's *t*-test showed that at this dose, post-weanling female *dcc* +/- mice displayed increased stereotypy scores, excluding the possibility that the difference in AMPH-induced locomotion between genotypes is an artefact of increased drug-induced stereotypy in +/+ animals ($t_{(7)} = 2.34$, $p = 0.05$) (Fig III-2d). To determine whether this effect was dependent on the dose of AMPH used, we tested a separate group of post-weanling female mice with a higher AMPH dose (3.5 mg/kg), based on our previous dose-response experiments conducted in adult mice (Flores et al. 2005). At this dose, neither the effect of enhanced AMPH-induced locomotion (main effect of genotype: $F_{(1, 13)} = 0.39$, $p = 0.54$; genotype by time interaction: $F_{(17, 221)} = 0.21$, $p = 0.99$) (Fig III-2c), nor the increased drug-induced stereotypy ($t_{(12)} = 0.88$, $p = 0.39$) (Fig III-2d) was observed. Moreover, and similar to what we observed in male mice, no differences in locomotor activity ($F_{(1,18)} = 0.06$, $p = 0.80$; genotype by time interaction: $F_{(17, 306)} = 0.59$, $p = 0.90$) or stereotypy counts ($t_{(18)} = 0.27$, $p = 0.79$) were observed between female peri-pubertal *dcc* +/- and +/+ mice in response to 2.2 mg/kg of AMPH (Fig III-2c & d).

The reduced response to amphetamine observed in peri-pubertal female mice, compared to other ages, is in agreement with other studies using similar doses that show that peri-pubertal rodents are less sensitive to the locomotor activating effects of amphetamine at lower doses; an effect that is greater in

female mice (Spear and Brake 1983; Adriani and Laviola 2000; Niculescu et al. 2005).

Together these data indicate that the reduced sensitivity to the effects of AMPH observed in adult male and female *dcc* +/- mice does not emerge prior to puberty. It is important to note that meaningful comparisons across ages and genders cannot be made because the behavioural experiments were conducted on different days and there is a near-doubling in body size between post-weanling and peri-pubertal animals.

dcc +/- mice do not exhibit increased mPFC DA concentrations prior to puberty

In our previous studies we showed that adult *dcc* +/- mice have large increases in baseline tissue concentrations of DA and DA metabolites (DOPAC and HVA) in the mPFC. In addition, baseline DOPAC and HVA levels in the NAcc were reduced in adult *dcc* +/- mice in comparison to +/+ littermates (Flores et al. 2005; Grant et al. 2007). Remarkably, in the present study repeated-measures ANOVAs revealed no significant differences in DA concentrations in *dcc* +/- and +/+ mice at either post-weaning or peri-pubertal ages (post-weaning: main effect of genotype $F_{(1,17)} = 1.93$, $p = 0.18$; peri-pubertal: main effect of genotype $F_{(1,13)} = 0.02$, $p = 0.88$; Fig III-3a). The apparent differences in DA concentrations in mPFC and NAcc observed between genotypes at post-weaning were not statistically significant (post-weanling: genotype by brain region interaction: $F_{(2,34)} = 2.34$, $p = 0.11$. Peri-pubertal: genotype by time interaction: $F_{(2, 13)} = 0.09$, $p = 0.92$).

Repeated measures ANOVAs did show a significant difference between +/- and +/+ groups in baseline DOPAC concentration at post-weaning, but not at peri-pubertal age (post-weaning: main effect of genotype: $F_{(1,17)} = 7.15$, $p = 0.016$; genotype by brain region interaction: $F_{(1,17)} = 6.65$, $p = 0.02$. Peri-pubertal: main effect of genotype: $F_{(1,13)} = 0.35$, $p = 0.57$; genotype by brain region interaction $F_{(1,13)} = 0.04$, $p = 0.84$*;* Fig III-3b). Although repeated measures ANOVA on baseline HVA concentrations, revealed no significant main effect of genotype at either post-weaning or peri-pubertal periods, there was a significant interaction between genotype and brain region in post-weanling, but not peri-pubertal animals (post-weaning: main effect of genotype $F_{(1,17)} = 2.42$, p = 0.14; genotype by brain region interaction: $F_{(2,34)} = 4.43$, p = 0.02. Peri-pubertal: main effect of genotype, $F_{(1,13)} = 1.10$, p = 0.31; genotype by brain region interaction $F_{(2,13)} = 0.97$, $p = 0.39$*;* Fig III-3c). Intriguingly, *post hoc* analyses of these significant interactions indicated that at post-weaning, *dcc* +/- mice, in comparison to +/+ controls, have significantly higher baseline concentrations of DOPAC and HVA in the NAcc, (Fig III-3b); a finding *opposite* to that observed in adult animals (Flores et al. 2005). Importantly, by peri-pubertal age, no differences in NAcc DOPAC (Fig III-3b) or HVA (Fig III-3c) concentrations were observed between genotypes. Finally, there was no effect of genotype on baseline concentrations of DA, DOPAC, or HVA in the dorsal striatum at either age. See Fig III-3 legend for *post hoc* comparisons.

The raw data of baseline concentrations of DA and DA metabolites for post-weanling and peri-pubertal *dcc* +/- and +/+ mice are shown in Table III-1. It is important to clarify, that we do not show mPFC DOPAC concentrations from

brains of post-weanling and peri-pubertal mice because the levels of this metabolite that were obtained were almost negligible and in many instances undetectable. This is to be expected considering that the density of the DA innervation in the mPFC during post-weaning and peri-pubertal ages is significantly low.

To gain insight into differences in baseline dopamine activity in NAcc and STR between *dcc* +/- and +/+ mice at post-weaning or peri-pubertal ages, we calculated the metabolite/dopamine ratios (see Table III-2). Statistical analysis, however, revealed no significant effect of genotype and no significant interaction for either of these regions at either age (DOPAC/DA post-weaning: main effect of genotype $F_{(1,15)} = 0.88$, p = 0.36; genotype by brain region interaction: $F_{(1,15)} = 0.06$, p = 0.80; DOPAC/DA peri-pubertal: main effect of genotype $F_{(1,10)} = 0.39$, p = 0.55; genotype by brain region interaction: $F_{(1,10)} = 1.01$, p = 0.34. HVA/DA post-weaning: main effect of genotype, $F_{(1,14)} <0.01$, p = 0.95; genotype by brain region interaction $F_{(1,14)} = 0.05$, p = 0.82; HVA/DA peri-pubertal: main effect of genotype, $F_{(1,10)} = 0.70$, p = 0.42; genotype by brain region interaction $F_{(1,10)} = 2.48$, p = 0.15).

dcc +/- mice do not exhibit changes in mPFC TH protein expression prior to puberty

Consistent with the finding of no differences between genotypes in baseline DA concentrations, repeated-measures ANOVAs revealed no significant differences between post-weanling or peri-pubertal +/- and +/+ mice in TH expression in any of the brain regions examined (post-weaning: main effect of

genotype: $F_{(1,15)} = 0.12$, $p = 0.74$. Peri-pubertal: main effect of genotype: $F_{(1,14)} = 0.20$, $p = 0.66$; Fig III-4). It can be seen, however, that this is in contrast to the large increases in TH expression observed only in the mPFC of adult *dcc* +/- mice (see Fig III-4 insert). It is important to note that both post-weanling and peri-pubertal *dcc* +/- mice do in fact express reduced levels of DCC in all brain regions as compared to +/+ controls (data not shown; post-weanling main effect of genotype: $F_{(1,14)} = 13.98$, $p = 0.0022$; peri-pubertal main effect of genotype: $F_{(1,6)} = 6.65$, $p = 0.04$).

dcc +/- mice do not exhibit reduced number of midbrain DA neurons before puberty

We showed previously that adult *dcc* +/- mice have ~ 20% fewer midbrain TH-immunoreactive neurons than adult +/+ mice (Flores et al. 2005). Repeated-measures ANOVAs of unbiased estimates of TH-positive neurons in VTA and SN *pars* compacta indicated no significant difference between +/- and +/+ at either post-weanling or peri-pubertal periods (post-weaning: main effect of genotype: $F_{(1,9)} = 0.76$, $p = 0.41$; genotype by brain region interaction $F_{(1,9)} = 1.30$, $p = 0.28$. Peri-pubertal: main effect of genotype: $F_{(1,8)} = 1.74$, $p = 0.22$; genotype by brain region interaction $F_{(1,8)} = 1.57$, $p = 0.25$; Fig III-5). At both ages, there was a significantly greater number of TH-positive cells observed in the SN *pars* compacta than in the VTA. However this effect was observed in both genotypes.

DISCUSSION

The major findings from this study are that the behavioural, neurochemical and molecular phenotypes exhibited by adult *dcc* heterozygous mice are not evident in the post-weaning or peri-pubertal periods. These findings are in agreement with our hypothesis, based on our previous findings in adult mice, that reduced DCC leads to selective alterations in the organization and function of mPFC DA circuitry. These results are novel, shedding light on mechanisms potentially involved in *protecting* against neurochemical and behavioural abnormalities associated with developmental disorders such as schizophrenia and drug abuse.

Our data collectively indicates that DCC plays an important role in the normal maturation of the mesocortical DA system and, perhaps, in the adolescent transition from a primarily NAcc function to a predominant mPFC DA function in adulthood (Kalsbeek et al. 1988; Leslie et al. 1991; Spear 2000; Teicher et al. 1991; Tseng and O'Donnell 2007). First, adolescence is a critical developmental period for the maturation and refinement of mPFC DA connectivity and function. This is in contrast to DA connectivity in the NAcc, which already reaches adult innervation patterns by the third week of life in rodents (Voorn et al. 1988). In our studies, we find changes in mPFC, but not in NAcc, DA function in *dcc* heterozygotes after puberty. Second, it has seen shown previously in rodents that there is a post-pubertal increase in mPFC DA fibre density (Benes et al. 1996; Kalsbeek et al. 1988), together with a loss of autoreceptor-like modulation of DA synthesis (Teicher et al. 1991), which lead to enhanced mPFC DA concentration,

turnover, and synthesis (Leslie et al. 1991; Spear 2000; Teicher et al. 1993). Indeed, we find changes in mPFC DA concentrations in adult, but not pre-pubertal *dcc* heterozygous mice. Third, the post-pubertal *increase* in baseline TH immunoreactivity in the mPFC together with the *reduced* number of VTA DA neurons in *dcc* heterozygous mice, suggest that *dcc*-haploinsufficiency may lead to enhanced axonal branching of DA fibres innervating the mPFC during puberty (Flores et al. 2005). Indeed, netrin-1 has been implicated in axonal branching of cortical neurons (Dent et al. 2004; Hutchins and Kalil 2008; Tang and Kalil 2005) and, interestingly, axonal branching is more pronounced at specific developmental periods (Gogolla et al. 2007; Halloran and Kalil 1996; Uesaka et al. 2006). Furthermore, upon target recognition, it is collateral branch formation, rather than extension of the primary growth cone, that appears to be the main mechanism underlying cortical innervation (Halloran and Kalil 1994). Finally, during the peri-pubertal age there is extensive synaptic reorganization of mPFC excitatory and inhibitory circuitries, including pruning, which is conserved across species (Andersen et al. 2000; Crews et al. 2007; Lewis 1997; van Eden et al. 1990). Recent studies have shown that, indeed, netrin-1 signalling is involved in the spatio-temporal organization of synaptic connectivity (Colon-Ramos et al. 2007; Mitchell et al. 1996; Poon et al. 2008; Winberg et al. 1998) and recent evidence implicates guidance cues in axon pruning (Luo and O'Leary 2005; Waimey and Cheng 2006; Xu and Henkemeyer 2009).

The mechanism underlying this DCC/DA interaction that, according to our findings, occurs during or around adolescence, is not known. Significantly, however, we have recently shown that there is a peri-pubertal switch in the

expression of DCC and UNC5H receptors by midbrain DA neurons. Using BL6 mice and Sprague-Dawley rats we showed that whereas VTA DA neurons express DCC from embryonic life to adulthood, these neurons only begin to co-express UNC5H receptors at the peri-pubertal period. Furthermore, western blot analysis revealed a significant *increase* in UNC5H immunoreactivity in the VTA of peri-pubertal animals and a sharp *down-regulation* of DCC levels at post-weaning (Labelle-Dumais and Flores 2008). This reciprocal developmental shift in the relative expression of DCC and UNC5H, previously described in the spinal cord (Manitt et al. 2004), suggests that the reduction in DCC may actually trigger the increase in UNC5H. An intriguing possibility is that the peri-pubertal emergence of UNC5H expression by DA neurons may be associated with a critical event in the developmental course and organization of mPFC DA circuitry, including modifications in fibre density, shape, and/or distribution. Because *dcc* heterozygotes develop with reduced DCC levels (Flores et al. 2005; Grant et al. 2007), it is possible that in these mice, the emergence of predominant UNC5H expression by DA neurons and, in turn, the critical modifications in the development of mPFC DA circuitry, occur earlier and/or are exaggerated. It is important to note that, as mentioned, adult *dcc* heterozygous mice exhibit alterations in dendritic spine density in layer V mPFC pyramidal neurons. Thus, altered mesocortical DA transmission in post-pubertal mice may be a consequence of prior changes in synaptic formation and/or maintenance within the mPFC. To this end, we are currently conducting a thorough characterization of differences between *dcc* heterozygous and wild-type mice in neuronal structure at

post-weaning, peri-pubertal, and adult ages (see supplementary discussion section of this Chapter for a discussion of the results of this study).

Although, it is intriguing that the post-pubertal reduction in DA cell numbers observed in the VTA of *dcc* heterozygotes appears to follow the emergence of UNC5H expression, at this point we cannot speculate whether and how these two events are mechanistically related. Furthermore, there is a reduction in the total number of midbrain DA neurons between the peri-pubertal period and adulthood in both *dcc* heterozygous and wild-type mice. Interestingly, UNC5H is a dependence receptor that has been shown to induce apoptosis in cells that lack availability to netrin-1 (Llambi et al. 2001). The sudden expression of UNC5H by DA neurons at peri-pubertal age may be associated with pruning and perhaps be related to the loss of DA neurons between peri-pubertal period and adulthood.

Whereas in all the experiments conducted in this study we found a complete lack of differences between *dcc*-heterozygous and wild-type mice at peri-pubertal age, some of the results obtained from experiments conducted in post-weanling mice suggest a transient increase in NAcc DA activity in *dcc* heterozygous mice. When post-weanling female *dcc* heterozygotes were given the same dose of amphetamine as the one used in the experiments with peri-pubertal and adult mice (2.2 mg/kg; (Grant et al. 2007), they displayed *higher* locomotor activity and stereotypy counts than wild-type controls. As mentioned, this effect was not evident with a higher dose. In addition, neurochemical analysis of brain from post-weanling *dcc* heterozygous mice revealed *greater* baseline concentrations of DA metabolites in the NAcc. The functional significance of this

effect appears to be subtle because differences in behaviour were only seen in female mice with the lower dose of amphetamine and only at post-weaning age. Nonetheless, this finding may reflect that DCC has an earlier, but transient, effect on mesolimbic DA function. Alternatively or additionally, because increased sensitivity to the locomotor-activating effect of the lower dose of amphetamine was seen in post-weanling female, but not male *dcc* heterozygous mice, the early phenotype observed may result from an interaction between genotype and sex differences in D2-like receptor overproduction, elimination and/or sensitivity (Andersen et al. 1997; Andersen et al. 2002). To address this issue we are currently conducting quantitative receptor autoradiography experiments in post-weanling, peri-pubertal and adult male and female *dcc* heterozygous and wild-type mice.

Finally, the precise developmental age at which the DA and behavioural phenotype observed in adult mice with *dcc* haplo-insufficiency emerges remains to be established. To date, we know that the phenotype is observed after PND90, but not before PND35. Identifying the period in which the phenotype first appears could potentially enable us to link specific cellular and/or molecular process(es) to the post-pubertal 'switch' in DA function and behaviour, such as alterations in intrinsic physiological properties of neurons comprising DA circuitry (Tseng & O'Donnell, 2005). In a pilot study conducted in PND60 ± 5 female mice, we observed that *dcc* heterozygous mice already exhibit reduced locomotor activity in response to a single injection of amphetamine (of 2.2 mg/kg) in comparison to wild-type mice (see Fig III-6 in the supplementary data section of this Chapter).

Intriguingly, the density of the DA innervation in the mPFC appears to be similar between PND60 and PND90 in the rat (Kalsbeek et al. 1988).

In conclusion, these results provide the intriguing possibility that variations in netrin-1 receptor levels may be one of the converging paths through which the diverse genetic and environmental risk factors exert their enduring effects on DA function and DA-related behaviours. In fact, we assessed recently the effects of ventral hippocampal lesions in neonatal rats, a putative neurodevelopmental model of schizophrenia (Lipska et al. 1992; Lipska and Weinberger 2000; Marcotte et al. 2001; Tseng et al. 2007), on DCC expression at different times in life. Neonatal lesions produced dynamic and opposite changes in DCC expression in mPFC and NAcc and, remarkably, for both regions a 'shift' in the direction of the effect was observed during adolescence Alterations in mPFC, but not NAcc, DCC expression lasted until adulthood (Flores et al. 2009). These findings suggest that variations in netrin-1 receptor may be a common mechanistic link between disruption of brain development by early adverse events and the delayed-onset of DA abnormalities associated with schizophrenia-like symptoms.

FIGURES AND TABLES

Fig. III-1. Baseline and AMPH-induced locomotion in post-weanling and peri-pubertal male *dcc* heterozygous and wild-type mice

Male *dcc* heterozygous (+/-) and wild-type (+/+) mice do not differ in baseline or amphetamine (AMPH)-induced locomotion at either post-weaning or the peri-pubertal ages. Data points represent the total distance (cm) travelled (mean ± SEM). Adult data presented in the inserts are reproduced from Grant et al, 2007. *(a)* Day 1: habituation response. There were no genotypic differences in habituation to the testing apparatus for either post-weanling or peri-pubertal groups. *(b)* Day 2: locomotor following an i.p. injection of saline. The arrow indicates the injection time point. No group differences were observed in either post-weanling or peri-pubertal mice. *(c)* Day 3: locomotor response following an injection of AMPH (2.5 mg/kg; i.p.). There were no differences between genotypes in AMPH-induced locomotion in post-weanling or peri-pubertal mice. *(d)* Amphetamine-induced stereotypy scores. There were no group differences in AMPH-induced stereotypy counts in either post-weanling or peri-pubertal mice. Post-weaning experiment: n = 8 per group; peri-pubertal experiment: +/-, n = 5 mice; +/+, n = 10.

* denotes significance of $p < 0.05$

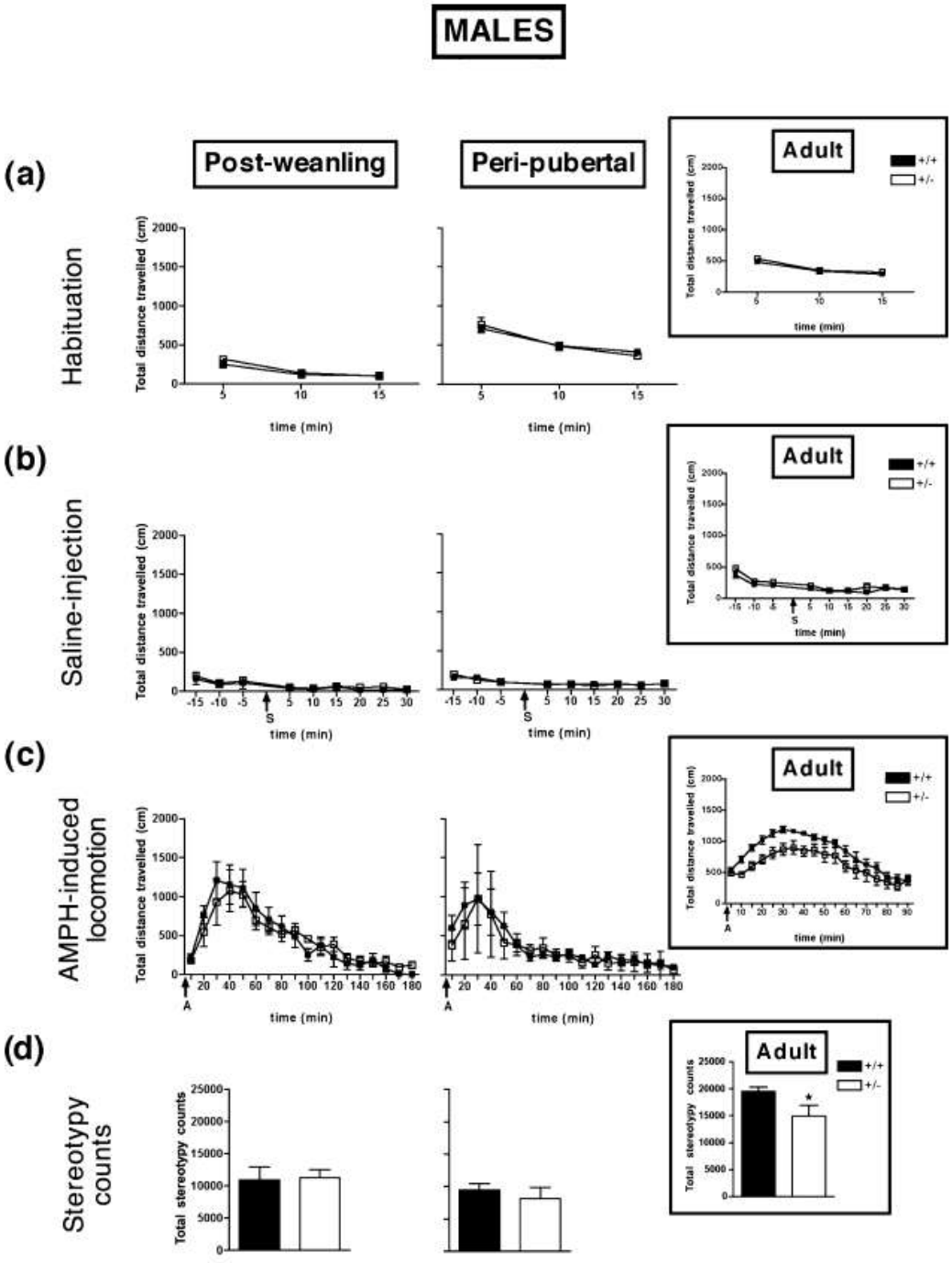

MALES
(a)
Post-weanling
Peri-pubertal
Adult
+/+
+/-
Habituation
Total distance travelled (cm)
time (min)
(b)
Saline-injection
S
(c)
AMPH-induced locomotion
A
(d)
Stereotypy counts
Total stereotypy counts
*

Fig. III-2. **Baseline and AMPH-induced locomotion in female post-weanling and peri-pubertal *dcc* heterozygous and wild-type mice**

Female *dcc* heterozygous (+/-) and wild-type (+/+) mice do not differ in basal locomotion at either the post-weaning or the peri-pubertal age, but do exhibit dose-dependent differences in amphetamine (AMPH)-induced locomotion at post-weaning. Data points represent the total distance (cm) travelled (mean ± SEM). Adult data presented in the inserts are reproduced from Grant et al, 2007. *(a)* Day 1: habituation response. There were no genotypic differences in habituation to the testing apparatus for either post-weanling or peri-pubertal groups. *(b)* Day 2: locomotor response following an i.p. injection of saline. The arrow indicates the injection time point. No group differences were observed in either post-weanling or peri-pubertal mice. *(c)* Day 3: locomotor response following an injection of AMPH. Post-weanling, but not peri-pubertal, *dcc* +/- mice showed significantly greater locomotor activity following administration of 2.2 mg/kg of AMPH. However, when post-weanling animals were challenged with 3.5 mg/kg of AMPH, there was no longer a significant difference between genotypes. *(d)* Amphetamine-induced stereotypy scores. There was a significant difference in AMPH-induced stereotypy counts in post-weanling groups at 2.2 mg/kg dose, but not at 3.5 mg/kg dose. No differences between genotypes were found in AMPH-induced stereotypy at the peri-pubertal age. Post-weaning experiment: Dose of 2.2 mg/kg: +/-, $n = 5$; +/+, n = 4. Dose of 3.5 mg/kg: +/-, $n = 8$; +/+, $n = 6$. Peri-pubertal experiment: +/-, $n = 12$; +/+, $n = 8$.

* denotes significance of $p < 0.05$, ** denotes significance of $p < 0.01$

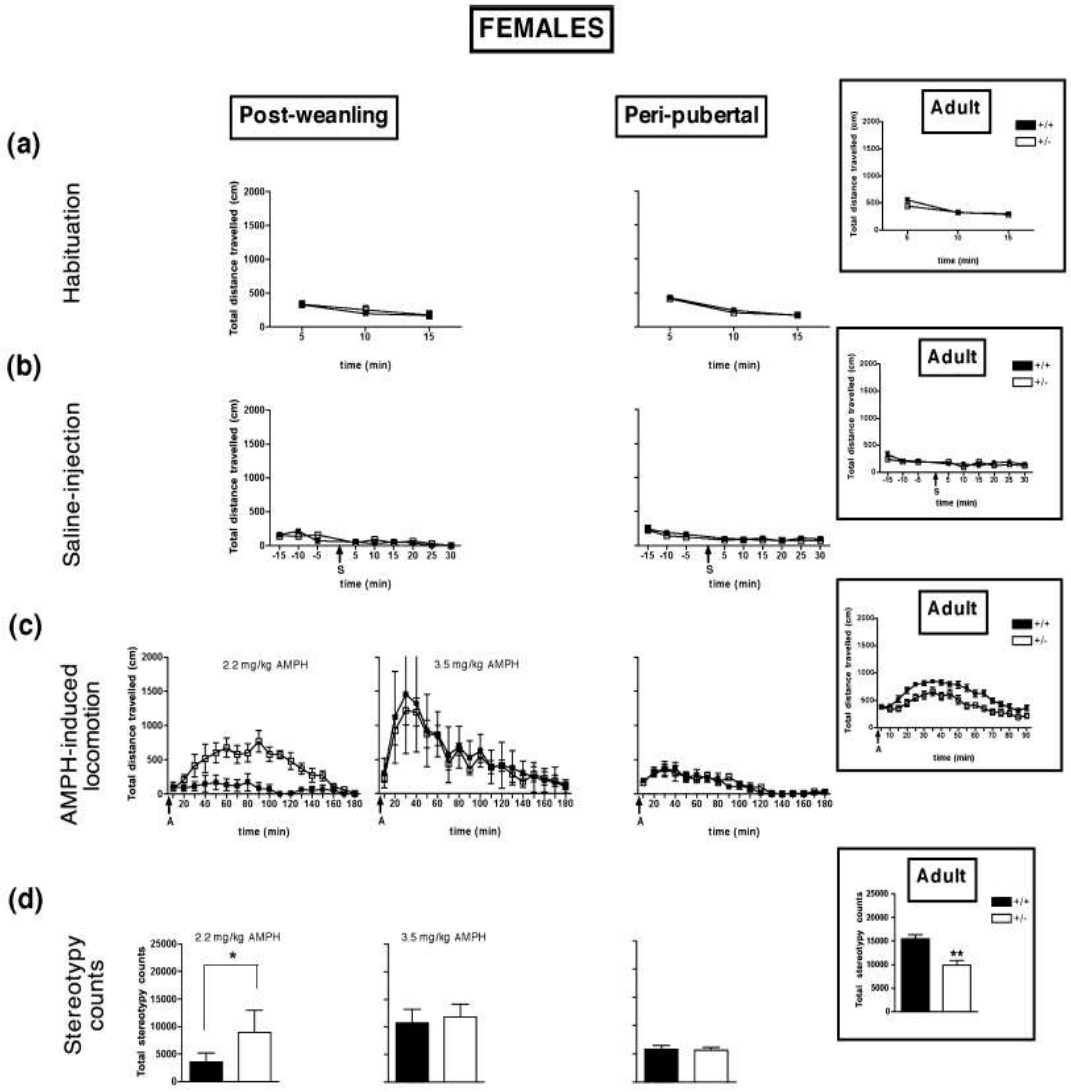
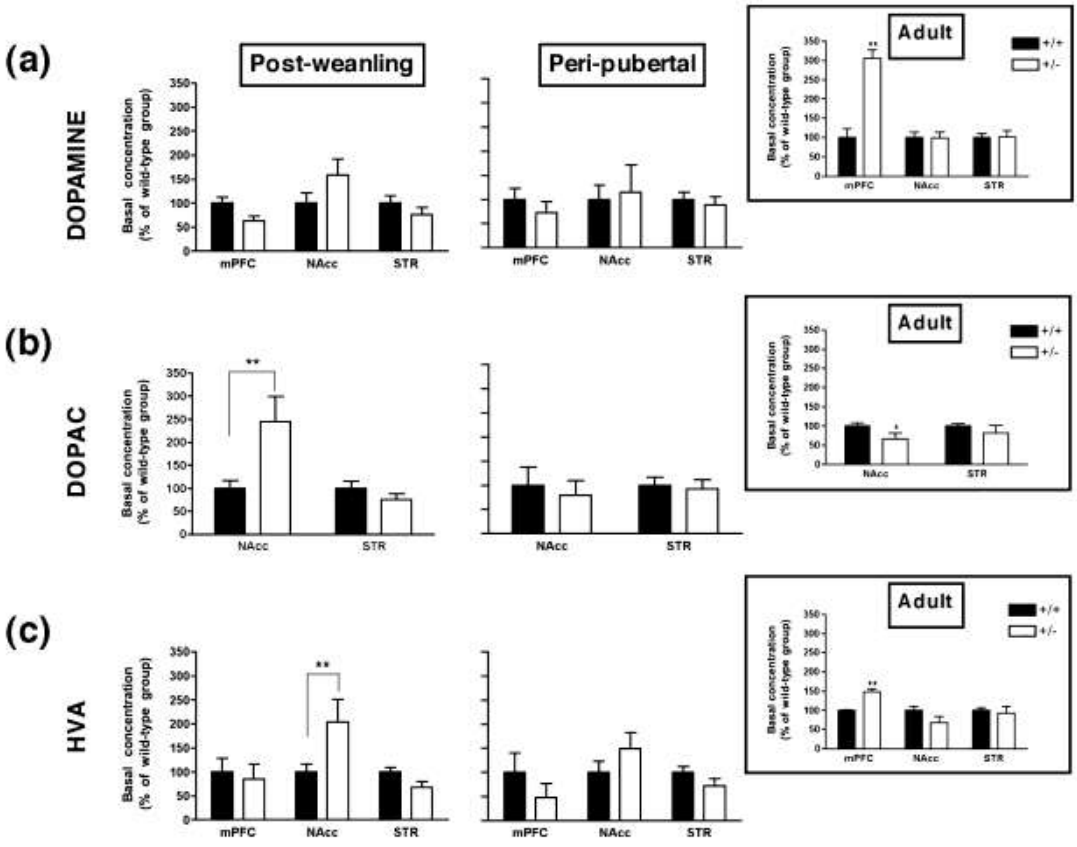

FEMALES
Post-weanling
Peri-pubertal
Adult
(a)
Habituation
(b)
Saline-injection
(c)
AMPH-induced locomotion
(d)
Stereotypy counts
Total distance travelled (cm)
time (min)
2.2 mg/kg AMPH
3.5 mg/kg AMPH
Total stereotypy counts
+/+
+/-
S
A
*
**

Fig. III-3. Baseline concentrations of DA and DA metabolites in DA terminal regions in post-weanling and peri-pubertal *dcc* heterozygous and wild-type mice

Baseline concentrations of mPFC DA and DA metabolites do not differ between *dcc* heterozygous (+/-) and wild-type (+/+) mice at post-weaning or peri-pubertal ages. All analyses were conducted on the raw data. Data points are expressed as percentage of +/+ group. Adult data presented in the inserts were adapted from Flores et al, 2005. *(a)* Baseline DA concentrations. The significant differences in DA concentrations in mPFC and NAcc seen in adult *dcc* +/- mice is not observed at either the post-weaning or peri-pubertal ages. *(b)* Baseline DOPAC concentrations. Repeated measures ANOVAs revealed a significant interaction between genotype and brain region on baseline DOPAC concentration at post-weaning, but not at peri-pubertal age. A *post hoc* ANOVA test for simple effects indicated that the significant interaction in DOPAC concentrations in post-weanling groups resulted from greater levels of this metabolite in the NAcc of +/- mice ($F_{(1,34)}$ = 11.39, p = .002). *(c)* Baseline HVA concentrations. Repeated measures ANOVAs revealed a significant interaction between genotype and brain region on baseline HVA concentrations at post-weaning, but not at peri-pubertal age. A *post hoc* ANOVA test for simple effects indicated that the significant interaction in HVA concentrations in post-weanling groups resulted from greater levels of this metabolite in the NAcc of +/- mice ($F_{(1,51)}$ = 10.99, p = 0.002). Post-weaning experiment: *n* = 9-10, per group; peri-pubertal experiment: +/-, *n* = 6-7; +/+ n = 8.

* denotes significance of $p < 0.05$, ** denotes significance of $p < 0.01$


(a)
DOPAMINE
Post-weanling
Peri-pubertal
Adult
+/+
+/-
Basal concentration (% of wild-type group)
mPFC
NAcc
STR
(b)
DOPAC
(c)
HVA

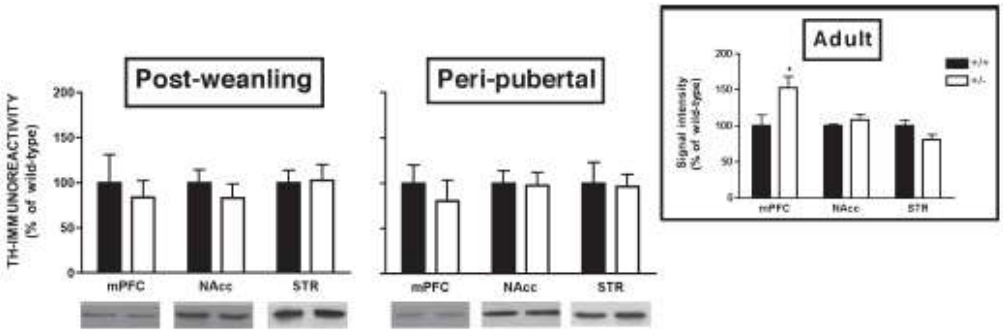


Fig. III-4. Expression of tyrosine hydroxylase in DA regions in post-weanling and peri-pubertal *dcc* heterozygous and wild-type mice

Baseline expression of tyrosine hydroxylase (TH) in DA regions does not differ between *dcc* heterozygous (+/-) and wild-type (+/+) mice at either post-weanling or peri-pubertal ages. Optical density data were normalized to tubulin and converted to percent of the +/+ group. Representative examples of western blots for +/- and +/+ mice are shown below the graphs. All analyses were conducted on the raw data. Adult data presented in the inserts were adapted from Flores et al, 2005. There were no differences between either post-weanling or peri-pubertal *dcc* +/- and +/+ mice in the expression of TH in any DA terminal regions examined. Post-weaning experiment: +/-, n = 9; +/+, n = 9; peri-pubertal experiment: +/-, n = 4-9; +/+, 4-7

* denotes significance of $p < 0.05$

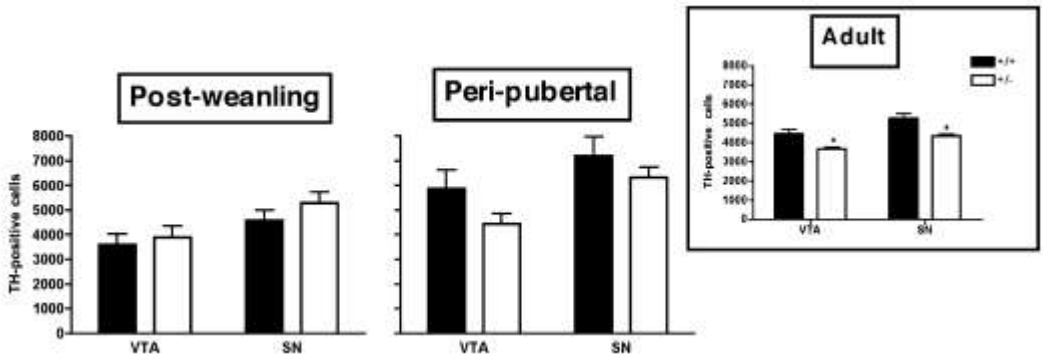


Fig. III-5. Stereological counts of TH-positive neurons in the VTA and SN of post-weanling and peri-pubertal *dcc* heterozygous and wild-type mice

Numbers of midbrain TH-positive neurons do not differ between *dcc* heterozygous (+/-) and wild-type (+/+) mice at either post-weanling or peri-pubertal ages. Adult data presented in the inserts were adapted from Flores et al, 2005. No differences in numbers of DA neurons in the VTA and SN *pars* compacta were detected between genotypes at the post-weaning or the peri-pubertal periods**.** Post-weaning experiment: +/-, n = 6, +/+ n=5; peri-pubertal experiment: n = 5 for both groups.

* denotes significance of $p < 0.05$

TABLE III-1.

Baseline concentration of DA and DA metabolites in DA terminal regions of post-weanling and peri-pubertal *dcc* heterozygous and wild-type mice (pg/µl/mg protein ± SEM)

	DA		**HVA**		**DOPAC**	
	+/-	+/+	+/-	+/+	+/-	+/+
Post-weanling						
mPFC	0.10 ± 0.01	0.15 ± 0.02	0.18± 0.08	0.24 ± 0.08	---	---
NAcc	17.19 ± 3.77	10.84 ± 2.46	5.16 ± 1.27	2.53 ± 0.43	7.80 ± 1.75	3.19 ± 0.54
STR	1.87 ± 0.37	2.45 ±0.38	1.34 ± 0.22	1.96 ± 0.18	3.26 ± 0.55	4.30 ± 0.66
Peri-pubertal						
mPFC	0.15 ± 0.05	0.21 ± 0.05	0.24 ± 0.15	0.33 ± 0.20	---	---
NAcc	11.45 ± 5.64	9.94 ± 2.97	2.78 ± 0.61	1.85 ± 0.40	4.00 ± 1.53	5.05 ± 1.78
STR	2.78 ± 0.57	2.99 ± 0.50	0.90 ± 0.19	1.24 ± 0.15	1.71 ± 0.33	1.93 ± 0.28

TABLE III-2.

Baseline DA turnover (metabolite : DA ratios) in *dcc* heterozygous and wild-type mice

	DOPAC : DA		**HVA : DA**	
	+/-	+/+	+/-	+/+
Post-weanling				
NAcc	0.61 ± 0.14	0.45 ± 0.18	0.27 ± 0.03	0.25 ± 0.04
STR	0.71 ± 0.11	0.61 ± 0.10	0.47 ± 0.05	0.48 ± 0.03
Peri-pubertal				
NAcc	0.34 ± 0.11	0.52 ± 0.12	0.45 ± 0.17	0.22 ± 0.08
STR	0.68 ± 0.09	0.60 ± 0.11	0.34 ± 0.07	0.43 ± 0.09

ACKNOWLEDGEMENTS

We would like to thank Dr. S. Ackerman (Jackson Laboratory) for providing the original *dcc* heterozygous breeders, Dr. T. Pawson (University of Toronto) for the pan-UNC5H antiserum, J. Stewart, C. Manitt and L. Yetnikoff for critical reading of the manuscript. This work was funded by the Canadian Institute for Health Research (C.F), the Natural Sciences and Engineering, Research Council of Canada (C.F), and the Fonds de la Recherche en Santé du Québec (C.F).

REFERENCES

Adriani, W. & Laviola, G. (2000) A unique hormonal and behavioral hyporesponsivity to both forced novelty and d-amphetamine in periadolescent mice. *Neuropharmacology*, **39**, 334-346.

Adriani, W. & Laviola, G. (2004) Windows of vulnerability to psychopathology and therapeutic strategy in the adolescent rodent model. *Behav Pharmacol*, **15**, 341-352.

Andersen, S.L., Rutstein, M., Benzo, J.M., Hostetter, J.C. & Teicher, M.H. (1997) Sex differences in dopamine receptor overproduction and elimination. *Neuroreport*, **8**, 1495-1498.

Andersen, S.L., Thompson, A.T., Rutstein, M., Hostetter, J.C. & Teicher, M.H. (2000) Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats. *Synapse*, **37**, 167-169.

Andersen, S.L., Thompson, A.P., Krenzel, E. & Teicher, M.H. (2002) Pubertal changes in gonadal hormones do not underlie adolescent dopamine receptor overproduction. *Psychoneuroendocrinology*, **27**, 683-691.

Andersen, S.L. (2003) Trajectories of brain development: point of vulnerability or window of opportunity? *Neurosci Biobehav Rev*, **27**, 3-18.

Banks, K.E. & Gratton, A. (1995) Possible involvement of medial prefrontal cortex in amphetamine-induced sensitization of mesolimbic dopamine function. *Eur J Pharmacol*, **282**, 157-167.

Barallobre, M.J., Pascual, M., Del Rio, J.A. & Soriano, E. (2005) The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Res Brain Res Rev*, **49**, 22-47.

Becker, J.B., Robinson, T.E. & Lorenz, K.A. (1982) Sex differences and estrous cycle variations in amphetamine-elicited rotational behavior. *Eur J Pharmacol*, **80**, 65-72.

Benes, F.M., Vincent, S.L., Molloy, R. & Khan, Y. (1996) Increased interaction of dopamine-immunoreactive varicosities with GABA neurons of rat medial prefrontal cortex occurs during the postweanling period. *Synapse*, **23**, 237-245.

Benes, F.M., Taylor, J.B. & Cunningham, M.C. (2000) Convergence and plasticity of monoaminergic systems in the medial prefrontal cortex during the postnatal period: implications for the development of psychopathology. *Cereb Cortex*, **10**, 1014-1027.

Boksa, P. (2004) Animal models of obstetric complications in relation to schizophrenia. *Brain Res Brain Res Rev*, **45**, 1-17.

Bunney, B.S. & Aghajanian, G.K. (1976) Dopamine and norepinephrine innervated cells in the rat prefrontal cortex: pharmacological differentiation using microiontophoretic techniques. *Life Sci*, **19**, 1783-1789.

Carr, D.B. & Sesack, S.R. (2000) Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci*, **15**, 3864-3873.

Cirulli, F. & Laviola, G. (2000) Paradoxical effects of D-amphetamine in infant and adolescent mice: role of gender and environmental risk factors. *Neurosci Biobehav Rev*, **24**, 73-84.

Colon-Ramos, D.A., Margeta, M.A. & Shen, K. (2007) Glia promote local synaptogenesis through UNC-6 (netrin) signaling in C. elegans. *Science*, **318**, 103-106.

Crews, F., He, J. & Hodge, C. (2007) Adolescent cortical development: a critical period of vulnerability for addiction. *Pharmacol Biochem Behav*, **86**, 189-199.

Dent, E.W., Barnes, A.M., Tang, F. & Kalil, K. (2004) Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J Neurosci*, **24**, 3002-3012.

Deutch, A.Y., Clark, W.A. & Roth, R.H. (1990) Prefrontal cortical dopamine depletion enhances the responsiveness of mesolimbic dopamine neurons to stress. *Brain Res*, **521**, 311-315.

Doherty, M.D. & Gratton, A. (1996) Medial prefrontal cortical D1 receptor modulation of the meso-accumbens dopamine response to stress: an electrochemical study in freely-behaving rats. *Brain Res*, **715**, 86-97.

Feinberg, I. (1982) Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? *J Psychiatr Res*, **17**, 319-334.

Finkelstein, D.I., Stanic, D., Parish, C.L., Tomas, D., Dickson, K. & Horne, M.K. (2000) Axonal sprouting following lesions of the rat substantia nigra. *Neuroscience*, **97**, 99-112.

Flores, C., Manitt, C., Rodaros, D., Thompson, K.M., Rajabi, H., Luk, K.C., Tritsch, N.X., Sadikot, A.F., Stewart, J. & Kennedy, T.E. (2005) Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Mol Psychiatry*, **10**, 606-612.

Flores, C., Bhardwaj, S.K., Labelle-Dumais, C. & Srivastava, L.K. (2009) Altered netrin-1 receptor expression in dopamine terminal regions following neonatal ventral hippocampal lesions in the rat. *Synapse*, **63**, 54-60.

Gogolla, N., Galimberti, I. & Caroni, P. (2007) Structural plasticity of axon terminals in the adult. *Curr Opin Neurobiol*, **17**, 516-524.

Gogos, J.A., Morgan, M., Luine, V., Santha, M., Ogawa, S., Pfaff, D. & Karayiorgou, M. (1998) Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. *Proc Natl Acad Sci U S A*, **95**, 9991-9996.

Gore, A.C., Roberts, J.L. & Gibson, M.J. (1999) Mechanisms for the regulation of gonadotropin-releasing hormone gene expression in the developing mouse. *Endocrinology*, **140**, 2280-2287.

Grant, A., Hoops, D., Labelle-Dumais, C., Prevost, M., Rajabi, H., Kolb, B., Stewart, J., Arvanitogiannis, A. & Flores, C. (2007) Netrin-1 receptor-deficient mice show enhanced mesocortical dopamine transmission and blunted behavioural responses to amphetamine. *Eur J Neurosci*, **26**, 3215-3228.

Gundersen, H.J., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B. & et al.

(1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Apmis*, **96**, 857-881.

Halloran, M.C. & Kalil, K. (1994) Dynamic behaviors of growth cones extending in the corpus callosum of living cortical brain slices observed with video microscopy. *J Neurosci*, **14**, 2161-2177.

Halloran, M.C. & Kalil, K. (1996) Selective neurite outgrowth of cultured cortical neurons on specific regions of brain cryostat sections. *J Comp Neurol*, **371**, 72-84.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M. & Stein, E. (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell*, **97**, 927-941.

Hutchins, B.I. & Kalil, K. (2008) Differential outgrowth of axons and their branches is regulated by localized calcium transients. *J Neurosci*, **28**, 143-153.

Kalsbeek, A., Voorn, P., Buijs, R.M., Pool, C.W. & Uylings, H.B. (1988) Development of the dopaminergic innervation in the prefrontal cortex of the rat. *J Comp Neurol*, **269**, 58-72.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. & Tessier-Lavigne, M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell*, **87**, 175-185.

Keleman, K. & Dickson, B.J. (2001) Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. *Neuron*, **32**, 605-617.

Knable, M.B. & Weinberger, D.R. (1997) Dopamine, the prefrontal cortex and schizophrenia. *J Psychopharmacol*, **11**, 123-131.

Labelle-Dumais, C. & Flores, C. (2008) Spatio-temporal pattern of netrin-1 receptor expression by mesocorticolimbic dopaminergic neurons. *Societly for Neuroscience Abstracts*.

Lammel, S., Hetzel, A., Hackel, O., Jones, I., Liss, B. & Roeper, J. (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. *Neuron*, **57**, 760-773.

Le Moal, M. & Simon, H. (1991) Mesocorticolimbic dopaminergic network: functional and regulatory roles. *Physiol Rev*, **71**, 155-234.

Leslie, C.A., Robertson, M.W., Cutler, A.J. & Bennett, J.P., Jr. (1991) Postnatal development of D1 dopamine receptors in the medial prefrontal cortex, striatum and nucleus accumbens of normal and neonatal 6-hydroxydopamine treated rats: a quantitative autoradiographic analysis. *Brain Res Dev Brain Res*, **62**, 109-114.

Lewis, D.A. (1997) Development of the prefrontal cortex during adolescence: insights into vulnerable neural circuits in schizophrenia. *Neuropsychopharmacology*, **16**, 385-398.

Lin, L., Rao, Y. & Isacson, O. (2005) Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons. *Mol Cell Neurosci*, **28**, 547-555.

Lipska, B.K., Jaskiw, G.E., Chrapusta, S., Karoum, F. & Weinberger, D.R. (1992) Ibotenic acid lesion of the ventral hippocampus differentially affects

dopamine and its metabolites in the nucleus accumbens and prefrontal cortex in the rat. *Brain Res*, **585**, 1-6.

Lipska, B.K., Jaskiw, G.E. & Weinberger, D.R. (1993) Postpubertal emergence of hyperresponsiveness to stress and to amphetamine after neonatal excitotoxic hippocampal damage: a potential animal model of schizophrenia. *Neuropsychopharmacology*, **9**, 67-75.

Lipska, B.K. & Weinberger, D.R. (2000) To model a psychiatric disorder in animals: schizophrenia as a reality test. *Neuropsychopharmacology*, **23**, 223-239.

Llambi, F., Causeret, F., Bloch-Gallego, E. & Mehlen, P. (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. *EMBO J*, **20**, 2715-2722.

Luo, L. & O'Leary, D.D. (2005) Axon retraction and degeneration in development and disease. *Annu Rev Neurosci*, **28**, 127-156.

Manitt, C. & Kennedy, T.E. (2002) Where the rubber meets the road: netrin expression and function in developing and adult nervous systems. *Prog Brain Res*, **137**, 425-442.

Manitt, C., Thompson, K.M. & Kennedy, T.E. (2004) Developmental shift in expression of netrin receptors in the rat spinal cord: predominance of UNC5 homologues in adulthood. *J Neurosci Res*, **77**, 690-700.

Marcotte, E.R., Pearson, D. & Srivastava, L.K. (2001) Animal models of schizophrenia: a critical review. *J Psychiatry Neurosci*, **26**, 395-410.

Margolis, E.B., Lock, H., Chefer, V.I., Shippenberg, T.S., Hjelmstad, G.O. & Fields, H.L. (2006) Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. *Proc Natl Acad Sci U S A*, **103**, 2938-2942.

Margolis, E.B., Mitchell, J.M., Ishikawa, J., Hjelmstad, G.O. & Fields, H.L. (2008) Midbrain dopamine neurons: projection target determines action potential duration and dopamine D(2) receptor inhibition. *J Neurosci*, **28**, 8908-8913.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S. & Dickson, B.J. (1996) Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron*, **17**, 203-215.

Morley, B.J. & Rodriguez-Sierra, J.F. (2004) A phenotype for the alpha 7 nicotinic acetylcholine receptor null mutant. *Brain Res*, **1023**, 41-47.

Moron, J.A., Brockington, A., Wise, R.A., Rocha, B.A. & Hope, B.T. (2002) Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. *J Neurosci*, **22**, 389-395.

Niculescu, M., Ehrlich, M.E. & Unterwald, E.M. (2005) Age-specific behavioral responses to psychostimulants in mice. *Pharmacol Biochem Behav*, **82**, 280-288.

Oakberg, E.F. (1957) Duration of spermatogenesis in the mouse. *Nature*, **180**, 1137-1138.

Osborne, P.B., Halliday, G.M., Cooper, H.M. & Keast, J.R. (2005) Localization of immunoreactivity for deleted in colorectal cancer (DCC), the receptor

for the guidance factor netrin-1, in ventral tier dopamine projection pathways in adult rodents. *Neuroscience*, **131**, 671-681.

Parish, C.L., Finkelstein, D.I., Drago, J., Borrelli, E. & Horne, M.K. (2001) The role of dopamine receptors in regulating the size of axonal arbors. *J Neurosci*, **21**, 5147-5157.

Paxinos, G. & Franklin, K.B.J. (2001) *The mouse brain in stereotaxic coordinates*. Academic Press, San Diego.

Piazza, P.V., Rouge-Pont, F., Deminiere, J.M., Kharoubi, M., Le Moal, M. & Simon, H. (1991) Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. *Brain Res*, **567**, 169-174.

Poon, V.Y., Klassen, M.P. & Shen, K. (2008) UNC-6/netrin and its receptor UNC5 locally exclude presynaptic components from dendrites. *Nature*, **455**, 669-673.

Sesack, S.R., Hawrylak, V.A., Matus, C., Guido, M.A. & Levey, A.I. (1998) Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J Neurosci*, **18**, 2697-2708.

Spear, L.P. & Brake, S.C. (1983) Periadolescence: age-dependent behavior and psychopharmacological responsivity in rats. *Dev Psychobiol*, **16**, 83-109.

Spear, L.P. (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*, **24**, 417-463.

Tam, S.Y. & Roth, R.H. (1997) Mesoprefrontal dopaminergic neurons: can tyrosine availability influence their functions? *Biochem Pharmacol*, **53**, 441-453.

Tang, F. & Kalil, K. (2005) Netrin-1 induces axon branching in developing cortical neurons by frequency-dependent calcium signaling pathways. *J Neurosci*, **25**, 6702-6715.

Teicher, M.H., Gallitano, A.L., Gelbard, H.A., Evans, H.K., Marsh, E.R., Booth, R.G. & Baldessarini, R.J. (1991) Dopamine D1 autoreceptor function: possible expression in developing rat prefrontal cortex and striatum. *Brain Res Dev Brain Res*, **63**, 229-235.

Teicher, M.H., Barber, N.I., Gelbard, H.A., Gallitano, A.L., Campbell, A., Marsh, E. & Baldessarini, R.J. (1993) Developmental differences in acute nigrostriatal and mesocorticolimbic system response to haloperidol. *Neuropsychopharmacology*, **9**, 147-156.

Teicher, M.H., Andersen, S.L. & Hostetter, J.C., Jr. (1995) Evidence for dopamine receptor pruning between adolescence and adulthood in striatum but not nucleus accumbens. *Brain Res Dev Brain Res*, **89**, 167-172.

Thompson, T.L. & Moss, R.L. (1995) In vivo stimulated dopamine release in the nucleus accumbens: modulation by the prefrontal cortex. *Brain Res*, **686**, 93-98.

Tseng, K.Y., Lewis, B.L., Lipska, B.K. & O'Donnell, P. (2007) Post-pubertal disruption of medial prefrontal cortical dopamine-glutamate interactions in a developmental animal model of schizophrenia. *Biol Psychiatry*, **62**, 730-738.

Tseng, K.Y. & O'Donnell, P. (2005) Post-pubertal emergence of prefrontal cortical up states induced by D1-NMDA co-activation. *Cereb Cortex*, 15, 49-57.

Tseng, K.Y. & O'Donnell, P. (2007) Dopamine modulation of prefrontal cortical interneurons changes during adolescence. *Cereb Cortex*, **17**, 1235-1240.

Uesaka, N., Ruthazer, E.S. & Yamamoto, N. (2006) The role of neural activity in cortical axon branching. *Neuroscientist*, **12**, 102-106.

van Eden, C.G., Kros, J.M. & Uylings, H.B. (1990) The development of the rat prefrontal cortex. Its size and development of connections with thalamus, spinal cord and other cortical areas. *Prog Brain Res*, **85**, 169-183.

Ventura, R., Alcaro, A., Cabib, S., Conversi, D., Mandolesi, L. & Puglisi-Allegra, S. (2004) Dopamine in the medial prefrontal cortex controls genotype-dependent effects of amphetamine on mesoaccumbens dopamine release and locomotion. *Neuropsychopharmacology*, **29**, 72-80.

Vezina, P., Blanc, G., Glowinski, J. & Tassin, J.P. (1994) Blockade of D-1 dopamine receptors in the medial prefrontal cortex produces delayed effects on pre- and postsynaptic indices of dopamine function in the nucleus accumbens. *Synapse*, **16**, 104-112.

Voorn, P., Kalsbeek, A., Jorritsma-Byham, B. & Groenewegen, H.J. (1988) The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience*, **25**, 857-887.

Waimey, K.E. & Cheng, H.J. (2006) Axon pruning and synaptic development: how are they per-plexin? *Neuroscientist*, **12**, 398-409.

West, M.J. & Gundersen, H.J. (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. *J Comp Neurol*, **296**, 1-22.

Winberg, M.L., Mitchell, K.J. & Goodman, C.S. (1998) Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell*, **93**, 581-591.

Xu, N.J. & Henkemeyer, M. (2009) Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning. *Nat Neurosci*, **12**, 268-276.

Yetnikoff, L., Labelle-Dumais, C. & Flores, C. (2007) Regulation of netrin-1 receptors by amphetamine in the adult brain. *Neuroscience*, **150**, 764-773.

III.3. Supplementary data and discussion

The main goal of the set of experiments described in this Chapter was to determine whether the behavioural and DA phenotypes observed in adult *dcc* +/- mice would be observed prior to adolescence. Thus, I was primarily interested in assessing if any of the phenotypic traits described in Chapter II, or in Flores et al. 2005, would be present in *dcc* +/- mice that were PND35 or younger. However, I did perform a pilot amphetamine-induced locomotion study in young adult (PND60 ± 5) female *dcc* +/- and +/+ mice. As shown in Figure III-6, the reduced sensitivity to the behavioural-activating effects of amphetamine previously reported in PND90 *dcc* heterozygous mice is already present by PND60.

dcc +/- mice do not exhibit changes in neuronal structure in the medial prefrontal cortex before puberty

As described in Chapter II, adult *dcc* +/- mice exhibit changes in spine density selective to layer V pyramidal neurons. A recent study conducted by our laboratory now reports that this alteration in synaptic connectivity observed in adult *dcc* +/- mice, as with all the findings presented in this Chapter, is not evident prior to puberty (Manitt et al. 2011).

In this study, the authors describe other changes that are selective to mPFC DA circuitry in adult *dcc* +/- mice. In comparison to their +/+ littermates, stereological counts of TH-positive varicosities are increased in the cingulate and prelimbic mPFC in adult *dcc* +/- mice. This increase in the number of TH

varicosities is accompanied by an increase in the volume of TH innervation to these regions. Presumably, the elevation in mPFC DA concentration seen in adult *dcc* +/- mice is a result from the increased TH innervation to this area. Remarkably *none* of these other structural phenotypes are observed in post-weanling *dcc* +/- mice (Manitt et al. 2011). Thus, these data corroborate my conclusion that the phenotype exhibited by adult *dcc* +/- mice has a post-pubertal emergence. Moreover, they support the hypothesis that the "protective" behavioural and DA phenotypes demonstrated in adult *dcc* +/- results from changes to the normal dynamics in mPFC DA synaptic connectivity occurring during adolescence.

The results of their study also indicate that DCC may have an important role in establishing (or maintaining) the normal topography of mesocorticolimbic DA circuitry. In both post-weanling and adult +/+ mice there is low expression of netrin-1 in the NAcc, but robust expression of DCC within TH-positive fibres of the NAcc. In contrast, the mPFC express high levels of netrin-1, but low levels of DCC within TH-positive fibres. Thus, it appears as though DA axons that express high levels of DCC may preferentially innervate regions of low levels netrin-1 (NAcc) and those expressing low levels of DCC innervate regions of intense netrin-1 expression (mPFC) (Manitt et al. 2011). Since the TH-positive fibres in *dcc* +/- mice invariably express less DCC, they may be more likely to fail to recognize a target area with less netrin-1 expression (i.e. NAcc) and instead localize to regions of high netrin-1 expression (i.e. mPFC). In support of this notion, the authors found a two-fold increase in the density of DCC/TH co-labelled varicosities in the cingulate and prelimbic

regions of mPFC of *dcc* +/- as compared to wild-type mice. This finding suggests that there may be abnormal innervation to the mPFC of DCC/TH fibres that normally project to other DA targets. These "extra" TH-positive fibres may contribute, in part, to the increased number of TH varicosities in and volume of TH innervation (and thus, DA innervation) to the mPFC of adult *dcc* +/- mice (Manitt et al. 2011).

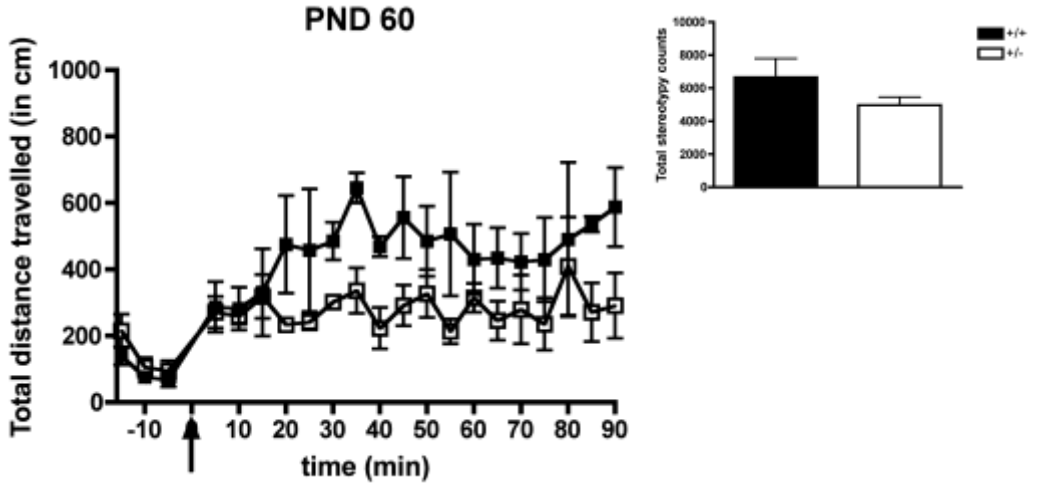


Fig. III-6. **AMPH-induced locomotion in PND60 ± 5 female *dcc* heterozygous (+/-) and wild-type (+/+) mice**

Data points represent the total distance (cm) travelled (mean ± SEM). (a) In comparison to +/+ controls, and as was previously reported in PND90 mice (Grant et al, 2007; see Chapter II), *dcc* +/- mice showed significantly blunted locomotor activity following a systemic injection of 2.2 mg/kg of AMPH (main effect of genotype: $F_{(1,17)} = 5.12$, $p = 0.04$). (b) Amphetamine-induced stereotypy scores. No differences between genotypes were found in AMPH-induced stereotypy at this age ($t_{(5)} = 1.79$, $p = 0.13$).

+/-, $n = 4$; +/+, n = 3

Chapter IV: A case-control association study of DCC polymorphisms with schizophrenia

IV.1. Preamble

In Chapters II and III, I describe the results from a series of experiments conducted in mice heterozygous for *dcc* gene. These results indicate that reduced expression of DCC during development and/or throughout life appears to confer resilience to the development of schizophrenia-like DA and behavioural abnormalities. Importantly, the emergence of this protective phenotype coincides with the mPFC maturational events that occur during the adolescent period. Therefore my results presented thus far not only implicate a potential novel role for DCC in the normal maturation of the mesocortical DA system during adolescence, but they also suggest that variations in DCC function may confer individual differences in susceptibility to the development of schizophrenia. The intriguing possibility that *DCC* may be a novel candidate gene associated with differential vulnerability to develop schizophrenia is the focus of the present chapter.

IV.2. Research Manuscript

A case-control association study of *DCC* polymorphisms with schizophrenia

Alanna Grant[1], Ferid Fathalli[2], Guy Rouleau[3], Ridha Joober[1,2], and Cecilia Flores[1,2]

[1] Integrated Program in Neuroscience , McGill University, Douglas Mental Health University Institute, 6875 LaSalle Boulevard, Montreal, Quebec, H4H 1R3, Canada

[2]Department of Psychiatry, McGill University, Douglas Mental Health University Institute, 6875 LaSalle Boulevard, Montreal, Quebec, H4H 1R3, Canada

[3]CHUM Research Centre Department of Medicine, Centre of Excellence in Neuromics, Universite de Montreal, Montreal, Quebec, H2L 2W5, Canada

Submitted

ABSTRACT

Schizophrenia is a highly heritable neurodevelopmental disorder associated with alterations in synaptic connectivity. Deleted in colorectal cancer (DCC), a receptor for the guidance cue netrin-1, has an important role in establishing neural connectivity. Results from experiments we previously conducted in *dcc*-heterozygous mice show that DCC plays a critical role in the developmental organization of the mesocorticolimbic dopamine (DA) circuitry. Furthermore we have shown that reduced expression of DCC during development and/or throughout life confers resilience to the development of schizophrenia-like DA and behavioural abnormalities. Importantly, this "protective" phenotype only emerges after puberty. Here we assess whether *DCC* may be a novel candidate gene associated with schizophrenia. We examined 10 single nucleotide polymorphisms (SNPs) located in the *DCC* gene for genetic association with schizophrenia using a case-control sample consisting of 556 unrelated schizophrenic patients and 208 healthy controls. We found one SNP, rs2270954, to be nominally associated with schizophrenia; patients were less likely to be heterozygous at this locus and more likely to be homozygous for the minor allele (χ^2 = 9.84, df = 2, nominal p=0.0071). Intriguingly, this SNP is located within the 3' untranslated region, an area known to contain a number of regulatory sequences that determine the stability and translation efficacy of mRNA. These results, together with our previous findings from studies in rodents, point at *DCC* as a promising novel candidate gene that may contribute to the genetic basis behind individual differences in susceptibility to schizophrenia.

INTRODUCTION

Schizophrenia is a complex neurodevelopmental disorder associated with altered synaptic connectivity (Harrison and Weinberger 2005; McGlashan and Hoffman 2000; Selemon and Goldman-Rakic 1999). Because it has heritability estimates between 80-90%, genetic factors undoubtedly play a major role in the susceptibility to this disease (Cardno et al. 1999). Not surprisingly, genes implicated in developmental processes required for the formation of brain circuitries have been identified as potential candidates (Harrison and Weinberger 2005; Impagnatiello et al. 1998; Jaaro-Peled et al. 2009; Mei and Xiong 2008; Millar et al. 2000; Ross et al. 2006; Stefansson et al. 2002; Straub et al. 2002). Deleted in colorectal cancer (DCC), a receptor for the developmental guidance cue netrin-1, plays a pivotal role in the guidance of growing axons and dendrites to their correct target area. Furthermore, once the processes reach their targets, DCC-mediated netrin-1 signalling also contributes to synaptic connectivity by influencing axonal arborization and synaptogenesis (Colon-Ramos et al. 2007; Dent et al. 2004; Hutchins and Kalil 2008; Lim et al. 1999; Manitt et al. 2009; Tang and Kalil 2005). Results from a large series of studies that we have conducted in recent years have led us to speculate that DCC may be a novel candidate gene associated with differential vulnerability to develop schizophrenia (Flores 2011).

We have shown that DCC is a critical determinant of the organization, plasticity, and function of the mesocorticolimbic dopaminergic (DA) system, a system tightly associated with the symptomatology of schizophrenia (Howes and Kapur 2009). We have reported that adult *dcc*-heterozygous mice exhibit reduced

amphetamine-induced locomotor activity, are resistant toward amphetamine-induced deficits in prepulse inhibition, and do not develop sensitization when repeatedly exposed to this drug. Correspondingly, these mice have blunted amphetamine-induced DA release in the ventral striatum, but exaggerated DA activity in the medial prefrontal cortex (mPFC) (Flores et al. 2005; Grant et al. 2007). Significantly, these phenotypes are present in adult, but not pre-pubertal mice, and most likely result from selective alterations in the reorganization of mPFC DA synaptic connectivity during adolescence (Grant et al. 2009; Manitt et al. 2011). Importantly, many of the DA and behavioural traits observed in *dcc*-heterozygous mice are opposite to those observed in a subset of patients with schizophrenia and in neurodevelopmental animal models of this illness (Abi-Dargham et al. 2009; Akil et al. 1999; Boksa 2004; 2010; Grillon et al. 1992; Kegeles et al. 2010; Laruelle et al. 1996; Tseng et al. 2009).

Here, we begin to assess the intriguing hypothesis that *DCC,* a candidate gene strongly implicated in the normal development of the mesocorticolimbic DA system, may be associated with individual differences in the vulnerability to develop schizophrenia. To this end we compare the genotypic and allelic frequencies of individual single nucleotide polymorphisms (SNPs) located in the *DCC* gene between schizophrenic patients and healthy control subjects. In addition, we also perform haplotype-based analysis on the selected SNPs.

MATERIALS AND METHODS

Subjects

Demographic characteristics of the patients and healthy control subjects are presented in Table IV-1. Written informed consent was obtained from all participants. The collection and use of participants' DNA for genotyping was approved by the Research Ethics Board of each participating institution.

Subjects Recruitment and psychiatric assessment

Subjects were recruited from the following facilities: the Douglas Hospital Mental Health University Institute (Montreal, Canada), the Clinique Jeunes Adultes of the Louis-H. Lafontaine Hospital (Montreal, Canada), the Schizophrenia Program of the Royal Ottawa Hospital (Ottawa, Canada), and the Razi Psychiatric Hospital (Tunis, Tunisia). Patients were administered the Diagnostic Interview for Genetic Studies (DIGS). Diagnoses were made according to the DSM-III-R or DSM-IV based on information extracted from the DIGS and from additional information in the patients' medical files. Control subjects were screened for a history of major psychiatric illness using the Structured Clinical Interview for DSM diagnoses (SCIDI and SCIDII). The sample analyzed in the present study consisted of 556 unrelated patients with a diagnosis of schizophrenia (or schizoaffective disorder) and 208 healthy controls.

SNP selection

We searched the National Center for Biotechnology Information dbSNP database for SNPs in *DCC* that were non-synonymous (i.e. resulted in an amino acid substitution), exonic (or otherwise transcribed), and with heterozygosity greater than or equal to 3%. These were: rs984274, rs2229080, and rs2270954. To capture the greatest degree of genetic variation within the *DCC* gene, we also genotyped tag SNPs in *DCC*. Tag SNPs are representative polymorphisms found in regions of DNA of high linkage disequilibrium (LD) and therefore enable for the identification of genetic variation over a larger span of a gene than a single nucleotide. Thus, the use of tag SNPs can reduce the number of SNPs needed to detect a linkage-based association between a region of the genome (i.e. *DCC* gene) and a trait of interest (i.e. schizophrenia). We selected tag SNPs using the genotype data of the Caucasian sample (CEU) of the International HapMap Project database, setting a minor allele frequency (MAF) of 0.1 and an R-squared cut-off of 0.80 as the criteria. The R-squared value reflects the degree to which captured alleles are in linkage disequilibrium with the tag SNP. The selected tag SNPs were rs2298606, rs2156283, rs17390646, rs8088048, rs11082990, rs2339639, and rs12953529. The chosen SNPs and their locations within the DCC gene are shown in Table IV-2. A cartoon depicting the location of the genotyped SNPs in *DCC* is provided in Figure IV-1.

Genotyping

Genomic DNA was isolated from blood or saliva samples that were obtained from the patients and healthy controls. The concentration (calculated

from the absorbance value measured at a wavelength of 260 nm) and purity (estimated from the ratio of the absorbance values measured at wavelengths of 260 nm and 280 nm [A260/A280]) of the DNA samples were assessed using a spectrophotometer. Only samples with sufficient purity, defined as an A260/A280 ratio between 1.8-2.0, were used. From the stock DNA solution, diluted samples (20 ng/ul, diluted in sterile water) were prepared and aliquoted in a blind fashion onto 96-well plates and stored at -20°C. Importantly, a single plate contained DNA from both cases and controls. SNPs were genotyped by Genome Quebec (Montreal, Canada) using Sequenom® iPLEX® Gold genotyping technology.

Statistical Analyses

Analysis for SNP association

Each SNP was tested for violation of the Hardy-Weinberg Equilibrium (HWE) using Chi-square tests; SNPs that were not in HWE were excluded from further analysis (see Table IV-2). We analyzed for differences in the allelic and genotypic distribution frequencies between schizophrenic patients and healthy controls for each SNP using Pearson's Chi-square tests (R software, version 2.6.1). In instances where the numbers in the contingency table were less than 5, we conducted Fisher's Exact two-tailed tests.

Analysis for haplotype association

Additionally, we identified LD blocks and tested for an association between haplotypes and affection status using the HaploView program (version 4.2). LD blocks were constructed according to the criteria used by Gabriel and

colleagues; an LD block was created if 95% of the informative comparisons were in strong LD (defined as D' values >0.95) (Gabriel et al. 2002). The haplotypes generated from each block were tested for an association with schizophrenia by assessing for differences in the haplotype frequencies between schizophrenic patients and healthy control subjects using Chi-square tests. Only those haplotypes with a frequency greater than .01 were examined.

RESULTS

We genotyped a total of 10 SNPs within the *DCC* gene. One of these SNPs deviated significantly from Hardy-Weinberg equilibrium and was not considered for further analyses (see Table IV-2). The observed allele and genotype frequencies of the remaining 9 SNPs are shown in Table IV-3. Upon testing for genotype frequency distribution differences, we found that there was a difference in the genotype distribution of the rs2270954 polymorphism between the schizophrenic patients and healthy control subjects (χ^2 = 9.84, df = 2, nominal p=0.0073). Contrasting heterozygous (A/C) to homozygous (C/C and A/A) genotypes revealed an excess of homozygosity in patients relative to controls (χ^2 = 7.02 df = 1, p=0.0081), with more patients being homozygous for the minor allele than controls (χ^2 = 3.76, df = 1, p=0.052). The excess of homozygosity of the major allele in patients did not reach statistical significance (χ^2 = 3.39, df = 1, p=0.066). As shown in Table IV-2 and Figure IV-1, this polymorphism is located in the beginning of the 3' untranslated region (3' UTR). No other significant differences in either the allelic or genotypic distributions for any of the other SNPs were observed between schizophrenic patients and healthy control subjects.

As shown in Figure IV-2, the Haplotype program generated three LD blocks (rs2229080 and rs17390646; rs11082990 and rs2339639; rs8088048 and rs2270954). Haplotype analysis did not reveal a significant association of any haplotype with schizophrenia (see Table IV-4).

DISCUSSION

Here we report evidence for an association between schizophrenia and the rs2270954 polymorphism in the 3' UTR of the *DCC* gene. We found that schizophrenic patients, in comparison to healthy control subjects, were less likely to be heterozygous at this locus and more likely to carry two copies of the minor allele of this polymorphism. To our knowledge, this is the first time an association between any *DCC* variant and schizophrenia has been reported. This finding suggests that *DCC* may be a novel candidate gene for schizophrenia and may represent a potential innovative line for pharmaceutical research. This idea is very well supported by our findings from studies in transgenic mice that show that variations in DCC function lead to differential vulnerability to develop schizophrenia-like DA traits in adulthood (Flores et al. 2005; Grant et al. 2007; Grant et al. 2009).

Role of DCC in the development and function of the mesocortical DA system

Schizophrenia is a neurodevelopmental disorder associated with alterations in synaptic connectivity. In the majority of cases, the full symptomatology of the disorder does not manifest until late adolescence or early adulthood. The delayed-onset of the disorder is intriguing in light of the fact that there is substantial reorganization of neuronal connectivity and functional refinement to the mesocortical DA circuitry during adolescence (Spear 2000). This observation has led to the hypothesis that the developmental events occurring

during this period may play a pivotal role in the emergence of the disorder (Feinberg 1982; Weinberger 1987).

Our studies conducted in *dcc* heterozygous mice indicate *(i)* that DCC is critically involved in the peri-adolescent functional reorganization of the mesocortical DA system (Grant et al. 2007; Grant et al. 2009; Manitt et al. 2011); *(ii)* that reduced expression of DCC during development and/or throughout life confers resistance to the manifestation of schizophrenia-like DA and behavioural abnormalities in adulthood (Flores et al. 2005; Grant et al. 2007); and *(iii)* that the protective phenotype observed in adult *dcc*-heterozygous mice results from selective changes to mPFC DA circuitry that occurs *during* adolescence (Manitt et al. 2011). Notably, whereas schizophrenia typically manifests in late adolescence or early adulthood, the protective phenotype of *dcc*-heterozygous mice only emerges after puberty (Grant et al. 2009). It is important to note that these mice retain one functional copy of the *dcc* gene and do, in fact, express DCC. Therefore, our findings from these mice reflect the effect of *variation* of DCC expression rather than the complete absence of the protein.

Within this context, it is intriguing that we find evidence for an association between schizophrenia and the rs2270954 polymorphism located within the initial portion of the 3' UTR. It is well established that the 3' UTR of genes contains a number of regulatory sequences that determine the stability, cellular localization, and translation of the mRNA (Chen et al. 2006; Xie et al. 2005). Thus, it is plausible that the allelic variants of the rs2270954 polymorphism confer differential stability and/or efficacy of translation of the *DCC* mRNA. Albeit speculative, it may be that inheriting two copies of the minor allele increases the

overall stability and/or translation of *DCC*, resulting in increased expression of DCC. Importantly, we have shown in rats that repeated exposure to amphetamine, a paradigm often used to model the sensitized mesolimbic DA function observed in some schizophrenic patients, upregulates DCC expression in the DA cell body region (Yetnikoff et al. 2007). Furthermore, reducing DCC at the time of amphetamine administration through the use of a functional-blocking antibody prevents the develoment of sensitized mesolimbic DA function in rats (Yetnikoff et al. 2010). Therefore, these data support the possibility that genetic variants which result in enhanced DCC function may be associated with an increased risk for developing a hypersensitive mesolimbic DA system in humans. Conversely, genetic variants that result in reduced DCC expression may lend protection against the development of positive systems of schizophrenia. Future studies assessing for allelic imbalances in mRNA expression between the two alleles are warranted.

Alterations in DCC in humans and disease

Mutations in *DCC* have been documented in humans; *DCC* heterozygosity has recently been shown to be associated with congenital mirror movements in otherwise healthy individuals (Depienne et al. 2011; Srour et al. 2010). In these subjects, there is an involuntary copying or "mirroring" of movement by the contralateral limb. Because DCC plays an important role in the crossing of commissural and corticospinal axons across the midline (Fazeli et al. 1997; Finger et al. 2002; Keino-Masu et al. 1996), a reduction in *DCC* gene dosage may result in less midline guidance. Such reduction could lead to the

formation of abnormal ipsilateral connections and, in turn, to the mirror movement phenotype (Cincotta et al. 1994; Depienne et al. 2011; Finger et al. 2002; Srour et al. 2010). This association of DCC and mirror movement highlights the pivotal role that DCC has in the development of lateralization of the human brain.

Interestingly, abnormal lateralization has been widely described in schizophrenia and related disorders (Crow 1990; Crow et al. 1989; DeLisi et al. 1994; Flor-Henry 1969; Petty 1999; Shirakawa et al. 2001). In healthy right-handed individuals, the left temporal lobe is typically larger in size and demonstrates a greater activation in response to auditory stimuli (Galaburda and Geschwind 1981; Geschwind and Levitsky 1968). However, as shown by functional imaging studies, this asymmetry is reduced in right-handed schizophrenic patients and in their first-degree relatives (Li et al. 2007; Oertel et al. 2010; Sommer et al. 2004; Whyte et al. 2006). Thus, an intriguing possibility is that the altered lateralization observed in some individuals with schizophrenia and their first-degree relatives may be associated with variations within the DCC gene.

In addition to congenital mirror movements, DCC is associated with cancer. Indeed, DCC was named based on the fact that it was first identified in, and cloned from a region of the 18q chromosome affected by a loss of heterozygosity (LOH) in over 70% of human colorectal tumours (Fearon et al. 1990; Vogelstein et al. 1988). The LOH of chromosome 18q and/or reduced DCC expression have been shown to also occur in several other cancers (Mehlen and Fearon 2004). Intriguingly, several studies have found decreased incidence of

various cancers, including colorectal cancer, in schizophrenic patients and their first-degree relatives (Barak et al. 2005; Catts et al. 2008; Cohen et al. 2002; Grinshpoon et al. 2005; Levav et al. 2007; Mortensen 1994). Although this observation has not been consistently replicated (Dalton et al. 2004; Goldacre et al. 2005; Lichtermann et al. 2001) it has been suggested that the genetic predisposition to schizophrenia may also be associated with reduced vulnerability to develop certain cancers (Lichtermann et al. 2001). The findings we report here suggest that, in some individuals, specific alterations in *DCC* may account for these simultaneous, but opposing, risks for the development of schizophrenia and cancer.

Conclusions and future directions

Previous results from our studies in laboratory animals suggest that variations in DCC function may lead to differential vulnerability to schizophrenia. To begin to address this idea, we examined for differences in the genotypic and allelic frequencies of individual single SNPs located in the *DCC* gene between schizophrenic patients and healthy control subjects. The findings of the present study provide evidence for an association between schizophrenia and the *DCC* rs2270954 polymorphism**,** supporting the idea that *DCC* may be a novel candidate gene. Future analysis of the rs2270954 polymorphism as well as other markers in *DCC* in additional independent sample sets should be conducted to corroborate and understand the functional implications these results.

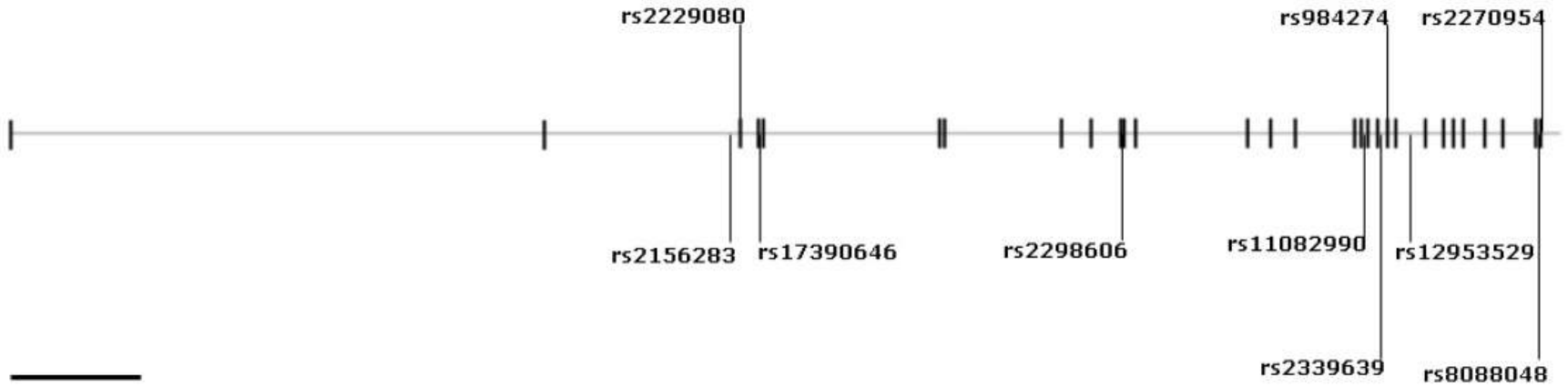


Fig. IV-1. Schematic of the *DCC* gene with introns and exons drawn to scale

The positions of the 10 single nucleotide polymorphisms (SNPs) examined in this study are indicated within the gene. Tag SNPs are presented below the cartoon of the gene; exonic SNPs are listed above the cartoon of the gene. The scale bar located at the bottom left represents 100 kb.

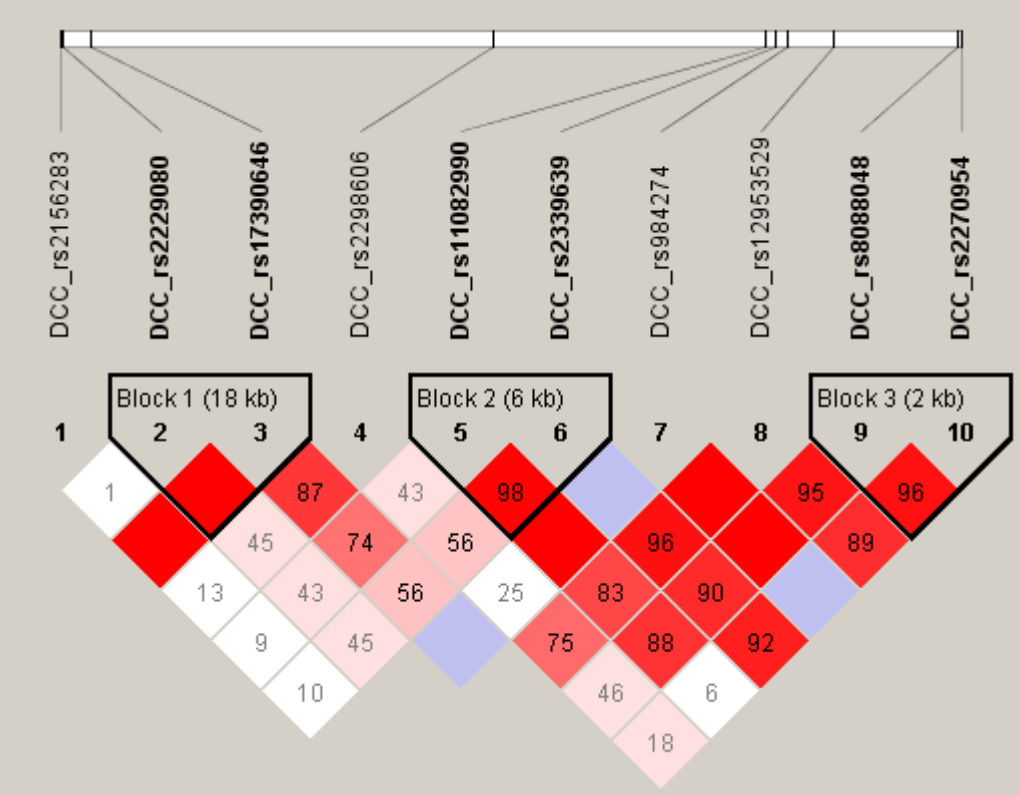


Fig. IV-2. Pairwise linkage disequilibrium and haplotype blocks constructed from the selected SNPs

The values in the diamonds represent the LD coefficient D' (x100). Each pairwise LD comparison is colour coded according to the D' and LOD scores for that comparison. *White*: LOD<2, D'<1; *shades of pink*: LOD>2, D'<1; *blue:* LOD<2, D'=1; *red*: LOD>2; D'=1; *grey:* uninformative.

Table IV-1.

Demographic characteristics of schizophrenic patients and control subjects

	Patients	Controls
Total	556	208
% males	72.30%	66.80%
Age (±SD)	36.66(±14.35)	34.57(±10.59)
Ethnicity		
African American	1.26%	1.92%
Asian	0.48%	0.36%
Caucasian	94.71%	96.40%
European descent	48.08%	56.48%
Middle eastern descent	25.48%	22.84%
Not otherwise specified	21.15%	17.09%

Table IV-2.

Genotyped SNPs and adherences to Hardy-Weinberg Equilibrium

SNP	Location	position	HWE (χ^2)	HWE (p)
rs11082990	Intron 17	50921646	0.75	0.687
rs12953529	Intron 21	50968973	0.02	0.990
rs17390646	Intron 4	50451397	0.04	0.980
rs2156283	Intron 2	50430275	1.32	0.517
rs2229080	Exon 3	50432602	0.25	0.882
rs2270954	3’ UTR	51057298	0.91	0.634
rs2298606	Intron 10	50731823	0.41	0.815
rs2339639	Intron 19	50928022	1.43	0.489
rs8088048	Intron 28	51054765	1.17	0.557
rs984274	Exon 20	50936935	10.23	0.006

Table IV-3.

Allele and genotype frequencies of the selected *DCC* polymorphisms in schizophrenic patients and healthy control subjects

SNP	MAF	Allele (frequency)		p	Genotype (frequency)			p
rs11082990	0.493	C	T	0.929	C/C	C/T	T/T	0.737
case		562 (.505)	550 (.495)		144 (.259)	274 (.493)	138 (.248)	
control		212 (.510)	204 (.490)		58 (.279)	96 (.462)	54 (.260)	
rs12953529	0.411	C	T	0.680	C/C	C/T	T/T	0.896
case		453 (.407)	659 (.593)		93 (.167)	267 (.480)	196 (.353)	
control		175 (.421)	241 (.579)		37 (.178)	101 (.486)	70 (.337)	
rs17390646	0.123	C	G	0.905	C/C	C/G	G/G	0.627
case		974 (.876)	138 (.124)		425 (.764)	124 (.223)	7 (.013)	
control		366 (.880)	50 (.120)		162 (.779)	42 (.202)	4 (.019)	
rs2156283	0.103	C	T	0.531	C/C	C/T	T/T	0.437
case		110 (.100)	990 (.900)		9 (.016)	92 (.167)	449 (.816)	
control		46 (.113)	362 (.887)		2 (.010)	42 (.206)	160 (.784)	
rs2229080	0.383	C	G	0.697	C/C	C/G	G/G	0.684
case		421 (.379)	689 (.621)		80 (.144)	261 (.470)	214 (.386)	
control		163 (.392)	253 (.608)		35 (.168)	93 (.447)	80 (.385)	

Table IV-3. con't

rs2270954	0.156	A	C	0.366	A/A	A/C	C/C	0.007
case		167 (.150)	945 (.850)		20 (.036)	127 (.228)	409 (.736)	
control		71 (.171)	345 (.829)		2 (.010)	67 (.322)	139 (.668)	
rs2298606	0.441	C	T	0.643	C/C	C/T	T/T	0.794
case		228 (.548)	188 (.452)		178 (.320)	270 (.486)	108 (.194)	
control		626 (.563)	486 (.437)		65 (.313)	98 (.471)	45 (.216)	
rs2339639	0.253	A	T	0.096	A/A	A/T	T/T	0.230
case		818 (.736)	294 (.264)		305 (.549)	208 (.374)	43 (.077)	
control		324 (.779)	92 (.221)		128 (.615)	68 (.327)	12 (.058)	
rs8088048	0.431	C	T	0.674	C/C	C/T	T/T	0.503
case		483 (.434)	629 (.566)		107 (.192)	269 (.484)	180 (.324)	
control		175 (.421)	241 (.579)		42 (.202)	91 (.438)	75 (.361)	

Table IV-4.

Frequency of observed haplotypes in schizophrenic patients and healthy control subjects

Block	Overall	Patients	Controls	χ^2	p
Block 1					
CG	0.495	0.495	0.496	0.000	0.993
CC	0.382	0.379	0.390	0.146	0.702
GG	0.123	0.125	0.114	0.305	0.581
Block 2					
TA	0.492	0.495	0.480	0.277	0.598
CA	0.255	0.248	0.286	2.193	0.139
CT	0.253	0.257	0.235	0.808	0.369
Block 3					
TC	0.420	0.414	0.407	0.055	0.815
CC	0.417	0.433	0.417	0.298	0.585
TA	0.161	0.152	0.170	0.722	0.395

REFERENCES

Abi-Dargham, A., van de Giessen, E., Slifstein, M., Kegeles, L.S. & Laruelle, M. (2009) Baseline and amphetamine-stimulated dopamine activity are related in drug-naive schizophrenic subjects. *Biol Psychiatry*, **65**, 1091-1093.

Akil, M., Pierri, J.N., Whitehead, R.E., Edgar, C.L., Mohila, C., Sampson, A.R. & Lewis, D.A. (1999) Lamina-specific alterations in the dopamine innervation of the prefrontal cortex in schizophrenic subjects. *Am J Psychiatry*, **156**, 1580-1589.

Barak, Y., Achiron, A., Mandel, M., Mirecki, I. & Aizenberg, D. (2005) Reduced cancer incidence among patients with schizophrenia. *Cancer*, **104**, 2817-2821.

Boksa, P. (2004) Animal models of obstetric complications in relation to schizophrenia. *Brain Res Brain Res Rev*, **45**, 1-17.

Boksa, P. (2010) Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain Behav Immun*, **24**, 881-897.

Cardno, A.G., Marshall, E.J., Coid, B., Macdonald, A.M., Ribchester, T.R., Davies, N.J., Venturi, P., Jones, L.A., Lewis, S.W., Sham, P.C., Gottesman, II, Farmer, A.E., McGuffin, P., Reveley, A.M. & Murray, R.M. (1999) Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. *Arch Gen Psychiatry*, **56**, 162-168.

Catts, V.S., Catts, S.V., O'Toole, B.I. & Frost, A.D. (2008) Cancer incidence in patients with schizophrenia and their first-degree relatives - a meta-analysis. *Acta Psychiatr Scand*, **117**, 323-336.

Chen, J.M., Ferec, C. & Cooper, D.N. (2006) A systematic analysis of disease-associated variants in the 3' regulatory regions of human protein-coding genes II: the importance of mRNA secondary structure in assessing the functionality of 3' UTR variants. *Hum Genet*, **120**, 301-333.

Cincotta, M., Ragazzoni, A., de Scisciolo, G., Pinto, F., Maurri, S. & Barontini, F. (1994) Abnormal projection of corticospinal tracts in a patient with congenital mirror movements. *Neurophysiol Clin*, **24**, 427-434.

Cohen, M., Dembling, B. & Schorling, J. (2002) The association between schizophrenia and cancer: a population-based mortality study. *Schizophr Res*, **57**, 139-146.

Colon-Ramos, D.A., Margeta, M.A. & Shen, K. (2007) Glia promote local synaptogenesis through UNC-6 (netrin) signaling in C. elegans. *Science*, **318**, 103-106.

Crow, T.J. (1990) Temporal lobe asymmetries as the key to the etiology of schizophrenia. *Schizophr Bull*, **16**, 433-443.

Crow, T.J., Ball, J., Bloom, S.R., Brown, R., Bruton, C.J., Colter, N., Frith, C.D., Johnstone, E.C., Owens, D.G. & Roberts, G.W. (1989) Schizophrenia as an anomaly of development of cerebral asymmetry. A postmortem study

and a proposal concerning the genetic basis of the disease. *Arch Gen Psychiatry*, **46**, 1145-1150.

Dalton, S.O., Laursen, T.M., Mellemkjaer, L., Johansen, C. & Mortensen, P.B. (2004) Risk for cancer in parents of patients with schizophrenia. *Am J Psychiatry*, **161**, 903-908.

DeLisi, L.E., Hoff, A.L., Neale, C. & Kushner, M. (1994) Asymmetries in the superior temporal lobe in male and female first-episode schizophrenic patients: measures of the planum temporale and superior temporal gyrus by MRI. *Schizophr Res*, **12**, 19-28.

Dent, E.W., Barnes, A.M., Tang, F. & Kalil, K. (2004) Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J Neurosci*, **24**, 3002-3012.

Depienne, C., Cincotta, M., Billot, S., Bouteiller, D., Groppa, S., Brochard, V., Flamand, C., Hubsch, C., Meunier, S., Giovannelli, F., Klebe, S., Corvol, J.C., Vidailhet, M., Brice, A. & Roze, E. (2011) A novel DCC mutation and genetic heterogeneity in congenital mirror movements. *Neurology*, **76**, 260-264.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M. & Weinberg, R.A. (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature*, **386**, 796-804.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W. & et al. (1990) Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science*, **247**, 49-56.

Feinberg, I. (1982) Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? *J Psychiatr Res*, **17**, 319-334.

Finger, J.H., Bronson, R.T., Harris, B., Johnson, K., Przyborski, S.A. & Ackerman, S.L. (2002) The netrin 1 receptors Unc5h3 and Dcc are necessary at multiple choice points for the guidance of corticospinal tract axons. *J Neurosci*, **22**, 10346-10356.

Flor-Henry, P. (1969) Psychosis and temporal loabe epilepsy: a controlled investigation. *Epilepsia*, **10**, 363-395.

Flores, C. (2011) Role of netrin-1 in the organization and function of the mesocorticolimbic dopamine system. *J Psychiatry Neurosci*, **36**, 100171.

Flores, C., Manitt, C., Rodaros, D., Thompson, K.M., Rajabi, H., Luk, K.C., Tritsch, N.X., Sadikot, A.F., Stewart, J. & Kennedy, T.E. (2005) Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Mol Psychiatry*, **10**, 606-612.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J. & Altshuler, D. (2002) The structure of haplotype blocks in the human genome. *Science*, **296**, 2225-2229.

Galaburda, A.M. & Geschwind, N. (1981) Anatomical asymmetries in the adult and developing brain and their implications for function. *Adv Pediatr*, **28**, 271-292.

Geschwind, N. & Levitsky, W. (1968) Human brain: left-right asymmetries in temporal speech region. *Science*, **161**, 186-187.

Goldacre, M.J., Kurina, L.M., Wotton, C.J., Yeates, D. & Seagroat, V. (2005) Schizophrenia and cancer: an epidemiological study. *Br J Psychiatry*, **187**, 334-338.

Grant, A., Hoops, D., Labelle-Dumais, C., Prevost, M., Rajabi, H., Kolb, B., Stewart, J., Arvanitogiannis, A. & Flores, C. (2007) Netrin-1 receptor-deficient mice show enhanced mesocortical dopamine transmission and blunted behavioural responses to amphetamine. *Eur J Neurosci*, **26**, 3215-3228.

Grant, A., Speed, Z., Labelle-Dumais, C. & Flores, C. (2009) Post-pubertal emergence of a dopamine phenotype in netrin-1 receptor-deficient mice. *Eur J Neurosci*, **30**, 1318-1328.

Grillon, C., Ameli, R., Charney, D.S., Krystal, J. & Braff, D. (1992) Startle gating deficits occur across prepulse intensities in schizophrenic patients. *Biol Psychiatry*, **32**, 939-943.

Grinshpoon, A., Barchana, M., Ponizovsky, A., Lipshitz, I., Nahon, D., Tal, O., Weizman, A. & Levav, I. (2005) Cancer in schizophrenia: is the risk higher or lower? *Schizophr Res*, **73**, 333-341.

Harrison, P.J. & Weinberger, D.R. (2005) Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol Psychiatry*, **10**, 40-68; image 45.

Howes, O.D. & Kapur, S. (2009) The dopamine hypothesis of schizophrenia: version III--the final common pathway. *Schizophr Bull*, **35**, 549-562.

Hutchins, B.I. & Kalil, K. (2008) Differential outgrowth of axons and their branches is regulated by localized calcium transients. *J Neurosci*, **28**, 143-153.

Impagnatiello, F., Guidotti, A.R., Pesold, C., Dwivedi, Y., Caruncho, H., Pisu, M.G., Uzunov, D.P., Smalheiser, N.R., Davis, J.M., Pandey, G.N., Pappas, G.D., Tueting, P., Sharma, R.P. & Costa, E. (1998) A decrease of reelin expression as a putative vulnerability factor in schizophrenia. *Proc Natl Acad Sci U S A*, **95**, 15718-15723.

Jaaro-Peled, H., Hayashi-Takagi, A., Seshadri, S., Kamiya, A., Brandon, N.J. & Sawa, A. (2009) Neurodevelopmental mechanisms of schizophrenia: understanding disturbed postnatal brain maturation through neuregulin-1-ErbB4 and DISC1. *Trends Neurosci*, **32**, 485-495.

Kegeles, L.S., Abi-Dargham, A., Frankle, W.G., Gil, R., Cooper, T.B., Slifstein, M., Hwang, D.R., Huang, Y., Haber, S.N. & Laruelle, M. (2010) Increased synaptic dopamine function in associative regions of the striatum in schizophrenia. *Arch Gen Psychiatry*, **67**, 231-239.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. & Tessier-Lavigne, M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell*, **87**, 175-185.

Laruelle, M., Abi-Dargham, A., van Dyck, C.H., Gil, R., D'Souza, C.D., Erdos, J., McCance, E., Rosenblatt, W., Fingado, C., Zoghbi, S.S., Baldwin, R.M., Seibyl, J.P., Krystal, J.H., Charney, D.S. & Innis, R.B. (1996) Single photon emission computerized tomography imaging of amphetamine-induced dopamine release in drug-free schizophrenic subjects. *Proc Natl Acad Sci U S A*, **93**, 9235-9240.

Levav, I., Lipshitz, I., Novikov, I., Pugachova, I., Kohn, R., Barchana, M., Ponizovsky, A. & Werner, H. (2007) Cancer risk among parents and siblings of patients with schizophrenia. *Br J Psychiatry*, **190**, 156-161.

Li, X., Branch, C.A., Ardekani, B.A., Bertisch, H., Hicks, C. & DeLisi, L.E. (2007) fMRI study of language activation in schizophrenia, schizoaffective disorder and in individuals genetically at high risk. *Schizophr Res*, **96**, 14-24.

Lichtermann, D., Ekelund, J., Pukkala, E., Tanskanen, A. & Lonnqvist, J. (2001) Incidence of cancer among persons with schizophrenia and their relatives. *Arch Gen Psychiatry*, **58**, 573-578.

Lim, Y.S., Mallapur, S., Kao, G., Ren, X.C. & Wadsworth, W.G. (1999) Netrin UNC-6 and the regulation of branching and extension of motoneuron axons from the ventral nerve cord of Caenorhabditis elegans. *J Neurosci*, **19**, 7048-7056.

Manitt, C., Mimee, A., Eng, C., Pokinko, M., Stroh, T., Cooper, H.M., Kolb, B. & Flores, C. (2011) The Netrin Receptor DCC Is Required in the Pubertal Organization of Mesocortical Dopamine Circuitry. *J Neurosci*, **31**, 8381-8394..

Manitt, C., Nikolakopoulou, A.M., Almario, D.R., Nguyen, S.A. & Cohen-Cory, S. (2009) Netrin participates in the development of retinotectal synaptic connectivity by modulating axon arborization and synapse formation in the developing brain. *J Neurosci*, **29**, 11065-11077.

McGlashan, T.H. & Hoffman, R.E. (2000) Schizophrenia as a disorder of developmentally reduced synaptic connectivity. *Arch Gen Psychiatry*, **57**, 637-648.

Mehlen, P. & Fearon, E.R. (2004) Role of the dependence receptor DCC in colorectal cancer pathogenesis. *J Clin Oncol*, **22**, 3420-3428.

Mei, L. & Xiong, W.C. (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nat Rev Neurosci*, **9**, 437-452.

Millar, J.K., Wilson-Annan, J.C., Anderson, S., Christie, S., Taylor, M.S., Semple, C.A., Devon, R.S., St Clair, D.M., Muir, W.J., Blackwood, D.H. & Porteous, D.J. (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum Mol Genet*, **9**, 1415-1423.

Mortensen, P.B. (1994) The occurrence of cancer in first admitted schizophrenic patients. *Schizophr Res*, **12**, 185-194.

Oertel, V., Knochel, C., Rotarska-Jagiela, A., Schonmeyer, R., Lindner, M., van de Ven, V., Haenschel, C., Uhlhaas, P., Maurer, K. & Linden, D.E. (2010) Reduced laterality as a trait marker of schizophrenia--evidence from structural and functional neuroimaging. *J Neurosci*, **30**, 2289-2299.

Petty, R.G. (1999) Structural asymmetries of the human brain and their disturbance in schizophrenia. *Schizophr Bull*, **25**, 121-139.

Ross, C.A., Margolis, R.L., Reading, S.A., Pletnikov, M. & Coyle, J.T. (2006) Neurobiology of schizophrenia. *Neuron*, **52**, 139-153.

Selemon, L.D. & Goldman-Rakic, P.S. (1999) The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol Psychiatry*, **45**, 17-25.

Shirakawa, O., Kitamura, N., Lin, X.H., Hashimoto, T. & Maeda, K. (2001) Abnormal neurochemical asymmetry in the temporal lobe of schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*, **25**, 867-877.

Sommer, I.E., Ramsey, N.F., Mandl, R.C., van Oel, C.J. & Kahn, R.S. (2004) Language activation in monozygotic twins discordant for schizophrenia. *Br J Psychiatry*, **184**, 128-135.

Spear, L.P. (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*, **24**, 417-463.

Srour, M., Riviere, J.B., Pham, J.M., Dube, M.P., Girard, S., Morin, S., Dion, P.A., Asselin, G., Rochefort, D., Hince, P., Diab, S., Sharafaddinzadeh, N., Chouinard, S., Theoret, H., Charron, F. & Rouleau, G.A. (2010) Mutations in DCC cause congenital mirror movements. *Science*, **328**, 592.

Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T.T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V.G., Gudmundsdottir, E., Bjornsson, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R.P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalo, A., Lemke, G., Sainz, J., Johannesson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M.L., Gurney, M.E., Kong, A., Gulcher, J.R., Petursson, H. & Stefansson, K. (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet*, **71**, 877-892.

Straub, R.E., Jiang, Y., MacLean, C.J., Ma, Y., Webb, B.T., Myakishev, M.V., Harris-Kerr, C., Wormley, B., Sadek, H., Kadambi, B., Cesare, A.J., Gibberman, A., Wang, X., O'Neill, F.A., Walsh, D. & Kendler, K.S. (2002) Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am J Hum Genet*, **71**, 337-348.

Tang, F. & Kalil, K. (2005) Netrin-1 induces axon branching in developing cortical neurons by frequency-dependent calcium signaling pathways. *J Neurosci*, **25**, 6702-6715.

Tseng, K.Y., Chambers, R.A. & Lipska, B.K. (2009) The neonatal ventral hippocampal lesion as a heuristic neurodevelopmental model of schizophrenia. *Behav Brain Res*, **204**, 295-305.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. & Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, **319**, 525-532.

Weinberger, D.R. (1987) Implications of normal brain development for the pathogenesis of schizophrenia. *Arch Gen Psychiatry*, **44**, 660-669.

Whyte, M.C., Whalley, H.C., Simonotto, E., Flett, S., Shillcock, R., Marshall, I., Goddard, N.H., Johnstone, E.C. & Lawrie, S.M. (2006) Event-related

fMRI of word classification and successful word recognition in subjects at genetically enhanced risk of schizophrenia. *Psychol Med*, **36**, 1427-1439.

Xie, X., Lu, J., Kulbokas, E.J., Golub, T.R., Mootha, V., Lindblad-Toh, K., Lander, E.S. & Kellis, M. (2005) Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature*, **434**, 338-345.

Yetnikoff, L., Eng, C., Benning, S. & Flores, C. (2010) Netrin-1 receptor in the ventral tegmental area is required for sensitization to amphetamine. *Eur J Neurosci*, **31**, 1292-1302.

Chapter V: Final summary and Conclusions

The work presented in the body of this Thesis firmly establishes that DCC is a key player in the development of mesocorticolimbic DA system. I have shown that mice that develop with reduced expression of DCC demonstrate functional reorganization of the mesocorticolimbic DA circuitry in adulthood. In comparison to wild-type littermates, adult *dcc* heterozygous mice show large increases in baseline as well as amphetamine-induced DA release in the mPFC, but significantly blunted amphetamine-induced DA release in the NAcc. These changes in mPFC and NAcc DA activity are accompanied by a behavioural phenotype that is opposite to that observed in putative neurodevelopmental animal models of schizophrenia and in the disorder itself; *dcc* heterozygous mice exhibit decreased sensitivity to the locomotor-activating and rewarding effects of amphetamine and show resistance to amphetamine-induced deficits in sensorimotor gating. I have also demonstrated that these mice display structural alterations, indicative of reorganization of local circuitry, in layer V, but not layer III, mPFC pyramidal neurons. Importantly, the neurons of mPFC layer V receive the densest DA innervation and are the ones that give rise to the mPFC projection to the NAcc. The selectivity of these structural alterations are intriguing and are consistent with the idea that reduced DCC function leads to changes that are specific to mPFC DA circuitry. Remarkably, and in corroboration with this conclusion, *none* of the phenotypic traits observed in adult *dcc*-heterozygous mice are present prior to maturation of the mPFC DA system. These data, therefore demonstrate a *novel* role for netrin-1 signalling in the normal functional organization of mesocortical DA circuitry during the adolescent period.

The fact that adult, but not post-weanling or peri-pubertal *dcc*-heterozgyous mice, show resistance to the development of DA and behavioural abnormalities associated with schizophrenia-like symptoms is intriguing in light of the fact that there is a delay in the emergence of these abnormalities in animal models of schizophrenia and in the clinical onset of the disorder itself. This delay coincides with mPFC DA maturational changes that occur during adolescence. Thus, a further implication of the results presented in this Thesis is that variations in DCC levels may confer differential risk to the development of psychopathology by reorganizing mPFC DA circuitry *during* adolescence, and hence, changing the function of this system in adulthood. They also point to the intriguing possibility that the diverse genetic and environmental risk factors associated with schizophrenia may exert their enduring effects on DA function and DA-related behaviours by altering the function DCC during adolescence. An exciting implication suggested by these data is that *DCC* may be a novel candidate gene associated with differential vulnerability to the development of schizophrenia. Indeed, in this Thesis, I report, for the first time, evidence for an association between a *DCC* genetic variant and schizophrenia.

Based on the findings described in this Thesis, I propose that altering DCC function during adolescence may result in a "protective" phenotype in adulthood by selectively influencing the mPFC DA developmental events that occur during this period (see Figure V-1). Importantly, and corroborating this conclusion, the effects of reduced DCC on mPFC DA synaptic connectivity observed in *dcc*-heterozygous mice are only present after puberty (Manitt et al. 2011b). In adulthood, these changes in mPFC DA circuitry lead to enhanced mPFC DA

function and may result in greater inhibitory influence over NAcc DA activity. As a result, the activity of the mesolimbic DA system is dampened and the animal becomes resistant to the development of schizophrenia-like behavioural abnormalities that are associated with enhanced NAcc DA function. Although it remains to be confirmed that the behavioural phenotype and blunted NAcc DA function observed in adult *dcc* heterozygous mice is, in fact, a *direct* result from increased mPFC DA function, these results suggest nonetheless that DCC participate in the differential organization and function of mesocortical and mesolimbic DA pathways.

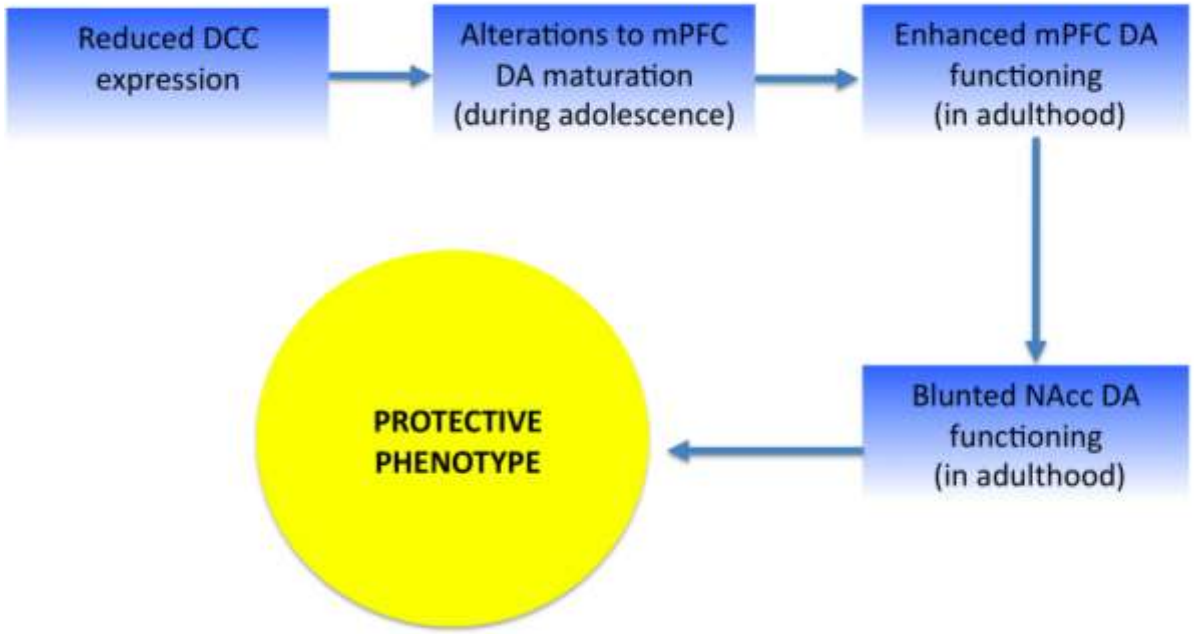
One way to ascertain the validity of the proposed model would be to study the effects of knocking down DCC during the adolescent period in mice using inducible knockout technologies. If my model is correct, reducing DCC expression during this period alone should be sufficient to reproduce the protective phenotype observed in *dcc*-heterozygous mice. The results of these experiments could potentially have exciting implications for the treatment and/or prevention of psychotic episodes in youth at ultra-high risk for developing schizophrenia (Correll et al. 2010; Yung et al. 1996).

My findings may also have important implications in understanding the genetic basis behind individual differences in the susceptibility to the development of substance use disorder (SUD) (Bierut 2011; Swendsen and Le Moal 2011). Like schizophrenia, SUD is associated with dysregulation of the mesocorticolimbic DA system and has a peri-adolescent onset (Chambers et al. 2003). It is intriguing that adult *dcc* heterozygous mice appear to show reduced sensitivity to the rewarding effects of amphetamine, and do not develop

behavioural sensitization when treated repeated (Flores et al. 2005a). In addition, it has been demonstrated in adult wild-type rodents that repeated exposure of amphetamine causes a significant upregulation of DCC expression selectively in the VTA (Yetnikoff et al. 2007), and that blocking DCC function in the VTA at the time of amphetamine administration prevents the development of behavioural sensitization (Yetnikoff et al. 2010). Taken together, the results of these studies point to the possibility that variation in DCC function in humans may be associated with differential vulnerability to the development of drug addiction. Future studies assessing whether genetic variants in *DCC* are associated with SUD are warranted.

To conclude, it remains to be determined whether alterations in DCC function affect the development and functioning of midbrain DA systems in humans. Significant findings of associations between *DCC* variants and Parkinson's disease suggest that this may be so (Kim et al. 2011; Lin et al. 2009). Importantly, *DCC*-heterozygous human individuals have been identified (Depienne et al. 2011; Srour et al. 2010). Assessment of whether these individuals exhibit comparable alterations in mesocorticolimbic DA function to those seen in *dcc*-heterozygous mice, would determine the extent to which the results and implications presented in this Thesis can be applied to humans.

Fig. V-1. General model of the effects of reduced DCC expression on mesocorticolimbic DA development and function



REFERENCES

Abi-Dargham, A. (2003) Probing cortical dopamine function in schizophrenia: what can D1 receptors tell us? *World Psychiatry*, **2**, 166-171.

Abi-Dargham, A., Gil, R., Krystal, J., Baldwin, R.M., Seibyl, J.P., Bowers, M., van Dyck, C.H., Charney, D.S., Innis, R.B. & Laruelle, M. (1998) Increased striatal dopamine transmission in schizophrenia: confirmation in a second cohort. *Am J Psychiatry*, **155**, 761-767.

Abi-Dargham, A., Rodenhiser, J., Printz, D., Zea-Ponce, Y., Gil, R., Kegeles, L.S., Weiss, R., Cooper, T.B., Mann, J.J., Van Heertum, R.L., Gorman, J.M. & Laruelle, M. (2000) Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. *Proc Natl Acad Sci U S A*, **97**, 8104-8109.

Abi-Dargham, A., van de Giessen, E., Slifstein, M., Kegeles, L.S. & Laruelle, M. (2009) Baseline and amphetamine-stimulated dopamine activity are related in drug-naive schizophrenic subjects. *Biol Psychiatry*, **65**, 1091-1093.

Ackerman, S.L., Kozak, L.P., Przyborski, S.A., Rund, L.A., Boyer, B.B. & Knowles, B.B. (1997) The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. *Nature*, **386**, 838-842.

Aguilar-Valles, A., Flores, C. & Luheshi, G.N. (2010) Prenatal inflammation-induced hypoferremia alters dopamine function in the adult offspring in rat: relevance for schizophrenia. *PLoS One*, **5**, e10967.

Akbarian, S., Bunney, W.E., Jr., Potkin, S.G., Wigal, S.B., Hagman, J.O., Sandman, C.A. & Jones, E.G. (1993a) Altered distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase cells in frontal lobe of schizophrenics implies disturbances of cortical development. *Arch Gen Psychiatry*, **50**, 169-177.

Akbarian, S., Vinuela, A., Kim, J.J., Potkin, S.G., Bunney, W.E., Jr. & Jones, E.G. (1993b) Distorted distribution of nicotinamide-adenine dinucleotide phosphate-diaphorase neurons in temporal lobe of schizophrenics implies anomalous cortical development. *Arch Gen Psychiatry*, **50**, 178-187.

Akil, M., Pierri, J.N., Whitehead, R.E., Edgar, C.L., Mohila, C., Sampson, A.R. & Lewis, D.A. (1999) Lamina-specific alterations in the dopamine innervation of the prefrontal cortex in schizophrenic subjects. *Am J Psychiatry*, **156**, 1580-1589.

Aksoy-Poyraz, C., Poyraz, B.C., Turan, S. & Arikan, M.K. (2011) Minor physical anomalies and neurological soft signs in patients with schizophrenia and their siblings. *Psychiatry Res*.

Altman, J. & Bayer, S.A. (1981) Development of the brain stem in the rat. V. Thymidine-radiographic study of the time of origin of neurons in the midbrain tegmentum. *J Comp Neurol*, **198**, 677-716.

Andersen, S.L. (2003) Trajectories of brain development: point of vulnerability or window of opportunity? *Neurosci Biobehav Rev*, **27**, 3-18.

Andersen, S.L., Rutstein, M., Benzo, J.M., Hostetter, J.C. & Teicher, M.H. (1997) Sex differences in dopamine receptor overproduction and elimination. *Neuroreport*, **8**, 1495-1498.

Andersen, S.L., Thompson, A.P., Krenzel, E. & Teicher, M.H. (2002) Pubertal changes in gonadal hormones do not underlie adolescent dopamine receptor overproduction. *Psychoneuroendocrinology*, **27**, 683-691.

Andersen, S.L., Thompson, A.T., Rutstein, M., Hostetter, J.C. & Teicher, M.H. (2000) Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats. *Synapse*, **37**, 167-169.

Baldwin, T.J., Fazeli, M.S., Doherty, P. & Walsh, F.S. (1996) Elucidation of the molecular actions of NCAM and structurally related cell adhesion molecules. *J Cell Biochem*, **61**, 502-513.

Banks, K.E. & Gratton, A. (1995) Possible involvement of medial prefrontal cortex in amphetamine-induced sensitization of mesolimbic dopamine function. *Eur J Pharmacol*, **282**, 157-167.

Bannon, M.J., Wolf, M.E. & Roth, R.H. (1983) Pharmacology of dopamine neurons innervating the prefrontal, cingulate and piriform cortices. *Eur J Pharmacol*, **92**, 119-125.

Barallobre, M.J., Pascual, M., Del Rio, J.A. & Soriano, E. (2005) The Netrin family of guidance factors: emphasis on Netrin-1 signalling. *Brain Res Brain Res Rev*, **49**, 22-47.

Bartoe, J.L., McKenna, W.L., Quan, T.K., Stafford, B.K., Moore, J.A., Xia, J., Takamiya, K., Huganir, R.L. & Hinck, L. (2006) Protein interacting with C-kinase 1/protein kinase Calpha-mediated endocytosis converts netrin-1-mediated repulsion to attraction. *J Neurosci*, **26**, 3192-3205.

Benes, F.M., Taylor, J.B. & Cunningham, M.C. (2000) Convergence and plasticity of monoaminergic systems in the medial prefrontal cortex during

the postnatal period: implications for the development of psychopathology. *Cereb Cortex*, **10**, 1014-1027.

Benes, F.M., Vincent, S.L., Molloy, R. & Khan, Y. (1996) Increased interaction of dopamine-immunoreactive varicosities with GABA neurons of rat medial prefrontal cortex occurs during the postweanling period. *Synapse*, **23**, 237-245.

Bertolino, A., Knable, M.B., Saunders, R.C., Callicott, J.H., Kolachana, B., Mattay, V.S., Bachevalier, J., Frank, J.A., Egan, M. & Weinberger, D.R. (1999) The relationship between dorsolateral prefrontal N-acetylaspartate measures and striatal dopamine activity in schizophrenia. *Biol Psychiatry*, **45**, 660-667.

Bierut, L.J. (2011) Genetic vulnerability and susceptibility to substance dependence. *Neuron*, **69**, 618-627.

Bjorklund, A. & Lindvall, O. (1984) Dopamine-containing systems in the CNS. In Bjorklund, A., Hokfelt, T. (eds) *Handbook of Chemical Neuroanatomy*. Elsevier Science Publishers, pp. 55-116.

Boks, M.P., Liddle, P.F., Burgerhof, J.G., Knegtering, R. & van den Bosch, R.J. (2004) Neurological soft signs discriminating mood disorders from first episode schizophrenia. *Acta Psychiatr Scand*, **110**, 29-35.

Boksa, P. (2004) Animal models of obstetric complications in relation to schizophrenia. *Brain Res Brain Res Rev*, **45**, 1-17.

Bouchard, J.F., Moore, S.W., Tritsch, N.X., Roux, P.P., Shekarabi, M., Barker, P.A. & Kennedy, T.E. (2004) Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: A novel mechanism regulating commissural axon extension. *J Neurosci*, **24**, 3040-3050.

Boyes, J., Whalley, H.C., Lawrie, S.M., Johnstone, E.C. & Best, J.J. (2001) A MRI study of ocular hypertelorism in individuals at high risk of developing schizophrenia. *Schizophr Res*, **50**, 1-2.

Brady, A.M., McCallum, S.E., Glick, S.D. & O'Donnell, P. (2008) Enhanced methamphetamine self-administration in a neurodevelopmental rat model of schizophrenia. *Psychopharmacology (Berl)*, **200**, 205-215.

Brake, W.G., Noel, M.B., Boksa, P. & Gratton, A. (1997) Influence of perinatal factors on the nucleus accumbens dopamine response to repeated stress during adulthood: an electrochemical study in the rat. *Neuroscience*, **77**, 1067-1076.

Brake, W.G., Sullivan, R.M. & Gratton, A. (2000) Perinatal distress leads to lateralized medial prefrontal cortical dopamine hypofunction in adult rats. *J Neurosci*, **20**, 5538-5543.

Breier, A., Su, T.P., Saunders, R., Carson, R.E., Kolachana, B.S., de Bartolomeis, A., Weinberger, D.R., Weisenfeld, N., Malhotra, A.K., Eckelman, W.C. & Pickar, D. (1997) Schizophrenia is associated with elevated amphetamine-induced synaptic dopamine concentrations: evidence from a novel positron emission tomography method. *Proc Natl Acad Sci U S A*, **94**, 2569-2574.

Brinschwitz, K., Dittgen, A., Madai, V.I., Lommel, R., Geisler, S. & Veh, R.W. (2010) Glutamatergic axons from the lateral habenula mainly terminate on GABAergic neurons of the ventral midbrain. *Neuroscience*, **168**, 463-476.

Broersen, L.M., Feldon, J. & Weiner, I. (1999) Dissociative effects of apomorphine infusions into the medial prefrontal cortex of rats on latent inhibition, prepulse inhibition and amphetamine-induced locomotion. *Neuroscience*, **94**, 39-46.

Brog, J.S., Salyapongse, A., Deutch, A.Y. & Zahm, D.S. (1993) The patterns of afferent innervation of the core and shell in the "accumbens" part of the rat ventral striatum: immunohistochemical detection of retrogradely transported fluoro-gold. *J Comp Neurol*, **338**, 255-278.

Brown, A.S., Begg, M.D., Gravenstein, S., Schaefer, C.A., Wyatt, R.J., Bresnahan, M., Babulas, V.P. & Susser, E.S. (2004) Serologic evidence of prenatal influenza in the etiology of schizophrenia. *Arch Gen Psychiatry*, **61**, 774-780.

Brown, V.J. & Bowman, E.M. (2002) Rodent models of prefrontal cortical function. *Trends Neurosci*, **25**, 340-343.

Brozoski, T.J., Brown, R.M., Rosvold, H.E. & Goldman, P.S. (1979) Cognitive deficit caused by regional depletion of dopamine in prefrontal cortex of rhesus monkey. *Science*, **205**, 929-932.

Bunney, W.E. & Bunney, B.G. (2000) Evidence for a compromised dorsolateral prefrontal cortical parallel circuit in schizophrenia. *Brain Res Brain Res Rev*, **31**, 138-146.

Cannon, M., Jones, P.B. & Murray, R.M. (2002) Obstetric complications and schizophrenia: historical and meta-analytic review. *Am J Psychiatry*, **159**, 1080-1092.

Cantor-Graae, E., McNeil, T.F., Torrey, E.F., Quinn, P., Bowler, A., Sjostrom, K. & Rawlings, R. (1994) Link between pregnancy complications and minor

physical anomalies in monozygotic twins discordant for schizophrenia. *Am J Psychiatry*, **151**, 1188-1193.

Carr, D.B., O'Donnell, P., Card, J.P. & Sesack, S.R. (1999) Dopamine terminals in the rat prefrontal cortex synapse on pyramidal cells that project to the nucleus accumbens. *J Neurosci*, **19**, 11049-11060.

Carr, D.B. & Sesack, S.R. (2000a) GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. *Synapse*, **38**, 114-123.

Carr, D.B. & Sesack, S.R. (2000b) Projections from the rat prefrontal cortex to the ventral tegmental area: target specificity in the synaptic associations with mesoaccumbens and mesocortical neurons. *J Neurosci*, **20**, 3864-3873.

Chambers, R.A., Krystal, J.H. & Self, D.W. (2001) A neurobiological basis for substance abuse comorbidity in schizophrenia. *Biol Psychiatry*, **50**, 71-83.

Chambers, R.A., Taylor, J.R. & Potenza, M.N. (2003) Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am J Psychiatry*, **160**, 1041-1052.

Charara, A., Smith, Y. & Parent, A. (1996) Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: Phaseolus vulgaris-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. *J Comp Neurol*, **364**, 254-266.

Chiodo, L.A., Bannon, M.J., Grace, A.A., Roth, R.H. & Bunney, B.S. (1984) Evidence for the absence of impulse-regulating somatodendritic and synthesis-modulating nerve terminal autoreceptors on subpopulations of mesocortical dopamine neurons. *Neuroscience*, **12**, 1-16.

Colamarino, S.A. & Tessier-Lavigne, M. (1995) The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell*, **81**, 621-629.

Colon-Ramos, D.A., Margeta, M.A. & Shen, K. (2007) Glia promote local synaptogenesis through UNC-6 (netrin) signaling in C. elegans. *Science*, **318**, 103-106.

Compton, M.T., Chan, R.C., Walker, E.F. & Buckley, P.F. (2011) Minor physical anomalies: potentially informative vestiges of fetal developmental disruptions in schizophrenia. *Int J Dev Neurosci*, **29**, 245-250.

Correll, C.U., Hauser, M., Auther, A.M. & Cornblatt, B.A. (2010) Research in people with psychosis risk syndrome: a review of the current evidence and future directions. *J Child Psychol Psychiatry*, **51**, 390-431.

Cox, S.M., Benkelfat, C., Dagher, A., Delaney, J.S., Durand, F., McKenzie, S.A., Kolivakis, T., Casey, K.F. & Leyton, M. (2009) Striatal dopamine responses to intranasal cocaine self-administration in humans. *Biol Psychiatry*, **65**, 846-850.

Creese, I., Burt, D.R. & Snyder, S.H. (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. *Science*, **192**, 481-483.

Dalley, J.W., Cardinal, R.N. & Robbins, T.W. (2004) Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neurosci Biobehav Rev*, **28**, 771-784.

Daly, M.P., Gooding, D.C., Jessen, H.M. & Auger, A.P. (2008) Indicators of developmental deviance in individuals at risk for schizophrenia. *Schizophr Res*, **101**, 152-160.

Davis, K.L., Kahn, R.S., Ko, G. & Davidson, M. (1991) Dopamine in schizophrenia: a review and reconceptualization. *Am J Psychiatry*, **148**, 1474-1486.

De Vries, M. & Cooper, H.M. (2008) Emerging roles for neogenin and its ligands in CNS development. *J Neurochem*, **106**, 1483-1492.

Depienne, C., Cincotta, M., Billot, S., Bouteiller, D., Groppa, S., Brochard, V., Flamand, C., Hubsch, C., Meunier, S., Giovannelli, F., Klebe, S., Corvol, J.C., Vidailhet, M., Brice, A. & Roze, E. (2011) A novel DCC mutation and genetic heterogeneity in congenital mirror movements. *Neurology*, **76**, 260-264.

Descarries, L., Lemay, B., Doucet, G. & Berger, B. (1987) Regional and laminar density of the dopamine innervation in adult rat cerebral cortex. *Neuroscience*, **21**, 807-824.

Deutch, A.Y., Clark, W.A. & Roth, R.H. (1990) Prefrontal cortical dopamine depletion enhances the responsiveness of mesolimbic dopamine neurons to stress. *Brain Res*, **521**, 311-315.

Doherty, M.D. & Gratton, A. (1996) Medial prefrontal cortical D1 receptor modulation of the meso-accumbens dopamine response to stress: an electrochemical study in freely-behaving rats. *Brain Res*, **715**, 86-97.

Doherty, P., Fazeli, M.S. & Walsh, F.S. (1995) The neural cell adhesion molecule and synaptic plasticity. *J Neurobiol*, **26**, 437-446.

Drevets, W.C., Gautier, C., Price, J.C., Kupfer, D.J., Kinahan, P.E., Grace, A.A., Price, J.L. & Mathis, C.A. (2001) Amphetamine-induced dopamine release in human ventral striatum correlates with euphoria. *Biol Psychiatry*, **49**, 81-96.

Duvauchelle, C.L., Fleming, S.M. & Kornetsky, C. (1998) Prefrontal cortex infusions of SCH 23390 cause immediate and delayed effects on ventral tegmental area stimulation reward. *Brain Res*, **811**, 57-62.

Elsworth, J.D., Leahy, D.J., Roth, R.H. & Redmond, D.E., Jr. (1987) Homovanillic acid concentrations in brain, CSF and plasma as indicators of central dopamine function in primates. *J Neural Transm*, **68**, 51-62.

Fallon, J.H. & Loughlin, S.E. (1987) Monoamine innervation of cerebral cortex and a theory of the role of monoamines in cerebral cortex and basal ganglia. In Jones, E., Peters, A. (eds) *Cerebral Cortex*. Plenum, New York, pp. 41-127.

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M. & Weinberg, R.A. (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature*, **386**, 796-804.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W. & et al. (1990) Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science*, **247**, 49-56.

Feinberg, I. (1982) Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? *J Psychiatr Res*, **17**, 319-334.

Finger, J.H., Bronson, R.T., Harris, B., Johnson, K., Przyborski, S.A. & Ackerman, S.L. (2002) The netrin 1 receptors Unc5h3 and Dcc are necessary at multiple choice points for the guidance of corticospinal tract axons. *J Neurosci*, **22**, 10346-10356.

Flagstad, P., Mork, A., Glenthoj, B.Y., van Beek, J., Michael-Titus, A.T. & Didriksen, M. (2004) Disruption of neurogenesis on gestational day 17 in the rat causes behavioral changes relevant to positive and negative schizophrenia symptoms and alters amphetamine-induced dopamine release in nucleus accumbens. *Neuropsychopharmacology*, **29**, 2052-2064.

Flores, C. (2011) Role of netrin-1 in the organization and function of the mesocorticolimbic dopamine system. *J Psychiatry Neurosci*, **36**, 100171.

Flores, C., Manitt, C., Rodaros, D., Thompson, K.M., Rajabi, H., Luk, K.C., Tritsch, N.X., Sadikot, A.F., Stewart, J. & Kennedy, T.E. (2005a) Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Mol Psychiatry*, **10**, 606-612.

Flores, G., Alquicer, G., Silva-Gomez, A.B., Zaldivar, G., Stewart, J., Quirion, R. & Srivastava, L.K. (2005b) Alterations in dendritic morphology of prefrontal cortical and nucleus accumbens neurons in post-pubertal rats after neonatal excitotoxic lesions of the ventral hippocampus. *Neuroscience*, **133**, 463-470.

Floresco, S.B., West, A.R., Ash, B., Moore, H. & Grace, A.A. (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci*, **6**, 968-973.

Fortier, M.E., Joober, R., Luheshi, G.N. & Boksa, P. (2004) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. *J Psychiatr Res*, **38**, 335-345.

Gad, J.M., Keeling, S.L., Wilks, A.F., Tan, S.S. & Cooper, H.M. (1997) The expression patterns of guidance receptors, DCC and Neogenin, are spatially and temporally distinct throughout mouse embryogenesis. *Dev Biol*, **192**, 258-273.

Gall, G.A.E. & Kyle, W.H. (1968) Growth of the laboratory mouse. *Theoretical and Applied Genetics*, **38**, 304-308.

Garris, P.A. & Wightman, R.M. (1994) Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. *J Neurosci*, **14**, 442-450.

Geddes, J.R. & Lawrie, S.M. (1995) Obstetric complications and schizophrenia: a meta-analysis. *Br J Psychiatry*, **167**, 786-793.

Geisler, S., Derst, C., Veh, R.W. & Zahm, D.S. (2007) Glutamatergic afferents of the ventral tegmental area in the rat. *J Neurosci*, **27**, 5730-5743.

Georges, F. & Aston-Jones, G. (2001) Potent regulation of midbrain dopamine neurons by the bed nucleus of the stria terminalis. *J Neurosci*, **21**, RC160.

Georges, F. & Aston-Jones, G. (2002) Activation of ventral tegmental area cells by the bed nucleus of the stria terminalis: a novel excitatory amino acid input to midbrain dopamine neurons. *J Neurosci*, **22**, 5173-5187.

Gitai, Z., Yu, T.W., Lundquist, E.A., Tessier-Lavigne, M. & Bargmann, C.I. (2003) The netrin receptor UNC-40/DCC stimulates axon attraction and outgrowth through enabled and, in parallel, Rac and UNC-115/AbLIM. *Neuron*, **37**, 53-65.

Gogos, J.A., Morgan, M., Luine, V., Santha, M., Ogawa, S., Pfaff, D. & Karayiorgou, M. (1998) Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. *Proc Natl Acad Sci U S A*, **95**, 9991-9996.

Gore, A.C., Roberts, J.L. & Gibson, M.J. (1999) Mechanisms for the regulation of gonadotropin-releasing hormone gene expression in the developing mouse. *Endocrinology*, **140**, 2280-2287.

Green, M.F., Satz, P., Gaier, D.J., Ganzell, S. & Kharabi, F. (1989) Minor physical anomalies in schizophrenia. *Schizophr Bull*, **15**, 91-99.

Gualtieri, C.T., Adams, A., Shen, C.D. & Loiselle, D. (1982) Minor physical anomalies in alcoholic and schizophrenic adults and hyperactive and autistic children. *Am J Psychiatry*, **139**, 640-643.

Gupta, S., Andreasen, N.C., Arndt, S., Flaum, M., Schultz, S.K., Hubbard, W.C. & Smith, M. (1995) Neurological soft signs in neuroleptic-naive and neuroleptic-treated schizophrenic patients and in normal comparison subjects. *Am J Psychiatry*, **152**, 191-196.

Guthrie, S. & Pini, A. (1995) Chemorepulsion of developing motor axons by the floor plate. *Neuron*, **14**, 1117-1130.

Hong, K., Hinck, L., Nishiyama, M., Poo, M.M., Tessier-Lavigne, M. & Stein, E. (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell*, **97**, 927-941.

Howes, O.D., Montgomery, A.J., Asselin, M.C., Murray, R.M., Valli, I., Tabraham, P., Bramon-Bosch, E., Valmaggia, L., Johns, L., Broome, M., McGuire, P.K. & Grasby, P.M. (2009) Elevated striatal dopamine function linked to prodromal signs of schizophrenia. *Arch Gen Psychiatry*, **66**, 13-20.

Huttenlocher, P.R. (1979) Synaptic density in human frontal cortex - developmental changes and effects of aging. *Brain Res*, **163**, 195-205.

Jaskiw, G.E., Weinberger, D.R. & Crawley, J.N. (1991) Microinjection of apomorphine into the prefrontal cortex of the rat reduces dopamine metabolite concentrations in microdialysate from the caudate nucleus. *Biol Psychiatry*, **29**, 703-706.

Jones, P.B., Barnes, T.R., Davies, L., Dunn, G., Lloyd, H., Hayhurst, K.P., Murray, R.M., Markwick, A. & Lewis, S.W. (2006) Randomized controlled trial of the effect on Quality of Life of second- vs first-generation antipsychotic drugs in schizophrenia: Cost Utility of the Latest Antipsychotic Drugs in Schizophrenia Study (CUtLASS 1). *Arch Gen Psychiatry*, **63**, 1079-1087.

Kalsbeek, A., Voorn, P., Buijs, R.M., Pool, C.W. & Uylings, H.B. (1988) Development of the dopaminergic innervation in the prefrontal cortex of the rat. *J Comp Neurol*, **269**, 58-72.

Kappler, J., Franken, S., Junghans, U., Hoffmann, R., Linke, T., Muller, H.W. & Koch, K.W. (2000) Glycosaminoglycan-binding properties and secondary structure of the C-terminus of netrin-1. *Biochem Biophys Res Commun*, **271**, 287-291.

Kegeles, L.S., Abi-Dargham, A., Frankle, W.G., Gil, R., Cooper, T.B., Slifstein, M., Hwang, D.R., Huang, Y., Haber, S.N. & Laruelle, M. (2010) Increased synaptic dopamine function in associative regions of the striatum in schizophrenia. *Arch Gen Psychiatry*, **67**, 231-239.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. & Tessier-Lavigne, M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell*, **87**, 175-185.

Keleman, K. & Dickson, B.J. (2001) Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. *Neuron*, **32**, 605-617.

Kellendonk, C., Simpson, E.H., Polan, H.J., Malleret, G., Vronskaya, S., Winiger, V., Moore, H. & Kandel, E.R. (2006) Transient and selective overexpression of dopamine D2 receptors in the striatum causes persistent abnormalities in prefrontal cortex functioning. *Neuron*, **49**, 603-615.

Kennedy, T.E. (2000) Cellular mechanisms of netrin function: long-range and short-range actions. *Biochem Cell Biol*, **78**, 569-575.

Kennedy, T.E., Serafini, T., de la Torre, J.R. & Tessier-Lavigne, M. (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell*, **78**, 425-435.

Keshavan, M.S., Sanders, R.D., Sweeney, J.A., Diwadkar, V.A., Goldstein, G., Pettegrew, J.W. & Schooler, N.R. (2003) Diagnostic specificity and neuroanatomical validity of neurological abnormalities in first-episode psychoses. *Am J Psychiatry*, **160**, 1298-1304.

Kim, J.M., Park, S.K., Yang, J.J., Shin, E.S., Lee, J.Y., Yun, J.Y., Kim, J.S., Park, S.S. & Jeon, B.S. (2011) SNPs in axon guidance pathway genes and susceptibility for Parkinson's disease in the Korean population. *J Hum Genet*, **56**, 125-129.

Lammel, S., Hetzel, A., Hackel, O., Jones, I., Liss, B. & Roeper, J. (2008) Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. *Neuron*, **57**, 760-773.

Larrivee, B., Freitas, C., Trombe, M., Lv, X., Delafarge, B., Yuan, L., Bouvree, K., Breant, C., Del Toro, R., Brechot, N., Germain, S., Bono, F., Dol, F., Claes, F., Fischer, C., Autiero, M., Thomas, J.L., Carmeliet, P., Tessier-Lavigne, M. & Eichmann, A. (2007) Activation of the UNC5B receptor by Netrin-1 inhibits sprouting angiogenesis. *Genes Dev*, **21**, 2433-2447.

Laruelle, M., Abi-Dargham, A., van Dyck, C.H., Gil, R., D'Souza, C.D., Erdos, J., McCance, E., Rosenblatt, W., Fingado, C., Zoghbi, S.S., Baldwin, R.M., Seibyl, J.P., Krystal, J.H., Charney, D.S. & Innis, R.B. (1996) Single photon emission computerized tomography imaging of amphetamine-induced dopamine release in drug-free schizophrenic subjects. *Proc Natl Acad Sci U S A*, **93**, 9235-9240.

Leighton, P.A., Mitchell, K.J., Goodrich, L.V., Lu, X., Pinson, K., Scherz, P., Skarnes, W.C. & Tessier-Lavigne, M. (2001) Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature*, **410**, 174-179.

Lewis, D.A. & Lieberman, J.A. (2000) Catching up on schizophrenia: natural history and neurobiology. *Neuron*, **28**, 325-334.

Leyton, M., Boileau, I., Benkelfat, C., Diksic, M., Baker, G. & Dagher, A. (2002) Amphetamine-induced increases in extracellular dopamine, drug wanting, and novelty seeking: a PET/[11C]raclopride study in healthy men. *Neuropsychopharmacology*, **27**, 1027-1035.

Lieberman, J.A., Kane, J.M. & Alvir, J. (1987) Provocative tests with psychostimulant drugs in schizophrenia. *Psychopharmacology (Berl)*, **91**, 415-433.

Lieberman, J.A., Stroup, T.S., McEvoy, J.P., Swartz, M.S., Rosenheck, R.A., Perkins, D.O., Keefe, R.S., Davis, S.M., Davis, C.E., Lebowitz, B.D., Severe, J. & Hsiao, J.K. (2005) Effectiveness of antipsychotic drugs in patients with chronic schizophrenia. *N Engl J Med*, **353**, 1209-1223.

Lim, Y.S., Mallapur, S., Kao, G., Ren, X.C. & Wadsworth, W.G. (1999) Netrin UNC-6 and the regulation of branching and extension of motoneuron

axons from the ventral nerve cord of Caenorhabditis elegans. *J Neurosci*, **19**, 7048-7056.

Lin, L., Lesnick, T.G., Maraganore, D.M. & Isacson, O. (2009) Axon guidance and synaptic maintenance: preclinical markers for neurodegenerative disease and therapeutics. *Trends Neurosci*, **32**, 142-149.

Lipska, B.K. (2004) Using animal models to test a neurodevelopmental hypothesis of schizophrenia. *J Psychiatry Neurosci*, **29**, 282-286.

Lipska, B.K., Jaskiw, G.E. & Weinberger, D.R. (1993) Postpubertal emergence of hyperresponsiveness to stress and to amphetamine after neonatal excitotoxic hippocampal damage: a potential animal model of schizophrenia. *Neuropsychopharmacology*, **9**, 67-75.

Livesey, F.J. & Hunt, S.P. (1997) Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. *Mol Cell Neurosci*, **8**, 417-429.

Llambi, F., Causeret, F., Bloch-Gallego, E. & Mehlen, P. (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. *EMBO J*, **20**, 2715-2722.

Lodge, D.J. & Grace, A.A. (2006) The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proc Natl Acad Sci U S A*, **103**, 5167-5172.

Lokwan, S.J., Overton, P.G., Berry, M.S. & Clark, D. (1999) Stimulation of the pedunculopontine tegmental nucleus in the rat produces burst firing in A9 dopaminergic neurons. *Neuroscience*, **92**, 245-254.

Louilot, A., Le Moal, M. & Simon, H. (1989) Opposite influences of dopaminergic pathways to the prefrontal cortex or the septum on the dopaminergic transmission in the nucleus accumbens. An in vivo voltammetric study. *Neuroscience*, **29**, 45-56.

Manitt, C. & Kennedy, T.E. (2002) Where the rubber meets the road: netrin expression and function in developing and adult nervous systems. *Prog Brain Res*, **137**, 425-442.

Manitt, C., Labelle-Dumais, C., Eng, C., Grant, A., Mimee, A., Stroh, T. & Flores, C. (2010) Peri-pubertal emergence of UNC-5 homologue expression by dopamine neurons in rodents. *PLoS One*, **5**, e11463.

Manitt, C., Mimee, A., Eng, C., Pokinko, M., Stroh, T., Cooper, H.M., Kolb, B. & Flores, C. (2011) The Netrin Receptor DCC Is Required in the Pubertal

Organization of Mesocortical Dopamine Circuitry. *J Neurosci*, **31**, 8381-8394.

Manitt, C., Nikolakopoulou, A.M., Almario, D.R., Nguyen, S.A. & Cohen-Cory, S. (2009) Netrin participates in the development of retinotectal synaptic connectivity by modulating axon arborization and synapse formation in the developing brain. *J Neurosci*, **29**, 11065-11077.

Manitt, C., Thompson, K.M. & Kennedy, T.E. (2004) Developmental shift in expression of netrin receptors in the rat spinal cord: predominance of UNC-5 homologues in adulthood. *J Neurosci Res*, **77**, 690-700.

Margolis, E.B., Lock, H., Chefer, V.I., Shippenberg, T.S., Hjelmstad, G.O. & Fields, H.L. (2006) Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. *Proc Natl Acad Sci U S A*, **103**, 2938-2942.

McNeil, T.F., Cantor-Graae, E. & Ismail, B. (2000) Obstetric complications and congenital malformation in schizophrenia. *Brain Res Brain Res Rev*, **31**, 166-178.

Mehlen, P. & Fearon, E.R. (2004) Role of the dependence receptor DCC in colorectal cancer pathogenesis. *J Clin Oncol*, **22**, 3420-3428.

Meyer-Lindenberg, A., Miletich, R.S., Kohn, P.D., Esposito, G., Carson, R.E., Quarantelli, M., Weinberger, D.R. & Berman, K.F. (2002) Reduced prefrontal activity predicts exaggerated striatal dopaminergic function in schizophrenia. *Nat Neurosci*, **5**, 267-271.

Mitchell, J.B. & Gratton, A. (1992) Partial dopamine depletion of the prefrontal cortex leads to enhanced mesolimbic dopamine release elicited by repeated exposure to naturally reinforcing stimuli. *J Neurosci*, **12**, 3609-3618.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S. & Dickson, B.J. (1996) Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron*, **17**, 203-215.

Morley, B.J. & Rodriguez-Sierra, J.F. (2004) A phenotype for the alpha 7 nicotinic acetylcholine receptor null mutant. *Brain Res*, **1023**, 41-47.

Moron, J.A., Brockington, A., Wise, R.A., Rocha, B.A. & Hope, B.T. (2002) Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. *J Neurosci*, **22**, 389-395.

Mrzljak, L., Uylings, H.B., Van Eden, C.G. & Judas, M. (1990) Neuronal development in human prefrontal cortex in prenatal and postnatal stages. *Prog Brain Res*, **85**, 185-222.

Muramatsu, R., Nakahara, S., Ichikawa, J., Watanabe, K., Matsuki, N. & Koyama, R. (2010) The ratio of 'deleted in colorectal cancer' to 'uncoordinated-5A' netrin-1 receptors on the growth cone regulates mossy fibre directionality. *Brain*, **133**, 60-75.

O'Callaghan, E., Larkin, C., Kinsella, A. & Waddington, J.L. (1991) Familial, obstetric, and other clinical correlates of minor physical anomalies in schizophrenia. *Am J Psychiatry*, **148**, 479-483.

Oakberg, E.F. (1957) Duration of spermatogenesis in the mouse. *Nature*, **180**, 1137-1138.

Olds, M.E. (1990) Enhanced dopamine receptor activation in accumbens and frontal cortex has opposite effects on medial forebrain bundle self-stimulation. *Neuroscience*, **35**, 313-325.

Omelchenko, N., Bell, R. & Sesack, S.R. (2009) Lateral habenula projections to dopamine and GABA neurons in the rat ventral tegmental area. *Eur J Neurosci*, **30**, 1239-1250.

Omelchenko, N. & Sesack, S.R. (2005) Laterodorsal tegmental projections to identified cell populations in the rat ventral tegmental area. *J Comp Neurol*, **483**, 217-235.

Ongur, D. & Price, J.L. (2000) The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. *Cereb Cortex*, **10**, 206-219.

Osborne, P.B., Halliday, G.M., Cooper, H.M. & Keast, J.R. (2005) Localization of immunoreactivity for deleted in colorectal cancer (DCC), the receptor for the guidance factor netrin-1, in ventral tier dopamine projection pathways in adult rodents. *Neuroscience*, **131**, 671-681.

Ozawa, K., Hashimoto, K., Kishimoto, T., Shimizu, E., Ishikura, H. & Iyo, M. (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. *Biol Psychiatry*, **59**, 546-554.

Paxinos, G. & Watson, C. (1998) *The rat brain in stereotaxic coordinates*. Academic Press, New York.

Phillips, A.G., Ahn, S. & Floresco, S.B. (2004) Magnitude of dopamine release in medial prefrontal cortex predicts accuracy of memory on a delayed response task. *J Neurosci*, **24**, 547-553.

Phillipson, O.T. & Pycock, C.J. (1982) Dopamine neurones of the ventral tegmentum project to both medial and lateral habenula. Some implications for habenular function. *Exp Brain Res*, **45**, 89-94.

Pickar, D., Breier, A., Hsiao, J.K., Doran, A.R., Wolkowitz, O.M., Pato, C.N., Konicki, P.E. & Potter, W.Z. (1990) Cerebrospinal fluid and plasma monoamine metabolites and their relation to psychosis. Implications for regional brain dysfunction in schizophrenia. *Arch Gen Psychiatry*, **47**, 641-648.

Pinto, A. & Sesack, S.R. (2000) Limited collateralization of neurons in the rat prefrontal cortex that project to the nucleus accumbens. *Neuroscience*, **97**, 635-642.

Poon, V.Y., Klassen, M.P. & Shen, K. (2008) UNC-6/netrin and its receptor UNC-5 locally exclude presynaptic components from dendrites. *Nature*, **455**, 669-673.

Pycock, C.J., Carter, C.J. & Kerwin, R.W. (1980) Effect of 6-hydroxydopamine lesions of the medial prefrontal cortex on neurotransmitter systems in subcortical sites in the rat. *J Neurochem*, **34**, 91-99.

Rapoport, J.L., Addington, A.M., Frangou, S. & Psych, M.R. (2005) The neurodevelopmental model of schizophrenia: update 2005. *Mol Psychiatry*, **10**, 434-449.

Rosa, A., Cuesta, M.J., Peralta, V., Zarzuela, A., Serrano, F., Martinez-Larrea, A. & Fananas, L. (2005) Dermatoglyphic anomalies and neurocognitive deficits in sibling pairs discordant for schizophrenia spectrum disorders. *Psychiatry Res*, **137**, 215-221.

Rosin, D.L., Clark, W.A., Goldstein, M., Roth, R.H. & Deutch, A.Y. (1992) Effects of 6-hydroxydopamine lesions of the prefrontal cortex on tyrosine hydroxylase activity in mesolimbic and nigrostriatal dopamine systems. *Neuroscience*, **48**, 831-839.

Sawaguchi, T. & Goldman-Rakic, P.S. (1991) D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science*, **251**, 947-950.

Sawaguchi, T. & Goldman-Rakic, P.S. (1994) The role of D1-dopamine receptor in working memory: local injections of dopamine antagonists into the

prefrontal cortex of rhesus monkeys performing an oculomotor delayed-response task. *J Neurophysiol*, **71**, 515-528.

Scarnati, E., Proia, A., Campana, E. & Pacitti, C. (1986) A microiontophoretic study on the nature of the putative synaptic neurotransmitter involved in the pedunculopontine-substantia nigra pars compacta excitatory pathway of the rat. *Exp Brain Res*, **62**, 470-478.

Scheffer, R.E. (2004) Abnormal neurological signs at the onset of psychosis. *Schizophr Res*, **70**, 19-26.

Seeman, P. & Lee, T. (1975) Antipsychotic drugs: direct correlation between clinical potency and presynaptic action on dopamine neurons. *Science*, **188**, 1217-1219.

Selemon, L.D. & Goldman-Rakic, P.S. (1999) The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol Psychiatry*, **45**, 17-25.

Selemon, L.D., Rajkowska, G. & Goldman-Rakic, P.S. (1995) Abnormally high neuronal density in the schizophrenic cortex. A morphometric analysis of prefrontal area 9 and occipital area 17. *Arch Gen Psychiatry*, **52**, 805-818; discussion 819-820.

Selemon, L.D., Rajkowska, G. & Goldman-Rakic, P.S. (1998) Elevated neuronal density in prefrontal area 46 in brains from schizophrenic patients: application of a three-dimensional, stereologic counting method. *J Comp Neurol*, **392**, 402-412.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C. & Tessier-Lavigne, M. (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell*, **87**, 1001-1014.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M. & Tessier-Lavigne, M. (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell*, **78**, 409-424.

Sesack, S.R., Hawrylak, V.A., Matus, C., Guido, M.A. & Levey, A.I. (1998) Dopamine axon varicosities in the prelimbic division of the rat prefrontal cortex exhibit sparse immunoreactivity for the dopamine transporter. *J Neurosci*, **18**, 2697-2708.

Simpson, E.H., Kellendonk, C. & Kandel, E. (2010) A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. *Neuron*, **65**, 585-596.

Smidt, M.P. & Burbach, J.P. (2007) How to make a mesodiencephalic dopaminergic neuron. *Nat Rev Neurosci*, **8**, 21-32.

Sowell, E.R., Thompson, P.M., Holmes, C.J., Jernigan, T.L. & Toga, A.W. (1999) In vivo evidence for post-adolescent brain maturation in frontal and striatal regions. *Nat Neurosci*, **2**, 859-861.

Spear, L.P. (2000) The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*, **24**, 417-463.

Srinivasan, K., Strickland, P., Valdes, A., Shin, G.C. & Hinck, L. (2003) Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis. *Dev Cell*, **4**, 371-382.

Srour, M., Riviere, J.B., Pham, J.M., Dube, M.P., Girard, S., Morin, S., Dion, P.A., Asselin, G., Rochefort, D., Hince, P., Diab, S., Sharafaddinzadeh, N., Chouinard, S., Theoret, H., Charron, F. & Rouleau, G.A. (2010) Mutations in DCC cause congenital mirror movements. *Science*, **328**, 592.

Swendsen, J. & Le Moal, M. (2011) Individual vulnerability to addiction. *Ann N Y Acad Sci*, **1216**, 73-85.

Tanda, G., Pontieri, F.E., Frau, R. & Di Chiara, G. (1997) Contribution of blockade of the noradrenaline carrier to the increase of extracellular dopamine in the rat prefrontal cortex by amphetamine and cocaine. *Eur J Neurosci*, **9**, 2077-2085.

Teicher, M.H., Andersen, S.L. & Hostetter, J.C., Jr. (1995) Evidence for dopamine receptor pruning between adolescence and adulthood in striatum but not nucleus accumbens. *Brain Res Dev Brain Res*, **89**, 167-172.

Thompson, T.L. & Moss, R.L. (1995) In vivo stimulated dopamine release in the nucleus accumbens: modulation by the prefrontal cortex. *Brain Res*, **686**, 93-98.

Tong, J., Killeen, M., Steven, R., Binns, K.L., Culotti, J. & Pawson, T. (2001) Netrin stimulates tyrosine phosphorylation of the UNC-5 family of netrin receptors and induces Shp2 binding to the RCM cytodomain. *J Biol Chem*, **276**, 40917-40925.

Tseng, K.Y., Chambers, R.A. & Lipska, B.K. (2009) The neonatal ventral hippocampal lesion as a heuristic neurodevelopmental model of schizophrenia. *Behav Brain Res*, **204**, 295-305.

Uylings, H.B., Groenewegen, H.J. & Kolb, B. (2003) Do rats have a prefrontal cortex? *Behav Brain Res*, **146**, 3-17.

Van Bockstaele, E.J. & Pickel, V.M. (1995) GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. *Brain Res*, **682**, 215-221.

van Eden, C.G., Kros, J.M. & Uylings, H.B.M. (1990) The development of the rat prefrontal cortex: Its size and development of connections with thalamus, spinal cord and other cortical areas. In Uylings, H.B.M., van Eden, C.G., J.P.C., D.B., Corner, M.A., Feenstra, M.G.P. (eds) *Progress in brain research, the prefrontal cortex: it's structure, function and pathology*. Elsevier, pp. 169-183.

Ventura, R., Alcaro, A., Cabib, S., Conversi, D., Mandolesi, L. & Puglisi-Allegra, S. (2004) Dopamine in the medial prefrontal cortex controls genotype-dependent effects of amphetamine on mesoaccumbens dopamine release and locomotion. *Neuropsychopharmacology*, **29**, 72-80.

Vezina, P., Blanc, G., Glowinski, J. & Tassin, J.P. (1991) Opposed Behavioural Outputs of Increased Dopamine Transmission in Prefrontocortical and Subcortical Areas: A Role for the Cortical D-1 Dopamine Receptor. *Eur J Neurosci*, **3**, 1001-1007.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. & Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, **319**, 525-532.

Voorn, P., Kalsbeek, A., Jorritsma-Byham, B. & Groenewegen, H.J. (1988) The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience*, **25**, 857-887.

Walker, E. & Lewine, R.J. (1990) Prediction of adult-onset schizophrenia from childhood home movies of the patients. *Am J Psychiatry*, **147**, 1052-1056.

Weinberger, D.R. (1987) Implications of normal brain development for the pathogenesis of schizophrenia. *Arch Gen Psychiatry*, **44**, 660-669.

Weinberger, D.R., Berman, K.F. & Zec, R.F. (1986) Physiologic dysfunction of dorsolateral prefrontal cortex in schizophrenia. I. Regional cerebral blood flow evidence. *Arch Gen Psychiatry*, **43**, 114-124.

Widerlov, E. (1988) A critical appraisal of CSF monoamine metabolite studies in schizophrenia. *Ann N Y Acad Sci*, **537**, 309-323.

Williams, M.E., Wu, S.C., McKenna, W.L. & Hinck, L. (2003) Surface expression of the netrin receptor UNC5H1 is regulated through a protein

kinase C-interacting protein/protein kinase-dependent mechanism. *J Neurosci*, **23**, 11279-11288.

Wilson, C. & Terry, A.V., Jr. (2010) Neurodevelopmental animal models of schizophrenia: role in novel drug discovery and development. *Clin Schizophr Relat Psychoses*, **4**, 124-137.

Winberg, M.L., Mitchell, K.J. & Goodman, C.S. (1998) Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell*, **93**, 581-591.

Yavich, L., Forsberg, M.M., Karayiorgou, M., Gogos, J.A. & Mannisto, P.T. (2007) Site-specific role of catechol-O-methyltransferase in dopamine overflow within prefrontal cortex and dorsal striatum. *J Neurosci*, **27**, 10196-10209.

Yetnikoff, L., Eng, C., Benning, S. & Flores, C. (2010) Netrin-1 receptor in the ventral tegmental area is required for sensitization to amphetamine. *Eur J Neurosci*, **31**, 1292-1302.

Yetnikoff, L., Labelle-Dumais, C. & Flores, C. (2007) Regulation of netrin-1 receptors by amphetamine in the adult brain. *Neuroscience*, **150**, 764-773.

Yui, K., Ikemoto, S., Ishiguro, T. & Goto, K. (2000) Studies of amphetamine or methamphetamine psychosis in Japan: relation of methamphetamine psychosis to schizophrenia. *Ann N Y Acad Sci*, **914**, 1-12.

Yung, A.R., McGorry, P.D., McFarlane, C.A., Jackson, H.J., Patton, G.C. & Rakkar, A. (1996) Monitoring and care of young people at incipient risk of psychosis. *Schizophr Bull*, **22**, 283-303.