Neurodevelopmental mechanisms underlying brain disorders

from loss-of-gene function to behavioral endophenotypes

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*From loss of gene function to behavioral endophenotypes*

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General Introduction
Neurodevelopmental mechanisms underlying brain disorders

The development of the brain involves an orchestration of a number of tightly regulated, genetically encoded processes with various influences from the environment. This multi-step process includes proliferation and migration of brain cells, axon guidance, dendritic arbor development, programmed neuron death, synaptogenesis and network formation (1-3). Disturbances or failures of any of these processes may underlie neurodevelopmental disorders that affect a large portion of the population worldwide; common disorders include Intellectual Disability (ID) (4-6), autism spectrum disorders (ASD) (7-9), attention deficit hyperactivity disorder (ADHD) (10, 11) and schizophrenia (SZ) (12, 13). Many symptoms accompanying these disorders, or brain disorders in general for that matter, cannot be treated effectively yet. Hence, more detailed and fundamental knowledge of the neurodevelopmental processes and the pathophysiology underlying these disorders is needed.

Early brain development

Brain development starts with the formation of a thickened neural plate that will fold to form a neural tube. In the proliferative zone lining the inner surface of the neural tube, neuronal progenitor cells are located, which cause regional expansion through division. This leads to a sequence of enlargements and constrictions along the anterior-posterior axis. The larger anterior portion of the neural tube develops into three primary vesicles; the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain), even before the neural tube has completely closed (Fig. 1A). The narrow caudal portion of the neural tube is last to close, and will develop into the spinal cord. Local signaling centers or ‘organizers’ such as the mid-hindbrain border (MHB; Fig. 1A) then provide the information for further patterning of the embryonic brain.

Signaling centers provide this positional information by timely secreting gradients of soluble morphogens including growth factors (Fig. 1A), such as Brain Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs), Sonic Hedgehog (SHH) and members of Wingless-type integration site family (Wnts) (14, 15). Gradients of the various morphogens are able to induce the graded expression of specific intrinsic transcription factors (TFs; Fig. 1B). This positional transcription code creates regional specification that is permissive for the development of a specific group of precursor cells (Fig. 1C). For example, the combined gradient of SHH expressed by the floorplate, Fgf8 secreted by the MHB and Fgf4 by the paraxial mesoderm (16) creates an area that is permissive for the expression of a group of TFs including the homeodomain transcription factor Nkx2.2 (17, 18), the zinc finger transcription factor Gata3 (19) and Plasmacytoma expressed transcription factor (Pet1) (20) that are specific for Serotonin (5-HT) neuron progenitor identity. Hence, the combinatorial expression of the TFs is crucial for the generation of specific neuronal subtypes that are positionally restricted. Markedly, the complex transcriptional regulation during development cannot be fully explained by induction and expression of area-specific transcription factors. These cell-intrinsic programs are regulated, in part, by epigenetic modification such as methylation, histone modifications and chromatin remodeling (21, 22). Therefore, epigenetic mechanisms form an additional layer of regulation of gene expression during normal brain development (22, 23).

Proliferation and migration

During the progression of brain development, the distinct neuronal cell types will express a diversity of proteins that are unique for each cell type, and hereby contribute to the formation of functionally discrete brain areas. Processes that are involved in the development of these brain areas include proliferation, migration, axonal targeting, synaptogenesis and network formation. Newborn neurons are generated by the (a)symmetric division of radial glia cells located within the (sub)ventricular zone (VZ/SVZ) of the developing brain areas (3, 24, 25). These newborn neurons then will commence to migrate from the VZ/SVZ to their final destination, a process that needs to be tightly regulated and
steered. The same radial glia cells that generated the newborn neurons additionally form a scaffold to assist the newborn neurons in their migratory activities. The radial glia cells, with their cell bodies located in the VZ, extend their rope-like processes all the way towards the pial surface where they attach to the basal membrane (Fig. 2). The newborn neurons will use this scaffold of glial processes to migrate to their destination by adhesion, somal translocation and locomotion (26). Eventually, the cytoarchitectural organization of a brain area can either be laminar, most common in the cortical areas, or the cells can be organized in clusters which can mostly be found in the more ventral and posterior brain regions. An example of these two types of organization will be described below.

**Laminar organization**

The cerebral cortex is a highly organized laminar structure consisting of a six-layered organization with a birth-date dependent ‘inside-out pattern’. The early-born projection neurons form the deeper layers, while the later-born projection neurons bypass these to form the upper layers. As mentioned above, cell divisions occur in the VZ and SVZ (Fig. 2A, Stage 1) (3, 24). After their final cell division, the future projection neurons start to migrate to the area just above the SVZ. Here they transform from a multipolar morphology to a bipolar morphology (Fig. 2A, Stage 2) (27, 28), characterized by a prominent leading process, and then migrate long distances through the intermediate zone (IZ) and the cortical plate (CP), using the scaffold of the radial glial fibers (Fig. 2A, Stage 3). This mode of migration is called locomotion (3, 27, 29). Eventually, when the neurons have almost reached their destination their leading processes attach to the most superficial layer of the developing cortex, the marginal zone (MZ). Next, their soma will detach from the radial glial fibers and move rapidly towards their destination close to the surface of the CP called terminal translocation (Fig. 2A, Stage 4) (30). Located in the MZ are the Cajal Retzius cells, which secrete the glycoprotein and chemoattractant Reelin. The gradient formed by Reelin provides positional information for the migrating neurons (31). The next wave of neurons will go through the same migration process, except that they will bypass the previous batch of already positioned neurons and contribute to the new layer above them. This continues until the well-known six-layered organization is achieved (Fig. 2B).
Figure 2. Formation of laminar organization of the cerebral cortex. (A) Stages in radial migration in the cerebral cortex. Stage 1: New-born neurons are generated by asymmetric divisions of the radial glia cells. Stage 2: The neurons start to migrate and have a multipolar morphology. Stage 3: After transition to a bipolar morphology with a prominent leading process the neurons follow the radial glia fibers during radial migration. Stage 4: Terminal translocations occur when the cells have almost reached their final destination in the cortical plate. (B) Radial migration contributes to the formation of the six-layered inside out pattern of the cerebral cortex. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; MZ, marginal zone; L-II/III – L-VI, cortical layer 2-6.

Cell-cluster organization
In contrast to the organization into distinct layers, in ventral brain areas the neurons are often arranged into a complex array of cell clusters. For example, the ventral midbrain dopaminergic (DA) neurons are subdivided into distinct and functionally characteristic cell groups, including the substantia nigra (SN) and ventral tegmental area (VTA). The DA neurons of the SN innervate the dorso-lateral striatum and are involved in controlling motor behavior (32). The VTA DA neurons innervate both cortical and limbic areas, and are mostly involved in emotional behaviors and cognition (32, 33). During development, the expression profiles of a set of TFs contribute to the generation of DA subtypes. The transcription factor SRY-box6 (Sox6) has been identified as a key factor for the early specification and development of SN DA neurons, and has been shown to be involved in suppressing VTA-specific characteristics (34). In addition, bicoid-related paired-like homeodomain 3 (Pitx3), a transcription factor expressed in both SN and VTA DA neurons, has been proven to be crucial for the maintenance of the SN neurons (35, 36). VTA identity is controlled by the expression of the TF orthodenticle homeobox 2 (Otx2), which is first expressed in the progenitor cells but is only maintained at high levels in a subset of differentiating and mature VTA neurons (34, 37, 38). Zincfinger protein 503 (Zfp503, Nolz1) expression additionally contributes to VTA DA neuron identity (34).

The newborn neurons of the future SN and VTA are generated in the VZ of the ventral midbrain, which after cell cycle exit will become postmitotic migratory DA precursor cells (Fig 3A). These cells will start to migrate radially, following a route stipulated by the radial glial scaffold and a group of molecules, collectively known as guidance cues. The migrating DA neurons express C-X-C chemokine receptor type 4 (CXCR-4), which is a receptor for the chemoattractant C-X-C motif chemokine 12 (CXCL12). CXCL12 is expressed by the meninges surrounding the ventral midbrain. Therefore, the structure of the radial glial scaffold together with CXCR4/CXCL12 signaling modulates this distinct radial migration behavior of the DA neurons (39, 40). The buildup of the radial glial scaffold is in principle the same as in the cortical region (Fig 3B) (41, 42). After radial migration, the DA neurons destined to form the SN will continue migrating, however this time in a tangential orientation. These neurons express Disabled-1 (DAB1), which is a downstream effector of Reelin. This large glycoprotein is expressed in an area just dorsally of the tangentially migrating SN DA neurons, attracting them to migrate to a more
lateral position (39, 43, 44). The VTA DA neurons do not respond to Reelin and remain in the central domain. Eventually the DA neurons of the ventral midbrain are organized in two anatomically distinct clusters, the VTA located medially, flanked on both sides by the SN (Fig3A).

**Figure 3. Formation of the cell cluster organization of the ventral midbrain.** (A) Migration of midbrain DA neurons. After initial radial migration the VTA DA neurons remain in the central domain while the SN neurons continue to migrate radially. Adapted from Panman et al. 2014 (34) (B) DA neurons use the radial glial scaffold during migration. Adapted from Tang et al. 2009 (42). E11.5, Embryonic day 11.5; E13.5 Embryonic day 13.5; E18.5, Embryonic day 18.5.

**Axon outgrowth, guidance and target selection**
An important feature of early brain development is the formation of a network of functional neuronal circuits, created by very precise synaptic connections between a variety of neuronal subtypes. In order to form these connections, developing neurons need to extend their axons over long distances, a process requiring navigation. Outgrowing axons are guided to their correct target by axonal guidance cues that can be perceived by the axons as either attractive or repulsive. In addition, the axonal guidance cues can be secreted, which allows them to signal over large distances, or they can be membrane-bound which permits a more restricted distribution and local signaling (45-48). Targeting axons sense the guidance cues with their growth cones; specialized, highly dynamic and motile structures at their foremost tip. Binding of the guidance molecules to their corresponding receptors present on the growth cone causes an activation of intracellular signaling cascades and induces the remodeling of the cytoskeleton resulting in growth cone steering (46, 49, 50). Prominent families among the axon guidance cues are the Netrins (51, 52); Slits, that signal through Roundabout (Robo) family receptors (53-55); Semaphorins, which signal through large multimeric receptor complexes (56-58), and lastly the Ephrins and their Eph receptors (59, 60). The expression of growth cone receptors is dynamic, enabling the growth cone to actively change its response to navigational cues that are present in the environment. The first-extending axons grow in a relatively axon-free environment on their way to their projection targets. The tracts they form will be used by the following extending axons as a scaffold for the various developing projection pathways (61, 62). These subsequent axons may associate in specific bundles or fascicles, a process that is facilitated by internal membrane-bound cues such as members of the neuronal cell adhesion molecule (NCAM) or the Semaphorin family (63-65). Additionally, soluble
guidance cues can contribute by creating a repulsive environment forcing the axon fibers together. Once the axonal growth cone has reached its proper target, synaptic contacts can be formed. The combinatorial expression of cell adhesion molecules facilitate the recognition and stabilization of matched synaptic contacts (66). By changing and refining their functional properties as development progresses, the synaptic contacts contribute to the maturation and development of the neuronal network (67, 68).

**Dysfunction of neurodevelopmental mechanisms contributes to brain disorders**

The neurodevelopmental mechanisms taking place in the various steps of brain formation need to be tightly regulated. Any alterations in these mechanisms could lead to maldevelopment of the brain and affect motor, cognitive, and behavioral development with lasting consequences. For many neurodevelopmental disorders, ongoing research has shed light on how defects in developmental processes contribute to their etiology. For example, in ASD defects in neurodevelopmental processes including proliferation, migration and synaptogenesis have been found. The essential clinical features of ASD are impairment in reciprocal social communication and social interaction, and restricted repetitive patterns of behaviors. Manifestations of the disorder vary significantly, hence the term Spectrum Disorder (Diagnostic and statistical manual of mental disorders -5th edition, DSM-V).

Common in ASD patients is macrocephaly, which is most likely a result of imperfect proliferation of neuronal progenitors (69, 70). Furthermore, defects in the migration of interneurons towards the cortical areas have been found in a rodent model of ASD (71-73). An abnormal neuronal connectivity is thought to underlie the combined cognitive and behavioral deficits of the etiology of ASD. A hyper connected network in the medial prefrontal cortex (mPFC) was found in a mouse model of ASD and ID. Additionally, the synapses of this network showed slower temporal processing (74), indicating an aberrant synapse formation and refinement, and thus network maturation. The neurodevelopmental mechanisms are guided by the expression of a multitude of genes which are expressed at a precise place and in a very specific time window. Essential genes expressed during brain formation thus act as a possible susceptibility factor for developing a brain disorder. Multiple genes have already been found to be associated with brain disorders; however, the specific functions of these genes in brain formation are still largely unknown.

**Functional neurodevelopmental studies**

To study the function of a gene, a so-called loss-of-function approach is often used. In this approach, the gene of interest is changed in such a way that the altered gene product lacks the function of the wild-type gene. The change can either be inherited, or can be temporally and spatially controlled by using various (site-directed) mutagenesis techniques. In early studies, the function of a gene of interest was completely abolished, either by spontaneous mutations or by homologous recombination. This results in a general absence of the gene from the very start of development. Engineering loss-of-function mutations by homologous recombination can be realized by various approaches. Classically, an essential part of the coding sequence was removed, resulting in a ‘knock-out mutant’. Other strategies include chemical mutagenesis induced by for example N-Ethyl-N-nitrosourea (ENU). ENU can transfer its ethyl group to nitrogen or oxygen radicals in DNA resulting in for instance base-pair substitutions or mispairing if not repaired (75). Loss-of-gene-function can also be achieved by replacing the coding sequence of the gene of interest with the coding sequence of a fluorescent marker such as green fluorescent protein (GFP), creating a ‘Knock-in mutant’. The original gene product is now nonfunctional, but the promoter activity can be monitored by GFP expression (76).

Although for some neurodevelopmental disorders complete abolishment of gene function may represent a suitable model, in many cases this approach is problematic. Compensatory mechanisms may be activated that might blur the effect of the loss-of-gene function that one is trying to elucidate. For
this reason, innovative new methods have been developed that allow for more spatial and temporal modulation of gene function. In utero electroporation (IUE)-mediated gene transfer is an example of such a method (77, 78). With this technique, various constructs regulating the expression of the gene of interest can be microinjected into the lateral ventricles of the developing brain (Fig. 4). The construct is then incorporated into the ventricular lining, the area where the dividing precursor cells are located. Subsequently, the effect of gene function modulation can be examined at various time points, by letting the embryos undisturbed for further development in the uterus until the preferred neurodevelopmental time point is reached.

Figure 4. Schematic representation of in utero electroporation-mediated gene transfer.
The uterus of an anaesthetized pregnant mouse is exposed through a small abdominal incision. A solution containing a construct and a fluorescent marker is microinjected in the lateral ventricles of the brain of the embryo through the uterine wall. Subsequently, tweezer-like electrodes placed over the embryonic head deliver electric pulses, causing incorporation of the construct in the ventricular lining.

Aim and outline of the thesis
The aim of this thesis is to study the developmental role of four distinct genes using a loss-of-function approach. Each of the four genes has been linked to a brain disorder and/or a brain area that is part of the etiology of a disorder. Studying the functions of these genes may provide a better understanding of the mechanisms underlying the associated neurodevelopmental disorders.

Chapter 2
Chapter 2 describes a study of the serotonin (5-hydroxytryptamine, 5-HT) projection system during development. Besides its well-known neurotransmitter function, 5-HT has been found to act as a neurodevelopmental signal. A key modulator of 5-HT levels, and thus of its neurodevelopmental role, is the serotonin transporter (5-HTT). This presynaptically located transporter terminates the 5-HT signal by facilitating reuptake for recycling or degradation (79, 80). The 5-HT projection system originates from the Raphe nuclei located within the brain stem. A distant target of the ascending 5-HT projection system is the mPFC. Improper 5-HTT functioning and altered 5-HT innervations of the mPFC have been implicated in several neurodevelopmental disorders. Therefore, we study the role of the 5-HTT in the formation of the rostral Raphe-prefrontal network. For this, we use a 5-HTT mutant rat model, generated by target-selected ENU-induced mutagenesis in a Wistar background. Lack of the 5-HTT affects the chemotrophic nature of the interaction of the 5-HT projection system between the rostral Raphe nuclei and the mPFC. Furthermore, without 5-HTT function, there is a decrease in 5-HT innervation of the mPFC, accompanied by an altered laminar cytoarchitecture.
Chapter 3
Chapter 3 provides new insights into the role of Pitx3 in the development of the VTA and its derived projections. The TF Pitx3 is expressed by a subset of midbrain DA neurons of the VTA and SN, and is important for their differentiation and maintenance. However, the various subsets of midbrain DA neurons show a differential dependency on Pitx3. The SN DA neurons are lost completely in the absence of Pitx3 in mice. The subsequent loss of dopamine in the SN target areas results in symptoms that resemble those of Parkinson’s disease (PD). However, the DA neurons of the VTA are less severely affected by loss of Pitx3, yet observations include an altered neuronal morphology. The DA projections originating from the VTA innervate both limbic and cortical structures, including the mPFC. Using a Pitx3-GFP knock-in mouse model, we investigate the role of Pitx3 in the development of the DA VTA and its mesoprefrontal-derived projections. In the Pitx3-deficient mice we find a defect in the migratory paths of the VTA, as well as aberrant fasciculation of its targeting axons. In accordance, we observe impaired targeting of the mPFC by the VTA-derived projections during early development. Subsequently, we analyze prefrontal-steered behavior in adult Pitx3-heterozygous GFP knock-in mice and find impairments in anxiety and social behavior.

Chapter 4
Chapter 4 demonstrates a role for HGprt in early dopamine system development in vivo. Mutations in the gene encoding the purine salvage enzyme hypoxanthine-guanine phosphoribosyl transferase (HGprt) cause Lesch-Nyhan disease (LND), an incapacitating disorder with a characteristic neurobehavioral phenotype. The underlying pathogenic mechanisms by which HGprt deficiency leads to the motor and behavioral abnormalities are poorly understood. It appears that dysfunction of the basal ganglia circuits due to impaired DA innervation by the SN and VTA underlies many of the symptoms, but the HGprt – dopamine connection remains elusive. Several studies suggest that HGprt deficiency might affect the neural programming of early midbrain DA neurons. To explore the role of HGprt during development, we use an HGprt knock-out mouse model. We show that HGprt deficiency causes an increase in cell division in the DA ventral midbrain, and that there is an abnormal cell alignment. The radial glial scaffold that mediates the migration of the developing DA neurons is abnormally structured and seems not in a proper place, with differences in the rostral to caudal axis. Finally, as HGprt is expressed in virtually all cells, HGprt deficiency might cause alterations in other brain regions as well. Indeed, we demonstrate an altered layer marker distribution in cortical targets of the dopamine system.

Chapter 5
Next-generation sequencing has revealed various genes that are associated with a wide range of neurodevelopmental disorders, but comprehensive knowledge of the associated protein dysfunction is mostly lacking. In this chapter switch-insensitive 3 family member A (Sin3A) is investigated. We identify dominant mutations in the gene encoding SIN3A in individuals with predominantly mild intellectual disability (ID) who display a strikingly similar facial gestalt and various similar additional features. Cerebral imaging revealed subtle but consistent brain abnormalities in these individuals, including corpus callosum hypoplasia and ventriculomegaly. Sin3A has the ability to bind to various members of a transcriptional regulatory complex to control a variety of developmental processes. To investigate the consequences of reduced SIN3A expression on brain development, we used IUE-mediated gene transfer to knock down Sin3a in mice. The amino acid sequence of mouse Sin3a protein closely resembles that of its human orthologue. Here, we find that Sin3a is crucial for the early stages in cortical development such as proliferation of cortical progenitors as well as the differentiation of the progenitors and thus influencing cell fate. Additionally, Sin3a is essential for cortico-cortical projections in the developing mouse brain.
Chapter 6
In this final chapter we discuss the main outcomes of this thesis and put the findings in a broader perspective.

It is our hope that by elucidating the early-developmental roles of the four genes reported in this thesis we provide a contribution to the understanding of the molecular mechanisms underlying the related brain disorders.
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Lack of serotonin reuptake during brain development alters rostral raphe-prefrontal network formation

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Abstract
Besides its ‘classical’ neurotransmitter function, serotonin (5-HT) has been found to also act as a neurodevelopmental signal. During development, the 5-HT projection system, besides an external placental source, represents one of the earliest neurotransmitter systems to innervate the brain. One of the targets of the 5-HT projection system, originating in the brainstem raphe nuclei, is the medial prefrontal cortex (mPFC), an area involved in higher cognitive functions and important in the etiology of many neurodevelopmental disorders. Little is known however about the exact role of 5-HT and its signaling molecules in the formation of the raphe-prefrontal network. Using explant essays, we here studied the role of the 5-HT transporter (5-HTT), an important modulator of the 5-HT signal, in rostral raphe-prefrontal network formation. We found that the chemotrophic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system was affected in rats lacking the 5-HTT (5-HTT\textsuperscript{-/-}). While 5-HTT deficiency did not affect the dorsal raphe 5-HT-positive outgrowing neurites, the median raphe 5-HT neurites switched from a strong repulsive to an attractive interaction when co-cultured with the mPFC. Furthermore, the fasciculation of the mPFC outgrowing neurites was dependent on the amount of 5-HTT. In the mPFC of 5-HTT\textsuperscript{-/-} pups, we observed clear differences in 5-HT innervation and the identity of a class of projection neurons of the mPFC. In the absence of the 5-HTT, the 5-HT innervation in all subareas of the early postnatal mPFC increased dramatically and the number of Satb2-positive callosal projection neurons was decreased. Together, these results suggest a 5-HTT dependency during early development of these brain areas and in the formation of the raphe-prefrontal network. The tremendous complexity of the 5-HT projection system and its role in several neurodevelopmental disorders highlights the need for further research in this largely unexplored area.
Introduction

It has become increasingly clear that several “classical” neurotransmitters, such as serotonin (5-HT), additionally act as neurodevelopmental signals to direct the assembly of the developing brain (1-9). Even before the raphe-derived neurites start extending, there is an external placental source of 5-HT (10). Furthermore, 5-HT signaling molecules such as enzymes responsible for 5-HT synthesis and breakdown, 5-HT receptors and the 5-HT transporter (5-HTT) are already expressed in the brain before 5-HT neurons are born (4, 10-13). The role of 5-HT and its signaling molecules during development is especially important in the light of recent discussions on the effect of serotonin-reuptake inhibitors (SSRIs) during pregnancy (14-18). SSRIs given to the pregnant mother to treat depression, will increase the extracellular 5-HT in not only the mother but also in the brains of the unborn child (17, 19). These children acquire an increased risk to develop reduced somatosensory responses (16) and/or psychomotor control (20), and appear to have a higher risk to develop autism-like symptoms (21).

The 5-HT projection system is one of the earliest neurotransmitter systems to develop and send out its projections to distant targets (22, 23). Specifically, the 5-HT neurons located in the rostral raphe cluster extend profuse axon tracts into the fore- and midbrain (24, 25). A distant target of the ascending 5-HT projection system within the forebrain is the medial prefrontal cortex (mPFC)(26, 27). The mPFC is the seat of our highest cognitive abilities and known to be involved in attentional processes, working memory and behavioral flexibility (28, 29). In rodents, the developing 5-HT-positive fibers reach the mPFC around embryonic day 16-17 (E16 in mouse and E17 in rats), where they initially innervate the marginal zone and the subplate, before massively innervating the cortical plate proper (30). The 5-HT fibers, found within the marginal zone of the mPFC, are thought to contact Cajal-Retzius (CR) cells, cortical layer I cells secreting the glycoprotein reelin crucial for the correct layering of the cortex (30, 31). These CR cells express 5-HT1A and 5-HT3A receptors (30, 32, 33) and differences in 5-HT input onto the latter could result in an altered reelin release, cortical layering and ultimately, PFC-mediated cognitive functioning. Indeed, altered 5-HT innervations of the mPFC have been implicated in the etiology of neurodevelopmental disorders such as schizophrenia, autism spectrum disorders (ASD) and intellectual disability (22, 34-41).

There are indications that 5-HT acts as a soluble cue and modulates the response of targeting axons to guidance cues (42, 43). Due to the important role of 5-HT in neurodevelopment, factors that influence 5-HT signaling may also have profound effects on the correct development of the brain. The presynaptically located 5-HTT is the primary regulator of 5-HT signaling, terminating the 5-HT signal by allowing reuptake for recycling or degradation (23, 44, 45). Apart from being expressed in 5-HT neurons, the 5-HTT is also transiently expressed in non-aminergic neurons, belonging to many topographically distinct brain areas (12, 46-48). In humans, the 5-HTT gene-linked polymorphic region (5-HTTLPR), composed of a short and a long version (34, 44, 49), affects 5-HTT expression and function. The short (s) variant has been associated with robust neurodevelopmental changes in corticolimbic structures, and increased risk for depression in the context of stress (34). The 5-HTT+/− rat model is also known to display anxiety- and depression-related responses to stressors (46, 50). Extracellular levels of 5-HT are increased throughout the brain of the 5-HTT+/− rodent, and affect 5-HT receptor expression, where the 5-HT1A is known to be down-regulated in both 5-HTT+/− rodents and s-variant carriers (7, 46, 51). Also, due to 5-HTT deficiency, increased activity at the 5-HT6 receptor affects proper cortical cytoarchitecture and interneuron migration (7, 52). The mechanisms by which a reduced 5-HTT function in humans, or reduction/deficiency of 5-HTT in rodents, and consequent
increased 5-HT levels, affects areal maturation, guidance and network formation are still not fully understood.

Here we report the results of our study of the chemotropic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system using the 5-HTT knockout rat model. Additionally, we have examined the ability of the outgrowing neurites to form fascicles, and whether differences in fasciculation could be due to 5-HTT deficiency. Moreover, in order to determine whether the early lack of the 5-HTT also affected the maturation of the 5-HT raphe-mPFC projection system, we examined the 5-HT innervation within various subareas of the mPFC in 5-HTT−/− and 5-HTT+/+ pups. Using the transcription factor Satb2 (special AT-rich sequence binding protein 2) as a marker for callosal projection neurons in cortical layers II-VI, we analyzed the number of Satb2-positive neurons in 5-HTT-deficient pups.

Materials and methods

Animals

All animal use and care were performed in accordance with the institutional and national guidelines and regulations of the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands. All animal experiments conformed to the relevant regulatory standards. The 5-HTT mutant rats (Slc6a4<sup>−/−</sup>) were generated in a Wistar background by target-selected ENU-induced mutagenesis (for detailed description, see (53). Timed-pregnant rats were individually housed in macrolon cages in a temperature- and humidity-controlled room (21 ± 1 °C and 60% relative humidity, respectively). The rats had <i>ad libitum</i> access to food and water and a normal light-dark cycle was maintained. Timed-pregnant rats were sacrificed by means of CO<sub>2</sub>/O<sub>2</sub>. The morning on which a vaginal plug was detected is considered E0.5. Genotyping of the embryos and pups was performed by KBioscience (Hoddesdon, United Kingdom).

Explant cultures

Three-dimensional collagen matrix explant assays were performed as described previously (54). Embryonic day 16.5 (E16.5) rat embryos were collected in ice-cold L15 medium (Leibovitz with L-glutamine, PAA, Austria) and brains were rapidly dissected. Explants (<300 µm) were microdissected from (1) the rostral cluster of raphe nuclei, in a rostral-to-caudal direction dividing it in a rostral, intermediate and caudal subarea, bisected along the midline; and (2) the mPFC. Rostral and intermediate subareas correspond to the dorsal raphe nucleus, and the caudal subarea corresponds to the median raphe nucleus (MnR; Fig. 1A, B; Suppl. Fig. 1A). The explants were collected in ice-cold L15 medium containing 10% fetal calf serum (FCS).

Combinations of the various raphe subareas and the mPFC were embedded in close proximity (~300 µm apart) in a collagen matrix (10% 10X MEM, Invitrogen; and 10% NaHCO<sub>3</sub> in diluted rat tail collagen, Invitrogen) in four-well culture dishes (Nunclon surface, Nunc, ThermoScientific). As controls, the various raphe subareas and the mPFC explants were cultured individually to check for their radial growth. Explants were cultured in growth medium (DMEM-F12 with 10% glutamine and antibiotics, 6% 1,7M glucose, and 10% FCS) in a humidified incubator at 37°C with 5% CO<sub>2</sub> for 4 days. Growth medium was renewed after 24 hrs. For each of the combinations of co-cultures mentioned above, at least four independent experiments were performed.
**Immunohistochemistry**

Brains were rapidly dissected from E16.5 embryos and postnatal day 6 (P6) pups, fixed by immersion for 90 min. in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed in PBS and cryoprotected in 30% sucrose in PBS. Brains were frozen in M-1 embedding matrix (Thermo Fisher Scientific) on dry ice in a plastic cup and stored at -80°C. Cryostat sections were cut at 16 μm, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -20°C.

Cryosections were stained immunohistochemically as described previously (55). Rabbit anti-5-HT (Sigma, 1:5000) and mouse anti-Satb2 (Abcam, 1:500) were diluted in blocking buffer (BB, 1,7% normal donkey serum, 1,7% normal goat serum, 1,7% normal horse serum, 1% BSA, 1% glycine, 0,1% lysine, 0,4% Triton X-100, in PBS) and incubated overnight at 4°C. Sections were incubated in species-specific Alexa-conjugated secondary antibody (Molecular Probes) generated in goat and diluted 1:500 in BB for 30 min. at RT. After washing in PBS, sections were counterstained with fluorescent Nissl stain (NeuroTrace; Invitrogen; 1:500), washed extensively in PBS, and embedded in 90% glycerol. Antibody specificity was tested by omitting the primary antibody resulting in no positive signal (negative control) and careful comparison of immuno-positive brain areas with the areas described before (7, 56)(positive control). The nomenclature to describe 5-HT-positive cells and fibers within various brain areas is as described by (54, 57).

The explants in their collagen matrix were quickly washed in PBS, fixed in buffered 4% PFA for 1.5 hr, and washed extensively o/n at 4°C before performing immunocytochemistry. Explants were incubated in BB for 6-8 hr at room temperature (RT). The explants were incubated with primary antibody diluted in BB o/n at 4°C. Rabbit anti-5-HT (Sigma, 1:5000) and mouse anti-Tuj1 (β-III tubulin, Covance, 1:1000) were used to visualize 5-HT or all outgrowing neurites, respectively. On the second day, explants were washed 4 times for a total of 4-5 hrs at RT. They were then incubated with species-specific Alexa-conjugated secondary antibody (Molecular Probes) generated in goat and diluted 1:500 in BB for 1 hr at RT. After washing extensively in PBS o/n at 4°C, the explants were embedded (Prolong Gold, antifade reagent, Invitrogen). For visualization, a Leica DMRA Fluorescence microscope with DFC340FX camera and LASAF software was used.

**Data analysis**

All data analysis was performed in a double-blind fashion. For quantification of the explants assays, the explants were divided in a proximal and distal quadrant of which images were captured as described by (54). The length of the 20 longest neurites was measured in both the proximal and distal quadrants of the culture using Neuron J (Image J plug-in) with an average of 5 explants per condition. The average value of length of each explant in both proximal and distal quadrants was used to determine the proximal/distal ratio (P/D ratio) per explant (54). The number, width and length of the neurite fascicles were analyzed in the proximal and distal quadrants of the culture using NeuronJ by tracing across and along the fascicle.

For assessing 5-HT fiber length and number of Satb2-positive neurons in the various subareas of the mPFC of 5-HT+/+, 5-HT+/− and 5-HT−/− rats, three to five pups of each genotype of at least three independent litters were analyzed and two to four well-spaced (120 μm) sections at the same neuroanatomical level were imaged. A 0.1-mm-wide rectangle spanning the cerebral wall was placed over the centre of the subarea (either infralimbic, IL, prelimbic, PL or cingulate cortex, Cg) of the mPFC. The overall cortical width of a subarea was divided into 10 equal bins [bin 1 within the deep cortical zone and bin 10 within the presumptive layer I] within this rectangle, and 5-HT-positive
Results

Presence of 5-HTT during early development modulates rostral raphe-mPFC directional responses in vitro.

The rostral cluster of raphe nuclei forms projections towards their targets in the fore- and midbrain (24, 27, 58, 59). One of the targets within the forebrain is the mPFC (26, 60-62). The 5-HT projections are guided along the way to their target by various cues, either soluble or membrane-bound, as they develop (43, 63, 64).

To identify the chemotropic nature of the interaction between the rostral cluster of raphe nuclei and the mPFC and to evaluate possible changes in the 5-HTT knockout model, we performed three-dimensional collagen co-cultures of the rostral cluster of raphe nuclei and the mPFC (Fig. 1; Suppl. Fig. 1A, B and D). Brain areas were microdissected from E16.5 5-HTT+/−, 5-HTT+/− and 5-HTT−/− embryonic brains (Suppl. Fig. 1A). Explants were taken from the rostral cluster of raphe nuclei and were divided into three subareas; rostral, intermediate and caudal (Fig. 1A, B; Suppl. Fig. 1A). The rostral and intermediate subareas correspond to the dorsal raphe nucleus (DR) which mainly projects to the forebrain, including the mPFC (27, 58). The caudal subarea corresponds to the median raphe nucleus (MnR) which innervates both the fore- and midbrain (Fig. 1A, B; Suppl. Fig. 1A-C) (60). The explants from the mPFC were co-cultured with one of the subareas of the raphe in a collagen hill for 4 days (Fig. 1; Suppl. Fig. 1A, B, D). After 4 days, the explants were fixed and immunostained for 5-HT and Tuj1 (β-III tubulin, a marker for outgrowing neurites). To measure the extent of attraction or repulsion of outgrowing neurites, revealing the chemotropic nature of the interaction between the two areas, the explants were divided into a proximal and a distal quadrant, with the proximal quadrant facing the co-cultured explant (Fig. 1C). Within the proximal and distal quadrant the lengths of the longest neurites were measured and averaged (Fig. 1D). The average length of the neurites on the proximal site was then divided by the average length of the distal site neurites giving the proximal/distal-ratio (P/D ratio). A P/D ratio above 1 indicates an attractive interaction, whereas a P/D ratio less than 1 denotes repulsion (54, 65, 66). As a control, explants of the various brain areas were cultured individually and divided into four quadrants (Fig. 1E). The lengths of the longest neurites were measured in each quadrant and statistical analysis revealed no significant differences between the 4 quadrants, indicating a radial neurite outgrowth when cultured individually (Fig. 1F).

Fig. 2A-F shows examples of the proximal and distal side of explants of subareas of the rostral raphe and the mPFC co-cultured together (arrowheads above the schematic indicate the displayed explant). P/D ratios of the 5-HT neurite outgrowth and the overall neurite outgrowth (Tuj1-positive) of subareas of the raphe co-cultured with the mPFC (examples in Fig. 2A-D) were calculated and depicted in Fig. 2G and H. The P/D ratios of 5-HT neurite outgrowth of the DR (rostral and intermediate subarea) indicated little attraction towards the mPFC (Fig. 2G). Lack of 5-HTT had no
Figure 1  Three-dimentional collagen co-cultures of explants taken from the mPFC, DR and MnR show trophic responses. (A) Schematic of an embryonic brain showing the position of the 5-HT-positive rostral cluster of raphe nuclei projecting to the mid- and forebrain. The dorsal raphe nucleus (DR) projects to forebrain regions (green arrow) including the prefrontal cortex (mPFC). The median raphe nucleus (MnR) projects (blue arrows) to fore- and midbrain regions. (B) Enlargement of the boxed area in (A). The rostral (R) and intermediate (I) subarea correspond to the DR and the caudal (C) subarea corresponds to the MnR. (C) Example of a 5-HTT+/− caudal subarea (MnR) co-cultured with mPFC, divided in proximal and distal quadrants and stained for 5-HT (5-HT neurites, green) and Tuj1 (β-III tubulin, all outgrowing neurites, red). (D) In the proximal (and distal, not shown) quadrants the neurites are traced and measured. (E) Control explants were cultured separately and neurite outgrowth was measured in the 4 quadrants (example of WT mPFC). (F) The average length of the neurites in quadrants A, B, C or D showed no significant (NS) difference. HB, hindbrain; MB, midbrain. Scale bar represents 80 μm.

The targeted neurite outgrowth from the DR (Fig. 2G). The P/D ratios of the 5-HT neurite outgrowth of the MnR (caudal subarea) showed significant differences due to 5-HTT deficiency (Fig. 2A, B and G). In the wild-type situation a repulsive interaction towards the mPFC was observed (P/D ratio, 0.53; Fig. 2A, G). However, the reduction or lack of the 5-HTT caused a significant attractive interaction (P/D ratios, 1.52 and 1.20 respectively (resp.); p = 0.0018 and 0.033 resp.; Fig. 2B,G). Since the fibers are no longer repulsed, we can speculate that an increased number of 5-HT fibers may now target the mPFC. The observed switch to an attractive interaction was not found in the overall neurite outgrowth (Tuj1) from the MnR, here the interaction remained repulsive (P/D ratios, 0.59, 0.49, 0.47 for HTT+/+, 5-HTT+/− and 5-HTT−/− resp.; Fig. 2H). In the wild-type situation, the overall neurite outgrowth of the DR (rostral and intermediate explants) seemed to be slightly attracted by the mPFC (P/D ratio, 1.10 and 1.01; Fig. 2C,H). However, the 5-HTT+/− situation resulted in a switch to significant repulsion, although with the complete lack of 5-HTT this repulsion became less obvious (P/D ratios, 0.57 and 0.74, resp.; p = 0.012 and 0.022 resp.), especially in the more caudal
(intermediate subarea) part of the DR (P/D ratios, 0.58 and 0.94, resp.; \( p = 0.022 \) between HTT\(^{+/+} \) and 5-HTT\(^{+/+} \); Fig. 2D,H).

Fig 2I shows the P/D ratios of overall neurite outgrowth from the mPFC which was co-cultured with either the rostral, intermediate or caudal subarea of the raphe. The mPFC neurite outgrowth in the wild-type and 5-HTT\(^{-/+} \) situation was attractive towards all subareas of the rostral raphe (P/D ratios, 1.19, 1.01 and 1.29 resp.; Fig. 2E, F and I). However, for the 5-HTT\(^{-/+} \) explants this interaction of the mPFC with all subareas of the rostral raphe switched to repulsive (P/D ratios, 0.81, 0.72 and 0.58 resp.). This phenomenon was most prominent when the mPFC was co-cultured with the MnR (caudal subarea) as shown in Fig. 2E,F and I (\( p = 0.017 \) between HTT\(^{+/+} \) and 5-HTT\(^{-/+} \)). In absence of the 5-HTT, the P/D ratios were comparable with the wild type situation (P/D ratios, 1.23, 1.06 and 1.22 resp.; Fig. 2I).

Taken together, these data show that the outgrowing neurites of the DR/MnR and from the mPFC, show directional responses when cultured together. The nature of this response is different for DR compared to MnR, and is affected by the level of 5-HTT expression.

**Presence of 5-HTT during early development moderates fasciculation of outgrowing mPFC neurites towards rostral raphe.**

During the formation of neuronal projection systems, outgrowing axons are guided to their distant targets by a variety of guidance cues (67, 68). Most axons grow alongside other axons for much of their lengths as pioneer axons create the first scaffold for the different projection pathways. Subsequent axons may associate in specific bundles or fascicles, and grow alongside this scaffold in order to reach their proper targets (69, 70). The process of fasciculation of axons requires internal membrane-bound cues, such as members of the neuronal cell adhesion molecule (NCAM) or semaphorin family (71). However, soluble guidance cues secreted from (intermediate) targets also modulate fasciculation (70).

When examining the outgrowing neurites of the explants from the various subareas and genotypes, we noticed differences in the number of outgrowing fascicles, especially from the mPFC (Fig. 3; Suppl. Fig. 2A). Therefore, we first determined in how many of the explants (proximal quadrant) of the different subareas (both raphe as well as mPFC), fascicles with a minimum width of 5 \( \mu \)m, were formed (Suppl. Fig. 2C-E). It became obvious that most of the mPFC explants exhibited fasciculation. Considering exclusively the 5-HT-positive neurites, no fascicles were formed in explants either from the DR or the MnR (Suppl. Fig. 2C-F). Lack of 5-HTT did not affect this deficient 5-HT fasciculation. However, in some cases, the 5-HT-positive neurites did grow alongside TuJ1-positive fascicles (Suppl. Fig. 1D). In most explants of the mPFC, fascicles were formed, although differences were found when co-cultured with the rostral raphe subareas and across genotypes (Suppl. Fig. 2C-E). For example, the percentage of mPFC explants co-cultured with the rostral subarea (DR) showing fascicles was increased in 5-HTT\(^{+/+} \) and 5-HTT\(^{-/+} \) as compared to the 5-HTT\(^{+/+} \) mPFC (Suppl. Fig. 2C). All mPFC explants of wild-type and 5-HTT\(^{-/+} \) animals co-cultured with the intermediate (DR) and caudal subarea (MnR) had formed fascicles. Notably, the 5-HTT\(^{-/+} \) situation resulted in a reduced number of mPFC explants with fascicles co-cultured with the intermediate (DR) and caudal subarea (MnR) (Suppl. Fig. 2D,E).

Furthermore, we noticed that the number of fascicles formed per explant varied and depended on the genotype. We therefore quantified the average number of fascicles per mPFC explant. Fig. 3A shows the number of fascicles in wild-type (upper panels) as compared to 5-HTT\(^{+/+} \) (middle panels) and 5-HTT\(^{-/+} \) (lower panels) in the proximal quadrants of mPFC explants co-cultured with either the
Figure 2. The chemotrophic nature of the interaction between the mPFC and the DR and MnR depends on 5-HTT during development. (A-F) High magnification photographs of proximal and distal quadrants of the raphe (positive for 5-HT, green and TuJ1, red) and the mPFC (TuJ1, red). Arrowhead in schematic representations indicate the example given. (G, H) Quantification of the length of 5-HT-positive and TuJ1-positive neurites in the proximal and distal quadrants of the DR and the MnR, co-cultured with mPFC. Graphs show average P/D ratios ± SEM. (I) Quantification of the length of TuJ1-positive neurites in the proximal and distal quadrants of the mPFC co-cultured with either the DR or MnR. Graph shows average P/D ratios ± SEM. One-way ANOVA (α=0.05), *p<0.05, **p<0.01. Grey oval in G-I indicate the example given on the left. Dist, distal quadrant; DR, dorsal raphe nucleus; mPFC, medial prefrontal cortex; MnR, median raphe nucleus; Prox, proximal quadrant.

DR (left panels) or the MnR (right panels). Lack of 5-HTT resulted in a significant increase in the number of fascicles in the proximal quadrant of the mPFC co-cultured with the rostral subarea (DR) of the raphe (average number is 3.33, 6.25 and 7.33 fascicles for HTT+/+, 5-HTT+/- and 5-HTT-/- resp.; p
= 0.023; Fig. 3A-D, left) and a significant decrease when co-cultured with the caudal subarea (MnR, Fig. 3A,D, right; average number is 9.50, 4.33 and 2.67 fascicles for HTT+/+, 5-HTT+/− and 5-HTT−/− resp.; p = 0.022). An increase in the number of fascicles per mPFC explant was also observed in the distal quadrant of the mPFC that was co-cultured with the rostral part of the DR upon (partial) lack of the 5-HTT (average number is 3.40, 4.40 and 5.40 fascicles for HTT+/+, 5-HTT+/− and 5-HTT−/− resp.; p = 0.036; Fig. 3B and data not shown).

The length and width of a fascicle may provide information about the number of neurites bundled together, the nature of internal membrane-bound cues, and the nature of (soluble) environmental cues. For example, a repulsive interaction between axon and environment may favor fasciculation by channeling axons in a common path (69, 70). To measure the length and width, we traced along and across the fascicle, resp. (Suppl. Fig. 2B). We found differences in fascicle length growing from the mPFC when co-cultured with either the DR or the MnR, which depended on the genotype. Although not significant in the proximal quadrant of the mPFC, Fig. 4A-D shows that in the distal quadrant of a wild-type or 5-HTT+/− mPFC co-cultured with DR significantly longer fascicles were formed compared to the 5-HTT deficient situation (average length of fascicles, 286.3, 234.8 and 137.3 µm for HTT+/+, 5-HTT+/− and 5-HTT−/− resp.; p = 0.0097 and 0.0012 resp. for the rostral subarea and the average length of fascicles, 279.4, 221.9 and 195.5 µm for HTT+/+, 5-HTT+/− and 5-HTT−/− resp.;

![Figure 3](image_url)

**Figure 3**  **The amount of 5-HTT during development influences mPFC fasciculation.** (A) Examples of differences in number of fascicles per explant in WT, 5-HTT+/+ and 5-HTT−/− mPFC (proximal quadrant) co-cultured with the rostral (DR, left panels) or caudal subarea (MnR, right panels). Scale bar represents 50 µm. (B-D) Quantification of the number of mPFC fascicles (>5µm) per explant, for the different subareas and genotypes in both the proximal (prox) and distal (dist) quadrant. Grey ovals in G-I indicate the example given on the left. Graphs show average number of fascicles (>5µm) per explant ± SEM. One-way ANOVA (α=0.05), *p<0.05.
Figure 4  Differences in length of fascicles from the mPFC controlled by the presence of 5-HTT during development. (A,B) Example of differences in fascicle length in the distal quadrant of 5-HTT+/+ and 5-HTT-/- mPFC co-cultured with the rostral subarea. Scale bar represents 100 μm. (C-E) Quantification of the length of fascicles in the proximal and distal quadrants of a subarea cocultured with mPFC. Graphs show average length of fascicles per explant ± SEM. One-way ANOVA (α=0.05), *p<0.05, **p<0.01.

Small differences between DR and MnR were observed as well. For example, when comparing the distal quadrant of the rostral subarea of the DR (Fig. 4C) with the MnR (Fig. 4E) immunostained for β-III tubulin (all outgrowing neurites), the average fascicle length of fascicles from the MnR was longer.

Quantification of the average fascicle width among the various subareas and genotypes showed little significant differences, except for the proximal quadrant of the mPFC co-cultured with the rostral subarea of the DR where the width of the fascicles was significantly lower in the 5-HTT-/- compared to fascicles of 5-HTT+/+ explants (average fascicle width is 12.03, 14.61 and 9.65 μm for 5-HTT+/+, 5-HTT-/- and 5-HTT-/- resp.; p = 0.034; Suppl. Fig. 3C). Suppl. Fig. 3A and B illustrates the fascicle width of mPFC co-cultured with the MnR in 5-HTT-/- compared to wild-type explants which was increased, although not significantly. Taken together these data suggest that there are
differences in the formation of fascicles by outgrowing neurites from the DR/MnR and from the mPFC, which seems to be affected by the (partial) lack of 5-HTT.

Figure 5  Serotonergic innervation of the mPFC is increased in absence of the 5-HTT during development. (A) Schematic showing the level of coronal sectioning (dotted line) within the mPFC comprised of the Infralimbic (IL), Prelimbic (PL), and Cingulate cortex (Cg). (B) Camera lucida drawings of 5-HT-positive fibers within the IL of 5-HTT+/+ (WT) and 5-HTT−/− mPFC. Inset shows 5-HT fiber density (green) in the deeper layers of the mPFC. (C) Camera lucida drawings of 5-HT-positive fibers within the PL of 5-HTT+/+ and 5-HTT−/− mPFC. Inset shows 5-HT fiber density (green) in the deeper layers of the mPFC. (D) Camera lucida drawings of 5-HT-positive fibers within the Cg of 5-HTT+/+ and 5-HTT−/− mPFC. Inset shows 5-HT fiber density (green) in the superficial layers of the mPFC. (E) Quantification of the total 5-HT fiber length in µm in 10 bins dividing the IL as indicated in (B) confirming the qualitative observations as seen in (B). (F) Quantification of the total 5-HT fiber length in µm in 10 bins dividing the PL as indicated in (C) confirming the qualitative observations as seen in (C). (G) Quantification of the total 5-HT fiber length in µm in 10 bins dividing the Cg as indicated in (D) confirming the qualitative observations as seen in (D). There is a significant increase in the most deep cortical layers of the IL, PL as well as the Cg of 5-HTT−/− compared to 5-HTT+/+ pups (p < 0.05-0.001). The grey boxes indicate the non-significant bins in the more superficial layers. Graphs show average length of 5-HT-positive fibers per bin ± SEM. One-way ANOVA (α=0.05), *p<0.05, **p< 0.01, ***p<0.001.
**Figure 6**  Projection neuron identity is altered in absence of the 5-HTT during development. (A) Coronal sections immunostained for 5-HT (green), Satb2 (red) and counterstained with fluorescent Nissl (blue) of 5-HTT+/+ and 5-HTT−/− IL showing the position of callosal projection neurons (presumptive layer II-V). (B) Coronal sections of the PL as in (A). (C) Coronal sections of the Cg as in (A). Arrowheads indicate clusters of misplaced neurons. (D) Quantification of the number of Satb2-positive cells in 10 bins dividing the IL confirming the qualitative observations as seen in (A). (E) Quantification of the number of Satb2-positive cells in 10 bins dividing the PL confirming the qualitative observations as seen in (B). (F) Quantification of the number of Satb2-positive cells in 10 bins dividing the Cg confirming the qualitative observations as seen in (C). There is a significant decrease in the number of Satb2-positive cells in the IL, PL and Cg of 5-HTT−/− compared to 5-HTT+/+ (p < 0.05-0.001). Graphs show average number of Satb2-positive cells per bin ± SEM. One-way ANOVA (α=0.05), *p<0.05, **p<0.01, ***p<0.001.

Serotonergic innervation of various subareas of the mPFC is modulated by 5-HTT expression during development

The ability of 5-HTT to regulate 5-HT levels during development (1, 48, 72) together with the different directional responses of both raphe- as well as mPFC-derived projections raises the possibility that 5-HT and its signaling molecules can play an important role in axon guidance events.
navigating 5-HT axons to their forebrain targets. Therefore, we studied 5-HT innervation of the mPFC in vivo in 6 days old 5-HTT⁻/⁻ pups (P6) and compared that to wild-type innervation. Coronal sections of P6 5-HTT⁻/⁻ rats (n=3) and wild-type littermate controls (n=4) were stained for 5-HT to visualize raphe-derived projections and the length of the 5-HT innervation within the various subareas of the mPFC was measured. The length of the projections was quantified in 10 bins comprising the cerebral grey matter width (Fig. 5). To better visualize 5-HT-positive fibers over the cerebral swatch containing the grey matter which included the deeper and most superficial cortical layers, we made camera lucida drawings (Fig. 5B-D). In 5-HTT⁻/⁻ rats compared to control littermates, the drawings showed a clear increase in the amount of prefrontal 5-HT innervation in all subareas (Fig. 5B-D). The average 5-HT-positive fiber length in the 5-HTT⁻/⁻ mPFC was higher as compared to the wild-type mPFC in every bin, except for bin 8 (IL) and bin 8 and 9 (PL), likely to represent layers II and III (Fig. 5B,C,E,F). Remarkably, the cingulate mPFC showed a higher 5-HT fiber density in the deeper layers in absence of 5-HTT, whereas reduced levels were found in the more superficial layers (Fig. 5D,G), suggesting another raphe-derived route of the developing 5-HT projections.

Overall, these results indicate a crucial developmental role for 5-HTT in the guidance of 5-HT projections to their targets in the mPFC.

**Figure 7** Proposed model of the effect of 5-HTT deficiency on DR and MnR serotonergic neurite outgrowth during development. Cg, cingulate cortex; DR, dorsal raphe nucleus; HB, hindbrain; IL, infralimbic; MB, midbrain; MnR, median raphe nucleus; PL, prelimbic.

**Absence of 5-HTT during development alters the identity of prefrontal projection neurons**

Cortical neurons migrate to the proper location within the cortical plate (CP) through cell-autonomous and non-autonomous mechanisms (54, 73, 74). To assess whether 5-HTT, either directly or indirectly, influences the identity and/or migration of prefrontal cortical neurons, we performed immunocytochemistry for both 5-HT and Satb2, a marker for upper-layer neurons (75-77). Although the total number of neurons within the different subareas of the mPFC did not differ, we observed a remarkable reduction of Satb2-positive cells in all subareas of the mPFC in 5-HTT⁻/⁻ animals (n=3) as compared to their wild-type littermates (n=4)(Suppl. Fig. 4). Surprisingly, Satb2-positive cells were no longer homogeneously restricted to layers II-VI, where a small percentage was scattered over the cerebral swatch in 5-HTT⁻/⁻ as compared to control mPFC (Fig. 6A-F). Furthermore,
it appeared that in the prefrontal but especially in the cingulate cortex of 5-HTT<sup>−/−</sup> animals the Satb2-positive cells were often positioned in patches (Fig. 6B,C, arrowheads). It remains to be established whether this reduction of Satb2-positive cells in the mPFC of rats lacking 5-HTT is cell-autonomous or due to increased 5-HT innervation, or both.

In sum, these results indicate that appropriate 5-HTT levels during early brain development are important for proper maturation of the raphe-prefrontal projection system (Fig. 7).

Discussion
Our data show that the 5-HTT knockout rat represents an excellent model to investigate the role of 5-HTT in the development of the rostral raphe-prefrontal network formation. The present study evaluates the trophic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system and how this interaction is modulated by the lack of the 5-HTT during development. Furthermore, we observed the ability of outgrowing neurites originating from the DR or MnR and mPFC to form fascicles, and once formed; we quantified the number, length and width of the fascicles to evaluate the effect of 5-HTT expression during outgrowth. The nature of the interaction appears to depend on 1) the origin of 5-HTT-positive projections within the rostral raphe cluster and 2) the presence of 5-HTT during development. In wild-type explants, the 5-HT fibers of the DR have a slightly attractive interaction with the mPFC, although not significant, while the 5-HT neurites of the MnR are repulsed by the mPFC. The most striking finding of this study shows that the 5-HTT projections from the MnR become strongly attracted by the mPFC instead of being repelled in the absence of 5-HTT during development. In vivo, this is paralleled by the fact that in the 5-HTT<sup>−/−</sup> mPFC the 5-HT innervation was significantly increased as compared to the wild-type situation. In addition we show that the number of Satb2-positive callosal projection neurons is reduced in absence of the 5-HTT. Together, these results lead us to hypothesize, as depicted in Fig.7, that due to lack of 5-HTT throughout development 1) the characteristics of the raphe neurons might have changed, 2) raphe neurons can have an altered guidance of their outgrowing neurites as well as 3) the identity of the neurons within the mPFC, which send out projections in their turn, may have changed.

The role of 5-HTT in the development of the raphe nuclei
The 5-HTT is expressed in serotonergic neurons of the rostral raphe cluster as early as E11.5 (mouse) and E12.5 (rat) but also in non-serotonergic fibers such as thalamocortical projections (12, 13, 48, 78-83). One possibility to explain the observed results is that the characteristics of the 5-HTT neurons within the rostral raphe have changed because of altered 5-HTT expression. Indeed, in absence of the 5-HTT, the DR neurons are fewer in number which could have an effect on the organization of the MnR and its projections (84). The 5-HT<sub>1A</sub> of the DR neurons shows furthermore a marked desensitization when 5-HTT is lacking which could lead to functional consequences for the areas these DR axons innervate (85-88).

Three-dimensional collagen explant assays, are an excellent way to study interactions between areas within a particular network (42, 54, 89, 90). When cultured alone, the various explants of the raphe and mPFC showed radial growth, indicating optimal growth conditions for the outgrowing neurites. Furthermore, we found specific and consistent outgrowth read-outs demonstrating the validity of the assay. Yet, there are several points of discussion when using sensitive in vitro assays like the explant assay. Although it was not measured in this particular study, we can assume that
extracellular levels of 5-HT were elevated when the 5-HTT was absent (7, 25, 52, 91, 92). Even though the extrasynaptic concentration of 5-HT can reach the millimolar range (93), the concentration and/or clearance of the released 5-HT within the medium was not measured over time in this experiment which could have had an effect on the observed results. In addition, the fact that a reduced 5-HT reuptake results in a increased 5-HT synthesis has been well documented (92, 94). Although raphe explants from 5-HTT+/−/5-HTT−/− animals showed 5-HT-positive outgrowing neurites, it is to be investigated how much 5-HT, or other soluble cues, is actually secreted by these neurons in vitro.

**Axonal guidance of serotonergic projections**

The 5-HT projection system is one of the earliest neurotransmitter systems to innervate the brain, but the last to innervate the hippocampus and the cortex (61). During development, 5-HT may complete the maturation of a variety of neuronal projections systems, including its own, and the progression of interneuronal contacts (60, 95, 96). The 5-HT neurons of the DR and MnR are known to innervate various subareas of the cortex including the mPFC (26, 27, 97-99). While it cannot be excluded that the 5-HT raphe neurons are functionally different due to lack of 5-HTT, guidance of these and other (e.g. thalamocortical) projections might have been affected as well in absence of 5-HTT during development of the raphe-prefrontal network.

The observed interaction of the DR when co-cultured with the mPFC being neutral was unexpected since the DR is known to strongly innervate the cortical regions including the mPFC (60, 61). Lack of 5-HTT did not affect the chemotropic nature of this interaction. This may indicate that another target of the 5-HT fibers lies beyond the mPFC and may encounter the mPFC as an intermediate target or needs an older mPFC to become attracted. Another possibility can be that the growing 5-HT projections need intermediate targets along its projection (eg thalamic regions) which is first encountered before projecting towards the cortical areas. An interesting question would be to evaluate whether the responses of the outgrowing neurites would change when E16.5 raphe tissue would be co-cultured with older mPFC explants or with other (intermediate) targets such as thalamus or hippocampus.

Interestingly, the interaction of the 5-HT fibers of the MnR towards the mPFC switched from a fairly strong repulsive to an attractive interaction in the absence of 5-HTT. In vivo, this could result in an increased innervation of the mPFC by the MnR. MnR-derived varicose M-fibers, which are believed to not express 5-HTT (100), mainly target layers II and III in the frontal parts of the cortex (101), layers where we found the smallest differences in 5-HT innervation. In order to exclude the possibility that the switch of MnR neurites to attraction is due to an altered mPFC, it would be of interest to culture wild-type cortex together with DR/MnR from 5-HTT+/− or 5-HTT−/− animals.

Furthermore, 5-HT has been shown to have a modulatory effect on outgrowing axons by affecting their response to classical guidance cues such as netrin-1 (Bonnin et al., 2007). This means that in absence of the 5-HTT during development, the responses of the outgrowing neurites from either the raphe and/or the mPFC to guidance molecules may have altered. This would furthermore explain the in vivo results in that the 5-HT projections coming from the MnR are highly attracted by the mPFC when 5-HTT is lacking during development. It is plausible that because of the diminished expression of Satb2 (and maybe other transcription factors as well such as Ctip2), the expression of guidance cues is influenced. Indeed, it has been shown that the expression of a variety of axonal guidance molecules is amended in the absent expression of Satb2 (Alcamo et al., 2008). It would therefore be
of interest to look for aberrant guidance cue expression in the 5-HTT\(^{-}\)/ animals and co-culture either the various subareas of the rostral raphe cluster, intermediate targets or the mPFC with HEK cells secreting an axonal guidance cue and to see whether the responsiveness of the outgrowing neurites would change in the absence of 5-HTT and/or could be modulated by 5-HT.

It has been shown that 5-HT plays a major role in the plasticity of the various other projection systems by modulating the guidance of their projections (2, 102, 103). Since the overall outgrowth of neurites from the DR and MnR is affected by a (partial) lack of the 5-HTT, neurotransmitter systems other than the 5-HT system may be affected as well. Future experiments with 5-HTT\(^{-}\) three-dimensional explants of nuclei from other neurotransmitter systems such as the dopaminergic or noradrenergic system and their targets may give insight into such additional modulatory actions of 5-HT during development. While the total (Tuj1-positive) raphe and mPFC neurite outgrowth in the wild-type and 5-HTT\(^{-}\) situation appeared to be attractive by nature, this interaction becomes repulsive in the heterozygous animal. Perhaps, in vitro, either too high or too low concentrations of 5-HT permit an attractive response of both raphe as well as mPFC outgrowing neurites due to counteracting effects of different subtypes of 5-HT receptors expressed by the respective neurons (7, 33, 51, 102, 104-108), while intermediate 5-HT levels might elicit effects only via the more sensitive of the receptors producing a repulsive interaction. These results are intriguing considering that the s-allele carriers of the human 5-HTT polymorphism have a reduction in the amount of 5-HT but never a complete lack (109-112).

The function of 5-HTT in cortical integrity

A distant target of the ascending 5-HT projection system is the mPFC (113), which is involved in working memory and behavioral flexibility (29) and is also one of the last brain areas to mature (74). The 5-HTT is the primary regulator of the 5-HT signal and may therefore affect the role of 5-HT in correct development of the brain. The presence of 5-HTT itself within the developing mPFC already starts from E14.5 onwards with 5-HTT-positive cells and later fibers innervating the mPFC in two parallel paths contacting many other cells (e.g. Cajal-Retzius cells) important for its correct development and layering (12, 47). Lack of 5-HTT during development could therefore have profound effects on the integrity of the mPFC itself.

The transcription factor Satb2 (special AT-rich sequence binding protein 2) is a marker exclusively expressed by callosal projection neurons from E13.5 onwards and Satb2-positive cells reside in cortical layers II-V and in subsets of neurons in layer VI (75-77, 114). Satb2-positive callosal projection neurons were also present in the mPFC in which they most likely need selective 5-HT excitation to communicate (115). In absence of the 5-HT transporter, the number of Satb2-positive neurons in the mPFC was decreased in all cortical layers, suggesting an altered identity of a set of prefrontal callosal projection neurons. Interestingly, there are indications that SSRIs given for the treatment of depression have an effect on the anatomy of the corpus callosum (15, 116, 117). In absence of Satb2, many downstream targets (e.g. guidance cues) are either up- or down-regulated in expression which can have an effect on the identity of a class of mPFC neurons themselves and/or have an effect on incoming projection systems (77). Since the 5-HT fibers are thought to contact the Cajal-Retzius (CR) cells in the marginal zone (presumptive cortical layer I) of the mPFC ([30, 32] and unpublished data], an increase in axonal innervation could result in an altered 5-HT signal onto the CR cells. The subsequent altered reelin release could have profound effects on the cortical layering resulting in a modified mPFC-mediated behavioral control. Therefore, we cannot rule out the
possibility that during development, the migration of various classes of cortical projection neurons is also affected. To verify whether the callosal trajectory and/or identity of other projection neurons is affected in the absence of 5-HTT further experiments using various layer- and cell type-specific markers combined with electrophysiological recordings are needed.

The excessive 5-HT fibers seen in the 5-HTT<sup>−/−</sup> cortical plate can also have an effect on the maturity and dendritic arborization of the pyramidal neurons it harbors (Fig. 6 and (118), this topic). Therefore it is likely that subsets of mPFC projections neurons have an altered identity due to altered expression levels of transcription factors (such as Satb2) in absence of 5-HTT during development. This can have consequences for later innervations of either serotonergic or other transmitter projections because of an altered permissive environment of the mPFC.

During the formation of long projections systems, scaffolds are formed by pioneer axons. The following axons can now trail these scaffolds towards their proper targets and associate together in fascicles (69, 70). This is modulated by intrinsic membrane-bound cues and extrinsic diffusible cues. The number of mPFC explants of which neurites had formed fascicles was affected in the 5-HTT<sup>−/−</sup> animals. This could indicate that the altered 5-HT signal influences the response of the mPFC neurites to the intrinsic membrane-bound and soluble cues influencing fasciculation. Additionally, the altered 5-HT signal could alter the actual cues, and thereby influence fasciculation (43). Altered fasciculation in the brain could have profound effects. For example in the case of defasciculation, fibers would be less densely packed and spread out over a larger area, with possible projections into surrounding areas that normally are not innervated. For future experiments, co-culturing mPFC of the different genotypes with wild-type raphe would shed some light on the role of an altered mPFC due to absence of 5-HTT in the development of the raphe-prefrontal network.

Because of the altered developmental 5-HT levels, one may wonder whether the expression of particular 5-HT receptors within the mPFC is altered (7, 32, 33, 119), including 5-HT<sub>1A</sub> and 5-HT<sub>3A</sub> that are expressed by the CR cells themselves (30). The prefrontal pyramidal neurons express 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub> and 5-HT<sub>6</sub> receptors (7, 102, 105, 108) and by changing the level of extracellular 5-HT, the conveyed signals differ and can result in an altered cell identity. The prefrontal neurons can show altered characteristics because of altered expression of 5-HTT in the mPFC itself (cell autonomous effect) or, due to the increased 5-HT innervation in the mPFC of 5-HTT<sup>−/−</sup> animals (non-autonomous effect) this can lead to differences in cortical identity. For example, the amount of brain-derived-neurotrophic factor (BDNF) is dramatically decreased in the mPFC of animals lacking the 5-HTT (120, 121), thereby perhaps changing the permissiveness of the mPFC environment to incoming projections. Therefore, experiments involving manipulations of 5-HT levels in the culture medium of the explants using receptor agonists (eg Flesinoxan or mCPBG), antagonists (eg WAY 100635 or tropisetron) or 5-HT itself, may provide additional information about the role of the 5-HT signal and the regulation hereof by the 5-HTT on the 5-HT neurite targeting and interaction with the mPFC (32, 104, 106).

In sum, we conclude that the 5-HT projections arising from the rostral cluster of raphe and which innervate the mPFC seem to depend on the presence of 5-HTT during development. These results indicate that appropriate 5-HTT levels are required for proper 5-HT guidance, fasciculation and innervation of the mPFC and that appropriate 5-HTT levels are important for proper development of the raphe-prefrontal projection system (Fig. 7). Nevertheless, decoding the molecular program of 5-HT neurons and their projections towards the mPFC is a challenging task,
and additional research is necessary. Given that 5-HT$^{+/}$ animal models share many behavioral aspects with those seen in human 5-HTTLPR s-allele carriers, our data may help to understand the neurodevelopmental foundations of 5-HT associated behavioral phenotypes.

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Supplemental Figure 1  **Explant assays as a tool to study raphe-prefrontal network formation in vitro.** (A) Schematic representation showing the explant microdissection of the subareas of the rostral raphe cluster and the mPFC. The rostral raphe cluster was divided in a rostral (R), intermediate (I) and caudal (C) subarea and duplicated across the midline. Explants in a collagen hill were co-cultured at approximately 300 μm distance from each other. (B) Dorsal view of the dorsal raphe (DR) in a large explant showing 5-HT-positive neurons (green). (B’) Enlargement of the boxed area in B showing the midline (dashed line) and individual 5-HT neurons sending out their projections. (C) Coronal cryosection showing 5-HT-positive neurons (green) in both the DR and MnR costained with Satb2 (red) and counterstained with fluorescent Nissl (blue). (D) DR explant stained for 5-HT (green) and Tuj1 (red) showing healthy axonal growth cones (asterisk and arrowheads).
Supplemental Figure 2  Number of fascicles from the mPFC is moderated by the presence of 5-HTT during development. (A) Fascicles coming from the mPFC and immunostained for TuJ1 (β-III tubulin) with a minimum width of 5 μm (arrows) the length and width were measured. Scale bar represents 50 μm. (B) Enlargement of the boxed area in A. To measure the length and width, we traced along and across the fascicle. (C-E) The percentage of explants with fascicles measured in the proximal quadrant per subarea and per genotype. (F) Examples of the proximal quadrant of the rostral subarea (DR) of the genotypes (5-HTT+/+, 5-HTT+/− and 5-HTT−/−) immunostained for 5-HT (green). No fascicles were formed by 5-HT-positive neurites growing from the DR.
Supplemental Figure 3  *Width of fascicles from the mPFC is modulated by the presence of 5-HTT during development.*  

(A,B) Examples of fascicles with a certain width in the distal quadrant of the MnR (caudal subarea) of wild-type and 5-HTT<sup>−/−</sup> animals. Scale bar represents 20 µm.  

(C-E) Quantification of fascicle width for the different subareas and genotypes. Graphs show average length of fascicles per explant ± SEM. One-way ANOVA (α=0.05), *p < 0.05.
Supplemental Figure 4  Number of Satb2-positive neurons in the mPFC decrease in contrast to total number of cells in absence of 5-HTT during development. (A) Schematic representation of the various subareas of the mPFC as quantified in (B). (B) Quantification of the total number of cells (Nissl-positive) and the Satb2-positive cells in the various subareas of the mPFC over a swatch of 100 μm in width. Cg, cingulate cortex; IL, infralimbic; PL, prelimbic. Graphs show average number of cells per cortical swatch ± SEM. One-way ANOVA (α=0.05), *p<0.05, **p<0.01, ***p<0.001.
Perturbed mesoprefrontal targeting contributes to enhanced anxiety and altered social behavior in a mouse model of Parkinson’s disease

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Abstract

Parkinson’s disease (PD) is pathologically characterized by a selective loss of midbrain dopaminergic neurons. However, it remains unclear whether dopaminergic pathways other than the nigrostriatal pathway are perturbed in PD; they may account for the many non-motor features of PD, including depression, anxiety and cognitive decline. Here, we focused specifically on the role of Pitx3 in the maturation of the mesoprefrontal projection system and associated prefrontal cortex (PFC) function. Using a Pitx3-GFP knock-in mouse model, we document the aberrant development of the mesodiencephalic dopaminergic cluster and show, for the first time, clear defects in the migratory paths of dopaminergic neurons within the ventral tegmental area (VTA) of Pitx3-deficient mice. Furthermore, the mesoprefrontal dopaminergic innervation was affected and Pitx3 heterozygous mice displayed increased anxiety-like and altered social prefrontal-steered behavior. Together, our data establish that Pitx3 is associated with the correct maturation of the mesoprefrontal dopaminergic projection system and that loss of Pitx3 causes alterations in PFC-mediated behavior resembling some of the non-motor symptoms in PD.
Introduction

Parkinson’s disease (PD) is pathologically characterized by a selective loss of dopaminergic substantia nigra pars compacta (SNc) neurons and a subset of ventral tegmental area (VTA) neurons (1, 2). The clinical phenotype comprises motor symptoms, including bradykinesia, rigidity, tremor, and postural instability, as well as cognitive dysfunction that affect executive functions, attention, mood and working memory. As a consequence of the neurodegeneration, there is a massive reduction in the dopaminergic innervation of the dorsomedial striatum, which accounts for the deficits in motor behavior (3, 4). Besides this affected nigrostriatal pathway, it remains unsure whether other dopaminergic pathways, such as the mesocorticolimbic pathway that runs from the VTA to the ventromedial striatum and thalamic and cortical areas, are perturbed in PD as well. In addition, it is still unclear whether this selective vulnerability of dopaminergic neurons in PD emerges later in life, i.e. preceding pathology, or that is present much earlier, e.g. during development of the dopamine system, as in many animal models of PD (5).

Based on the clinical PD phenotype, and resemblance with other neuropsychiatric disorders, the mesocorticolimbic – and specifically the mesoprefrontal connectivity – is perturbed as well (3, 6-9). These aberrations might then account for the many non-motor features of PD, including depression, anxiety and cognitive decline (10-13). The prefrontal cortex controls many of our key cognitive functions and is formed by an intricate sequence of developmental events such as areal specification, proliferation of prefrontal progenitors, migration of prefrontal neurons to the proper cortical layers and differentiation (14, 15). Furthermore, the proper assembly of neuronal circuits requires correct positioning of GABAergic interneurons (16, 17) and guidance of afferents derived from various connected brain areas (18-21). As such, incoming dopaminergic axons from the VTA sculpt these developmental processes in the prefrontal cortex and other cortical areas (22-24). However, the underlying molecular mechanisms are not very well understood.

The bicoid-related paired-like homeodomain 3 (PITX3) is a member of the homeobox RIEG/PTIX family, located on chromosome 10q24.32, which functions as a transcription factor (25, 26) and is considered to be a key molecular player in the selective dopaminergic vulnerability in PD (27-30). Pitx3 is expressed in mesodiencephalic dopaminergic neurons of both the SNc as well as a subset of the VTA (31-34). Dopaminergic neurons of the SNc are specifically lost during embryonic development in Pitx3-deficient mice, resulting in a partial loss of motor control due to decreased dopamine levels in the dorsal striatum (34-36), resembling some symptoms of patients with PD (28-30, 37). Interestingly, a subset of VTA neurons are also affected in this mouse model and degenerate postnatally (34). Early observations indicated an altered neuronal morphology of dopaminergic neurons in the VTA of animals lacking Pitx3 (38), suggesting a distorted substrate for the development of dopaminergic projections to the ventromedial striatum, thalamic and cortical areas.

The present study focuses specifically on the role of Pitx3 in the development of VTA-derived mesoprefrontal projections and associated prefrontal cortex function. Using a validated Pitx3-GFP knock-in mouse model (39, 40), we document the aberrant development of the mesodiencephalic dopaminergic cluster and show, for the first time, clear defects in the migratory paths of dopaminergic neurons within the VTA in Pitx3-deficient mice. Furthermore, the mesoprefrontal dopaminergic innervation was reduced. To delineate the consequences of these neurodevelopmental changes on prefrontal-steered behavior, we subjected Pitx3 heterozygous mice to a battery of behavioral tests, and observed that Pitx3 heterozygous mice display increased anxiety-like behavior and altered social behavior, resembling some of the non-motor symptoms of PD (2, 11, 13). These findings further increase
our understanding of the variable vulnerability of SN versus VTA dopaminergic neurons, and the eventual consequences of its perturbed development and function in neurological disorders.

Materials and methods

Animals and tissue treatment
Wild type C57Bl6-Jico were obtained from Harlan Laboratories (The Netherlands), and Pitx3\(^{+/+}\), Pitx3\(^{GFP/+}\) and Pitx3\(^{GFP/GFP}\) mice on a C57Bl/6J background were either generously provided by Dr M. Smidt or bred locally (40, 41). In addition to animals that provided tissue specimens, 24 adult (7-12 months, median = 10 months old) male Pitx3\(^{+/+}\)\((n=12)\) and Pitx3\(^{GFP/+}\)\((n=12)\) were subjected to behavioral testing to assess whether prefrontal-dependent behavior is affected by a heterozygous loss of Pitx3 gene expression during brain development. These animals were group-housed (2-4 animals per cage) in a pathogen-free temperature-controlled (22°C ± 1) mouse facility on a reverse 12/12h light/dark cycle (lights on at 20:00h) with ad libitum access to food and water. An additional twenty-four naive adult (3 months old) male BALB/cByJ mice, obtained from Charles River (France) were used in the social interaction test. Housing conditions matched those of the Pitx3\(^{+/+}\) and Pitx3\(^{GFP/+}\) animals. All animal use and care were in accordance with institutional and European guidelines, and approved by the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands.

Tissue treatment
Timed-pregnant mice were sacrificed by means of cervical dislocation. The morning on which a vaginal plug was detected was considered embryonic day 0.5 (E0.5). Brains of E14.5 and E17.5 were carefully dissected, fixed in 4% PFA for a maximum of one hour, quickly washed in PBS and cryoprotected overnight in 30% sucrose in PBS at 4°C. Brains were frozen in M-1 embedding matrix (ThermoShandon) on dry ice and stored at -80°C. Coronal and sagittal cryostat sections were cut at 16 μm, mounted on Superfrost Plus slides (Menzel-Gläzer), air-dried, and stored desiccated at -20°C. Tail cuts were further processed for genotype PCR using both Pitx3\(^{GFP}\) spanning (FW: 5’-AGTTCGGTGCGGAGAGTAAG-3’, RV: 5’-TAGACACAGGGAGTTGTTGGG-3’) and individual primers (Pitx3 FW: 5’-CCCTCCGCTTCCAGAACATG-3’, Pitx3 RV: 5’-GTCCACACCGCCATCTTC-3’, GFP FW: 5’-CCTACGGCGTGCAGTGCTTCAGC-3’, GFP RV: 5’-CGGCGAGCTGACGCGTGCAGC-3’).

Quantitative RT-PCR
Total RNA from whole brains at various developmental ages (E10.5-P60) was extracted with TRI Reagent (Sigma Aldrich) and 1-2 μg total RNA was used for cDNA synthesis using the RevertAid H-minus first strand cDNA synthesis kit (Thermo scientific). The experiment was performed in triplicate with independent whole brain RNA pools derived from three non-related animals each. A 1:15 dilution of the cDNA pool was used in a 10 μl reaction for quantitative PCR (qPCR) analysis using the Sensifast SYBR no ROX qPCR kit (Bioline) and a Rotor-Gene\textsuperscript{TM} 6000 real-time analyzer (Qiagen, Hilden, Germany). The qPCR program used was (2 min. 95 °C, (10 sec 95 °C, 5 sec. 60 and 15 sec. 72 °C) x 40 cycles). Two intron-spanning primer sets were used: FW1: CGTGCGGGTGTGGTCAAG, RV1: TAGACACAGGGAGTTGTTGGG; FW2: GCTGTCGTTATCGGACGCA, RV2: AGGCCCTTCTCCGAGTGCTAGC. Peptidyl prolyl isomerase (PPIA) and β-actin were used as reference transcripts. qPCR data were analyzed by using comparative quantitation and the relative Q-values of the genes of interest calculated by equalizing the lowest Ct value to 1. The normalization factor for the reference genes was determined using the GeNORM program (medgen.ugent.be/genorm) and used to normalize the Q-values.
**Immunohistochemistry**

Cryosections were subjected to immunohistochemistry as described previously (22, 42). Sections were counterstained with fluorescent Nissl stain (NeuroTrace; Invitrogen; 1:500), washed extensively in PBS, and embedded in 90% glycerol in PBS. Staining was visualized using epifluorescent illumination on a Leica DMRA fluorescence microscope equipped with a DFC360FX camera and LASAF software or by confocal laser-scanning microscopy (Olympus FV1000) equipped with an argon/krypton laser, using a 63 x 1.4 NA oil objective. The primary antibodies used, their suppliers, and dilutions were as follows: rabbit anti-TH (Pel-Freez; 1:1000); rabbit anti-GFP (Molecular Probes; 1:3000); chicken anti-GFP (Abcam; 1:750). Species-specific secondary antibodies were either Alexa488 or Alexa555-conjugated (Molecular Probes; 1:500). The nomenclature to describe TH-positive cells and axons within different brain areas was applied as described previously by (22).

**Quantification and data analyses**

To quantify the total number of cells and the number of elongated nuclei in the VTA of Pitx3<sup>+/+</sup> and Pitx3<sup>GFP/GFP</sup> brains (4 embryos per genotype), two to four sections of the VTA at comparable anatomical positions were imaged and analyzed. The border defining the VTA area was based on TH expression and divided in three subareas; the rostral linear nucleus (RLi) combined with the interfascicular nucleus (IF), the parabrachial pigmented nucleus (PBP) and the paranigral nucleus (PN). Subsequently, the VTA was divided along the midline, generating two mirror duplicates per section. The nuclei were identified using TH and fluorescent Nissl staining. Data were averaged per genotype and expressed as means ± SEM and tested for significance by one-way ANOVA (α = 5%).

To assess the width, length and diameter of the individual fascicles of the medial forebrain bundle (MFB), images were captured of immunostained coronal cryosections of E17.5 Pitx3<sup>+/+</sup> and Pitx3<sup>GFP/GFP</sup> brains (4 per group). Subsequently, axon tract width was determined as described by (22) using NeuronJ software (NIH ImageJ, Bethesda, Maryland, USA). In brief, in each animal 2 to 3 well-spaced coronal sections were selected at the same anatomical level with the fasciculus retroflexus (fr) as a landmark in which the bilateral MFB was comparable. In each image, lines were drawn perpendicular to the direction of axon extension within the MFB at the location indicated in Figure 2. The average of the lines was designated as the width of the TH-positive MFB per embryo. To determine the length of the MFB, lines were drawn perpendicular on the lines of the width. To measure the diameter of axon fascicles in the MFB, the mean width of the 20 largest TH-positive axon fascicles in the MFB of Pitx3<sup>+/+</sup> and Pitx3<sup>GFP/GFP</sup> mice was measured. Data were tested for significance by one-way ANOVA (α = 5%) and expressed as means ± SEM.

For assessing TH-positive axon density in the medial PFC (mPFC) of Pitx3<sup>+/+</sup> and Pitx3<sup>GFP/GFP</sup> brains, three embryos per genotype were measured as described in (22). Two to three sections of the cingulate cortex (Cg), prelimbic cortex (PL) and the infralimbic cortex (IL) per animal were imaged and embryonic cortical zones were identified using blue fluorescent Nissl. A rectangle was placed over the center of the neuroanatomical locations spanning the whole cerebral thickness, and was divided into ten equal bins. TH-positive axon density was assessed within each bin by tracing the TH-positive fibers using NeuronJ (NIH ImageJ) software (Figure 3). The total fiber length per bin was averaged per genotype and tested for significance by one-way ANOVA (α = 5%) expressed as means ± SEM.

**Behavioral phenotyping**

All behavioral tests took place in the animals’ active phase (at least 4hrs after lights off). The animals were tested in several behavioral paradigms to assess differential aspects of prefrontal cortex function, including tests for long-term memory (43) (i.e., object recognition (44)), regulation of anxiety and vigilance (45) (i.e., light-dark transfer (46), elevated plus maze (EPM) (47), marble burying (48), and
acoustic startle (49), sensorimotor gating (i.e., pre-pulse inhibition (PPI) (50), and social behavior (51, 52) (i.e., the tube test of social dominance (53), and social interaction test (54)). A detailed description and protocol of each test can be found in the supplemental material.

Results

Pitx3 as a tool to study the selective vulnerability of dopaminergic neurons

The developmental transcription factor Pitx3 is expressed in SNc neurons and in a subset of VTA neurons and expression peaks around E13.5 (Supplemental Figure 1C). In order to investigate the role of Pitx3 in the differential vulnerability of SNc and VTA neurons and its impact on the development of mesoprefrontal dopaminergic innervation, we used the Pitx3-GFP knock-in mouse model (39, 40). In this model, the Pitx3 locus was targeted using an En2SAeGFP-IRES-P-HygroTK cassette as was previously described (40) (Supplemental Figure 1A). In order to compare Pitx3 expression in relation to TH in both the SNc and VTA, we examined the expression of the eGFP reporter in the developing brain of heterozygous Pitx3 mice. A ventral view of a E17.5 dissected brain showed Pitx3-directed GFP labeling within the dopaminergic midbrain, and clearly visible descending axonal projections to the striatum and basal forebrain through the medial forebrain bundle (MFB), confirming the correct targeting of the GFP reporter (Supplemental Figure 1D). As shown in Supplemental Figure 1E, the majority of GFP-positive cells within the SNc were labeled with TH, and a subset of VTA neurons were labeled as well.

VTA neurons differ in their morphology and migratory paths in the absence of Pitx3

To confirm the selective loss or absence of SNc neurons within the mesodiencephalic midbrain and determine possible differences in VTA development in absence of Pitx3, we examined the TH immunohistochemistry in E14.5 and E17.5 mouse brains. At E14.5, differences in the positioning of the TH-positive neurons could be observed in Pitx3-deficient (Pitx3<sup>GFP/GFP</sup>) embryos compared to wild-type (Pitx3<sup>+/+</sup>) controls. In wild-type embryos, TH-positive neurons migrated tangentially, subsequently forming the SN, whereas in Pitx3<sup>GFP/GFP</sup> these neurons were absent (Figure 1A). In the area closer to the midline in the vicinity of the ventricle, TH-positive neurons seemed to be morphologically different in Pitx3<sup>GFP/GFP</sup> animals. At E17.5, the absence of TH-positive neurons within the SNc was clearly visible within the Pitx3-deficient embryos (Figure 1B). However, within the VTA of the Pitx3<sup>GFP/GFP</sup> mice, TH-positive neurons seemed to be more packed. To examine whether these neurons within the dopaminergic midbrain were in another maturational state, we stained for the marker Tuj1 to identify immature neurons. Within the dorsal part of the VTA there was a higher level of Tuj1 in Pitx3<sup>GFP/GFP</sup> VTA as compared to Pitx3<sup>+/+</sup> VTA (Figure 1C), suggesting the Pitx3-deficient VTA neurons are within another developmental state.

Normally, nuclei of migrating neurons have an elongated shape (55-57). To explore whether the migration of progenitors destined for the VTA was affected, we counted the number of elongated nuclei. The percentage of elongated nuclei was drastically diminished within all aspects of the Pitx3-deficient VTA (Figure 1D, F), while the total number of nuclei was similar between Pitx3<sup>+/+</sup> and Pitx3<sup>GFP/GFP</sup> VTA (Figure 1E). Taken together, these data indicate that the maturational state and the migration of dopaminergic progenitors to the VTA subareas are severely affected by loss of Pitx3.
Figure 1. Loss of Pitx3 results in morphological and migrational defects of dopaminergic VTA neurons. (A) On the left, E14.5 coronal cryosections of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for TH (green) and counterstained with fluorescent Nissl (blue). On the right, enlargement of the dopaminergic area including the substantia nigra (SNc) and the ventral tegmental area (VTA). (B) On the left, E17.5 coronal cryosections of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for TH (green) and counterstained with fluorescent Nissl (blue). On the right, enlargement of the dopaminergic area including the SNc and the VTA. (C) Enlargements of the SNc/VTA area immunostained for TH (green), the immature neuron marker Tuj1 and counterstained with fluorescent Nissl (blue). (D) Left, schematic of the VTA subareas, IF, interfascicular subnucleus; PBP, parabracial nucleus; PN, paranigral nucleus; RLi, rostral linear nucleus. E17.5 coronal cryosections of the VTA subareas of Pitx3+/+ and Pitx3GFP/GFP brains stained with Nissl (white/grey). (E) Enlargement of the box in D showing the shape of the individual nuclei. (F) Quantification of the number of nuclei of Pitx3+/+ (n = 5) and Pitx3GFP/GFP (n = 5) animals. Graph represents average number ± SEM. One-way ANOVA (α = 0.05). (G) Quantification of the percentage of elongated nuclei of Pitx3+/+ (n = 4) and Pitx3GFP/GFP (n = 4) animals. Graph represents average percentage of nuclei ± SEM. One-way ANOVA (α = 0.05), ***P < 0.001.

Forebrain targeting through the medial forebrain bundle is perturbed when Pitx3 is absent

The rostral part of the VTA targets the striatum and prefrontal regions (22) while the more caudal aspects target the habenula thought the fasciculus retroflexus (fr) (58). As the developmental organization of the VTA seemed to be affected by the absence of Pitx3, axonal projections from the VTA to the forebrain regions in the MFB were assessed for changes. In E17.5 wild-type sagitally sectioned
mouse brains, the dopaminergic axons that target the forebrain is well defined. However, in Pitx3<sup>GFP/GFP</sup> brains this bundle of TH-positive fibers through the MFB was severely diminished. More specifically, in sagittal sections, the thick fascicles as observed in wild-type controls were absent and only thin fiber bundles and individual TH-positive fibers could be detected instead (Figure 2A). And, while in coronal sections the MFB appears as a triangular-shaped area with thick dopaminergic fascicles, Pitx3<sup>GFP/GFP</sup> MFB was less pronounced with thinner fascicles and more individually organized (instead of bundled) fibers (Figure 2C). Quantification of the width and length of the MFB and the diameter of the individual fascicles (Figure 2B) revealed an increase in width of the MFB in Pitx3<sup>GFP/GFP</sup> mice (Figure 2D), whereas the length seemed to be only slightly shorter, not reaching significance (Figure 2E). The diameter of the individual fascicles was significantly decreased in mutants compared to controls (Figure 2F), suggesting defasciculation of the dopaminergic fibers. Moreover, the rostrally oriented outgrowing neurites towards the forebrain regions were less compact and their extension seemed to be stalled along the way in the absence of Pitx3.

![Figure 2](image)

**Figure 2. MFB is perturbed in absence of Pitx3.** (A) E17.5 sagittal cryosections of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for TH (green) and counterstained with fluorescent Nissl (blue). Dashed line indicates the level of the coronal sections shown in (C). (B) Schematic showing a coronal view at the levels of the medial forebrain bundle (MFB) and the fasciculus retroflexus (fr). The MFB is enlarged showing the width, length and the diameter of the individual fascicles (FD). (C) Left, E17.5 coronal cryosections of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for TH (green) and counterstained with fluorescent Nissl (blue). Right, enlargement of the box on the left with the individual fascicles shown in green (see also inset). (D) Quantification of the width of the MFB of Pitx3+/+ (n = 4) and Pitx3GFP/GFP (n = 4) animals. Graph represents average width ± SEM. One-way ANOVA (α = 0.05), *P < 0.05. (E) Quantification of the length of the MFB of Pitx3+/+ (n = 4) and Pitx3GFP/GFP (n = 4) animals. Graph represents average length ± SEM. (F) Quantification of the average fascicle diameter (FD) of the individual MFB fascicles of Pitx3+/+ (n = 4) and Pitx3GFP/GFP (n = 4) animals. Graph represents average diameter ± SEM. One-way ANOVA (α = 0.05), *P < 0.05.
Figure 3. PFC dopaminergic innervation is perturbed in Pitx3GFP/GFP animals. (A) Schematic of the dopaminergic projection in a sagittal view of a developing brain (upper panel) and a coronal view of the mPFC region showing the subareas infralimbic (IL), prelimbic (PL) and the cingulate cortex (Cg). (B) E17.5 coronal cryosections of the PFC of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for TH (green) and counterstained with fluorescent Nissl (blue). (C) Cortical swatch of the IL immunostained for TH (grey/white) of Pitx3+/+ and Pitx3GFP/GFP brains divided into 10 bins. Dashed lines indicate the edge of ventricular (VZ) and marginal zone (MZ). (D) Cortical swatch of the PL immunostained for TH (grey/white) of Pitx3+/+ and Pitx3GFP/GFP brains divided into 10 bins. Dashed lines indicate the edge of the VZ and MZ. (E) Cortical swatch of the Cg immunostained for TH (grey/white) of Pitx3+/+ and Pitx3GFP/GFP brains divided into 10 bins. Dashed lines indicate the edge of the VZ and MZ. (F) Quantification of the fiber length per cortical bin within the IL of Pitx3+/+ (n = 5) and Pitx3GFP/GFP (n = 5) animals. Graph represents average fiber length per µm2 ± SEM. One-way ANOVA (α = 0.05), *P < 0.05; **P < 0.01. (G) Quantification of the fiber length per cortical bin within the PL of Pitx3+/+ (n = 5) and Pitx3GFP/GFP (n = 5) animals. Graph represents average fiber length per µm2 ± SEM. One-way ANOVA (α = 0.05), *P < 0.05; **P < 0.01. (H) Quantification of the fiber length per cortical bin within the Cg of Pitx3+/+ (n = 5) and Pitx3GFP/GFP (n = 5) animals. Graph represents average fiber length per µm2 ± SEM. One-way ANOVA (α = 0.05), *P < 0.05; **P < 0.01.
*Pitx3-deficiency leads to less dopaminergic innervation of the mPFC*

The defasciculation of the MFB in animals lacking Pitx3 supports the notion that the dopaminergic projections to the forebrain are hampered in their targeting. To test this hypothesis, we quantified the TH-positive fibers present in various regions of the mPFC in Pitx3<sup>GFP/GFP</sup> animals at E17.5 -- when dopaminergic projections are penetrating the cortical plate (CP) (22) -- to compare with Pitx3<sup>+/+</sup> controls (Figure 3A, B). At E17.5, in all prefrontal areas, i.e. the infralimbic (IL, Figure 3C), prelimbic (PL, Figure 3D) and cingulate cortex (Cg, Figure 3E), we observed a marked reduction in dopaminergic projections in Pitx3<sup>GFP/GFP</sup> animals in comparison to their wild type littermates. The total length of the dopaminergic fibers was significantly reduced within each area (Figures 3F, G and H), with the strongest effects in the higher bin number comprising the CP.

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**Figure 4.** Pitx3<sup>+/GFP</sup> mice display increased anxiety-like and compulsive behavior, but no differences in long-term memory and sensorimotor gating behavior. (A) The discrimination index (Y/(X+Y)) in the object recognition test, indicative of memory for a familiar vs. novel object, is not different between Pitx3<sup>+/GFP</sup> and Pitx3<sup>+/+</sup> mice. (B) Pitx3<sup>+/GFP</sup> mice tended to travel smaller distance in the lit compartment of the light-dark transition box (left), and entered the lit compartment significantly sooner. (C) Mutant animals spent less time and traveled smaller distance in the open arms of the elevated plus maze (EPM). (D) Mutant animals buried more marbles over time as compared to wildtype littermates. (E) Responses in the acoustic startle and prepulse inhibition (PPI) assay of Pitx3<sup>+/+</sup> and Pitx3<sup>+/GFP</sup> animals were not different. Graphs represent averages ± SEM. *P < 0.05; **P < 0.01.

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**Pitx3 heterozygous animals show impaired prefrontal-mediated behaviors**

To test whether the severely perturbed dopaminergic innervation of the PFC in Pitx3-deficient mice has behavioral consequences; we applied a battery of tests used for assessing PFC-mediated behaviors. As full Pitx3<sup>GFP/GFP</sup> mutants are blind, and show reduced spontaneous locomotion (34, 38) that could
interfere with our prefrontal assessment, we used heterozygous Pitx3+/GFP mice to compare with wild-type mice.

The loss of prefrontal dopaminergic innervation may imply potential effects on cognitive, emotional and social behavior (59-61) and relate to the co-morbidities observed in PD (62, 63). Several tests were used to address the effect of reduced presence of Pitx3 on these functions. To first assess the locomotor activity in a novel environment, we exposed the animals to an open field. In line with what has previously been found (64, 65), Pitx3 heterozygous mice were hardly hyper- or hypoactive (F(1,22) = 1.198, P = 0.286; Supplemental Figure 2).

Next, mice were subjected to the object recognition test assessing long-term memory function, known to be mediated by the PFC (66, 67). The discrimination index (discriminating the exploration of a novel vs. a familiar object) indicated no significant differences in memory performance between Pitx3+/GFP and Pitx3+/+ mice (F(1,22) < 1, Figure 4A). Next, since the PFC is a critical site for the regulation of anxiety and vigilance (6, 59), we tested for potential differences between genotypes in tests assessing anxiety-like behavior. Pitx3+/GFP mice entered the light compartment sooner (F(1,22) = 4.927, P = 0.038) in the dark-light transition test, indicative of a reduction in risk assessment (68), but tended to travel smaller distance in the lit compartment (F(1,20) = 3.891, P = 0.063; Figure 4B), suggesting increased anxiety levels. In the EPM, the Pitx3+/GFP mice also travelled a shorter distance (F(1,17) = 8.632, P = 0.009) and spent less time (F(1,19) = 4.448, P = 0.048) in the open arms (Figure 4C), confirming their anxious phenotype. Moreover, Pitx3+/GFP mice buried significantly more marbles at all time points tested compared to their wild type counterparts (main effect of genotype; F(1,22) = 9.053, P = 0.006, Figure 4D), consistent with a higher level of anxiety and compulsive behavior (69). No differences were observed in the acoustic startle reflex of the mice (Vmax; F(1,21) < 1) or in the PPI assessing sensorimotor gating (% PPI; F(1,21) < 1, Figure 4E).

Figure 5. Pitx3+/GFP mice display increased social dominance. (A) Photograph of the social interaction tube test. (B) Quantification of the percentage of won interactions within the tube. (C-F) Four of the main social interaction scores such as (C) aggressive behavior, (D) defensive behavior, (E) solitary behavior and (F) non-aggressive behavior. Graphs represent averages ± SEM. *** P = 0.001.
Social behavior is also, in part, directed by the PFC (70-72). Therefore, we also tested the social characteristics of the Pitx3<sup>+/GFP</sup> mice, and exposed them to the social interaction tube test (Figure 5A) to assess social dominance. A significantly higher percentage of social confrontations was won by the Pitx3<sup>+/GFP</sup> mice ($F_{1.21} = 14.261, P = 0.001$), suggestive of increased social dominance of Pitx3<sup>+/GFP</sup> mice. Lastly, to test a broader array of social behaviors in a less constraint environment, mice were also subjected to a social interaction test with an unfamiliar (non-aggressive) BALB/cByJ mouse. No clear differences were observed in the various types of social interactions between genotypes (i.e., aggressive, defensive, solitary or non-aggressive social behavior) (duration aggressive behavior; $F_{1.20} = 1.775, P = 0.198$, all other $F$'s $< 1$, Figure 5C-F).

Together, these data indicate that heterozygous Pitx3 animals show increased anxiety- and compulsive-like behaviors as well as social dominance.

**Discussion**

The selective vulnerability of dopamine neurons in PD is currently poorly understood (2, 27, 73, 74). Here, we are first to show that besides the well-described degeneration of dopaminergic SNc neurons also the dopaminergic VTA neurons projecting to the mPFC are distorted in a mouse model for PD, resulting in altered PFC-mediated behavior.

**The validity of the Pitx3 mouse model for PD**

Well-known clinical motor signs in PD include bradykinesia, rigidity, tremor, and postural instability (75, 76). PITX3 has been considered to be a key molecular player in PD and therefore a good candidate to functionally dissect in the development of the dopaminergic system (27-30, 77, 78) Previous studies on the (full) Pitx3 mouse mutant have reported on altered levels of spontaneous motor activity levels (34, 35, 64, 79), but there is still ongoing debate whether the mouse model can be considered hyper- or hypoactive. Additionally, the onset of the dopaminergic neurodegeneration underlying the clinical manifestations in patients is rather late, whereas in the Pitx3 mouse model, or any other PD genetic animal model for that matter, the cellular developmental defects appear to be much earlier. Yet, it remains to be determined whether there are already neurodevelopmental aberrations within the human brain before any clinical signs become overt.

For a long time, the heterozygous Pitx3<sup>+/GFP</sup> mice were considered to be normal on both cellular and behavioral levels (39, 79, 80). However, it has become apparent that this is not true, as they show increased sensitivity to neurodegenerative stress (32). In the present study, also changes in endophenotypic domains were demonstrated. As some of the behavioral tasks we applied required use of visual cues, we could not use Pitx3<sup>GFP/GFP</sup> mice, as they have no eye lens. Even though many of the genetic mouse models of PD, including the Pitx3 model, display the neurodegeneration within the dopaminergic midbrain, they have not been able to recapitulate all motor and non-motor symptoms in a progressive age-related way.

**Selective vulnerability of midbrain dopaminergic neurons**

Throughout the maturation and differentiation stage, there is a high level of diversity of midbrain dopamine neurons at the molecular, cellular level and functional level (81-83). Not only large differences exist between the dopamine neurons within the SNc and VTA but also within the VTA area itself there is a high level of heterogeneity in cellular phenotypes (36, 58, 84, 85), as highlighted by the existence of specific subareas (e.g. PBP, PN, RLi and IF). In contrast to previous reports stating that Pitx3-deficient VTA neurons are normal and not susceptible to neurodegeneration (34, 35, 86), it is now well established that a subset of dopaminergic VTA neurons lacking Pitx3 in rodents are morphologically different from...
their wildtype counterparts and will eventually degenerate postnatally (32, 38, 39, 79). Similarly, it is exactly this group of neurons comprising the SNc and a subpopulation of the VTA that degenerates in the progression of PD (1-3, 87, 88). In mice, Pitx3 is expressed within the midbrain from E11.5 onwards (31, 39). At this time, dopaminergic progenitors that are born at the ventricular zones migrate ventrally and laterally to form the various subgroups of dopaminergic neurons; the so-called fountain model (38, 89). They combinatorially express a plethora of intrinsic (e.g., transcription factors) as well as extrinsic factors (e.g., signaling molecules) in a spatially and temporally restricted manner (81, 83, 90-93). Genes that are regulated by Pitx3 include Th, Vmat2, Drd2, Slc6a3, Ahd2, Dlk, Cck, Vip, Bdnf, Gdnf, Nurr1 and En1/2 (81, 82, 94-98). Due to absence of Pitx3 during development this molecular program is likely altered, affecting the identity of dopaminergic progenitors and neurodevelopmental events such as the migratory trajectories of both SNc and VTA dopaminergic neurons. As SNc neurons are required to travel larger distances in the absence of Pitx3, these might be affected more severely than the later born VTA neurons, resulting in more pronounced effects on the initial migration, cell-cell interaction and the eventual survival of the dopaminergic neurons. The halted migration could result in incorrect positional information as these neurons do not reach their final position within the required time frame, which puts them at ‘the wrong place at the wrong time’. This could contribute to the impaired condition of these neurons, their aberrant projections and ultimately to the process of neuronal death. In the Pitx3<sup>GFP/GFP</sup> VTA neurons appeared to group together, as if cells were piling up on top of cells that were no longer migrating. This could indicate that the decrease in migrating cells is caused not only by the loss of Pitx3 but also by surrounding neurons. In the MFB, defasciculation of the TH positive fibers was seen. The dopaminergic innervation of the mPFC subareas by TH-positive fibers originating from the VTA was decreased due to loss of Pitx3. Further research on the birthdating, migration and radial scaffolding of developing dopaminergic neurons, and whether this has an effect on behavior, is needed to dissect this complex neurodevelopmental feature.

**VTA targets and co-morbidities of PD**

The VTA – including its subareas (IF, PBP, PN, CLI and RLi) – is a heterogeneous area that establishes connections with many distant targets such as PFC (22, 84, 99), but also medial and lateral aspects of the nucleus accumbens (99-101) and the habenulae (Hb) (58, 85, 99, 102). It therefore remains to be established whether the altered morphology and migratory paths of the neurons residing in the VTA of Pitx3-deficient mice also affect dopaminergic innervations of these distant targets. Because of the involvement of the PFC in many of the non-motor clinical signs, we here focused on the PFC and observed altered dopaminergic innervation within the IL, PL and Cg aspects of the PFC, most likely resulting in the prefrontal-mediated behavioral aberrations observed in the Pitx3 heterozygous mice. However, we cannot exclude the possibility that the behavioral differences observed in terms of anxiety/compulsivity/sociability of the heterozygous animals may be instructed by alterations elsewhere in the brain as well. Like serotonin, dopamine might be able to act as a neurodevelopmental signal instructing other neuronal cell types during development (103-106). It furthermore remains to be elucidated whether the eventual behavioral outcome is an effect of both the neurodegeneration of the dopaminergic midbrain as well as the misdevelopment of the dopaminergic system and its targets. As the clinical manifestations of PD are rather diverse and include non-motor symptoms such as depression, anxiety, apathy and cognitive decline (62, 63, 107), more brain areas are suspected to be involved as well. However, little is known about the full extent to which other brain areas are affected in PD. Neuromaging studies have shown that a rather extensive set of brain areas such as the caudate nucleus, limbic system, thalamus but also frontal areas such as the (m)PFC display aberrations in PD (108-111), many of which are target areas of the dopaminergic midbrain. As the PFC is involved in many behavioral functions, controlling stress and anxiety levels, mediating working memory, attention, conflict resolution and decision making (6, 14, 59, 67, 112, 113) and it a plausible neural substrate for
the PD non-motor symptoms. Some of these alterations of PFC-mediated behavior we also see in our Pitx3 heterozygous mice. Although one could question whether PITX3 is the best gene to study the mechanisms underlying PD (77, 114), genetic anomalies within the PITX3 locus or polymorphisms within PITX3 promoter elements are associated with an elevated risk of PD (28-30, 77, 78), making it an intriguing target for further investigation.

Conclusion
Together, our observations shed light on a previously unexplored area within PD research. Our study establishes the involvement of the affected mesoprefrontal projection pathway in the non-motor comorbid manifestations in PD. Yet, questions remain to what extent other brain areas are affected as well, highlighting the need for further studies to unravel the molecular underpinnings of maturation and maintenance of dopaminergic neurons in health and disease.

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Conflicts of interests
The authors declare no competing financial interests.
References


Supplemental material

Material and methods

Object recognition
To assess long-term memory function using the object recognition test, mice were first habituated to the test cage (day 1). The animals were placed in the corner of a lit (100-120 lux) white Plexiglas open field box (50 x 50 x 40 cm) to initiate a 10-min session. A camera was placed above to record the location of the animal to measure overall locomotion. The data were analyzed using Ethovision 9 XT (Noldus Information Technology, Wageningen, The Netherlands) to assess the total distance moved. On day 2, the animals were placed in the exact same box, but now with two identical objects (X) placed in opposite corners at approximately 10 cm distance from each wall. Each mouse was placed individually in a third corner, and was allowed to explore the objects during a 10-min training session. After an inter-trial interval (ITI) of 3 hours, the mouse was reintroduced in the set-up with one identical object (X) and the other (in counterbalanced position) replaced by a novel object (Y). In this test session, the mouse was again allowed to explore both objects for 10 min. Object X consisted of a grassed silver bowl (diameter 7.5 cm, height 4.5 cm) standing upside down and filled with marbles for counterweight, and object Y was a grey plastic tube with a lid on top (diameter bottom 4.5 cm, diameter top 6.5 cm, height 5.5 cm) filled with plaster bandage. The objects were available in triplicate and cleaned with water between sessions to avoid olfactory trails. Object exploration was defined as directing the nose to the object at a distance of less than 2 cm and/or touching the object with the nose, and manually scored by an experimenter blind to the genotype of the mouse. Exploration time of object X and Y was determined. The discrimination index was calculated using the formula Y/(X+Y) (44).

Dark-light transfer
General anxiety and risk assessment were measured using the dark-light transfer test, which makes use of the innate aversion of rodents to brightly illuminated areas and their spontaneous exploratory behavior. The test apparatus consisted of a box partitioned into two environments: a dark covered compartment (15 x 20 x 25 cm) and a brightly illuminated (1000–1500 lux) light compartment (30 x 25 x 25 cm). The compartments were connected by a small passage (6 cm in width) at the bottom center of the partition. The mice were placed in the dark compartment to initiate a 5 minutes test session. The animals were monitored using a camera mounted above the apparatus and data were analyzed by Ethovision 9 XT software (Noldus Information Technology). For the analyses, an arena of 3 cm lengthwise by 6 cm width-wise surrounding the passage to the light area was defined as risk assessment zone (68). Time spent in the risk assessment zone, the latency to enter the surrounding light zone, and the time spent and distance traveled in it were quantified.

Elevated plus maze
As a second test for anxiety-like behavior, probing the animals’ aversion to open spaces, the animals were tested in the EPM. The maze comprised a central area (5 x 5 cm), two opposing open arms (30.5 x 5 cm) and two opposing closed arms (30.5 x 5 x 15 cm), at a height of 53.5 cm, and was lit with ~5 lux. To initiate the 5 min test, the animals were placed in a closed arm facing the center. A camera was placed above the elevated plus maze to record the mice and the data were analyzed using Ethovision 9 XT software (Noldus Information Technology). Time spent in the open arms, distance travelled in the open arms, and total distance travelled, were analyzed.
Marble burying
To measure hypervigilance and compulsive behavior, marble burying behavior was assessed. Mice were placed in a compartment (30 × 27 × 26 cm) illuminated by ~5 lux containing 5 cm autoclaved bedding with 20 marbles centrally arranged 4 by 5. Mice were placed in the corner of the box and videotaped for 25 min (115). The number of marbles buried was assessed every 5 minutes until the end of the test by two independent raters, blind to the genotype of the mice. Averaged data were analyzed.

Acoustic startle and pre-pulse inhibition
Measures of acoustic startle (ASR) and pre-pulse inhibition (PPI) were obtained to assess hypervigilance and sensorimotor gating capabilities, respectively. A SR-LAB™ startle response system (San Diego Instruments) was used for doing so. A high precision sensor, integrated into the measuring platform, detected the movement of the animal during the trials. Inside the chamber were two high-frequency loudspeakers, which produced all the audio stimuli. The acoustic startle session consisted of seventy-two trials, and began with 5 min acclimation to white background noise (70 dB), maintained through the whole session. Twenty-four startle stimuli (120 dB, 40 ms in duration, with randomly varying inter-trial-intervals of 12-30 ms) were presented. These were presented in three time bins, where both the first time bin and the third time bin consisted of six of these trials in a row. The second time bin was interspersed with thirty-six additional startle stimuli, randomly preceded by prepulses of either 75 dB, 80 dB, or 85 dB, 40 ms in duration. Twelve additional trials did not contain any stimuli, and were used to measure background movement. Maximal ASR and latency to peak startle amplitude were measured both in response to individually presented startle stimuli and in response to startle stimuli preceded by pre-pulses. PPI was calculated as the percent difference between the maximal ASR to startle stimuli preceded by pre-pulses relative to that without, averaged over all pre-pulse intensities.

Tube test of social dominance
To measure social dominance, mice were tested in the tube test (adapted from (53)). The test employed a transparent Plexiglas tube 30 cm in length with a 3 cm inside diameter, which was sufficient to permit one adult mouse to pass through without reversing direction. To reduce novelty and ensure rapid entry and progress forward through the tube, mice were preconditioned to the tube in training sessions on two consecutive days. Each mouse was released at alternating ends of the tube to run through the tube, for four training trials per day. Mice that did not initially enter the tube were encouraged to run forward with a gentle pull of the tail. On the third and fourth day, the ‘match’ days, mice were given the test trials. Two unfamiliar mice of different genotypes (one Pitx3+/+ and one Pitx3^{GFP/+} (n = 12) were placed at the opposite ends of the tube, at alternating ends, and began to explore in a forward direction. A gate at the middle of the tube was only opened once both animals had entered the tube, and the two mice were allowed to approach each other. If one mouse was dominant and the other subordinate, the dominant mouse approached, while the subordinate backed away, quickly forcing the subordinate out of the tube. The matches were performed with a novel unfamiliar competitor each time for a total of eight times per mouse (four times on each match day). The percentage of wins was calculated from the total number of encounters per test subject.

Social interaction
To assess social behavior in a more natural setting, mice were tested in a social interaction test with an (unfamiliar) adult male BALB/cByJ mouse. The test mouse was first placed alone in a novel, neutral environment (Phenotyper 4500 box, 45 x 45 cm; Noldus Information technology), for exploration during a 15 min period. Then, the novel mouse was introduced into the cage, and behavior was recorded for 10 min (protocol adapted from (116)). Social investigation, including approaching, following, and social
nose contact (nose-to-nose sniffing, nose-to-anogenital sniffing), defensive behavior (either active avoidance of partner or freezing), aggression (threat, attack, bite, chase), and non-social behavior (such as self-grooming, cage exploration, digging and rearing) were analyzed. Videos were manually scored using Observer 11 XT software (Noldus Information Technology) by an experimenter blind to the genotype of the mouse.

Statistical analyses
Data were tested for significant effects of genotype by one-way ANOVAs ($\alpha = 5\%$) and expressed as means ± SEM. Marble burying results were analyzed using a repeated measures ANOVA, implementing time and genotype as within- and between-subject variables, respectively. Data points deviating >2 SDs from the average were considered outliers and excluded from analyses. Data on one animal ($Pitx3^{GFP/+}$) was removed from the analyses of the EPM, since this subject was hyperactive and qualified as an outlier on overall distance travelled.
Supplemental Figure 1. Validation of the Pitx3-GFP knock-in mouse model. (A) Schematic overview of the construction of the targeted Pitx3 allele. (B) Genotyping examples of the Pitx3GFP/GFP, Pitx3+/+ and Pitx3+/GFP as shown by PCR. (C, D) Ventral view of a dissected Pitx3+/GFP brain showing the GFP expression within the Pitx3 locus. L, lateral; MFB, medial forebrain bundle; OB, olfactory bulb; R, rostral; SNC, substantia nigra pars compacta; VTA, ventral tegmental area. (E) E17.5 coronal cryosection of Pitx3+/+ and Pitx3GFP/GFP brains immunostained for GFP (red), TH (green) and counterstained with fluorescent Nissl (blue). (E’-E’’) Enlargement of the box in (E) showing the VTA area stained for GFP (red, E’), TH (green, E’’) and GFP, TH and counterstained with fluorescent Nissl (E’’).
Supplemental Figure 2. Less Pitx3 shows no differences in overall locomotor behavior as assessed in the open-field test. Graphs represent averages ± SEM.
Deficiency of hypoxanthine guanine phosphoribosyl transferase affects early development of the dopamine system in a mouse model of Lesch-Nyhan disease

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Abstract

Lesch-Nyhan disease (LND) is caused by a mutation in the gene encoding the purine salvage enzyme hypoxanthine guanine phosphoribosyl transferase (HGprt) and associated with a loss of dopamine in the basal ganglia. Patients develop an incapacitating clinical phenotype dominated by dystonia, cognitive dysfunction and severe self-injurious behavior, usually within four years after birth. There is no cure and the mechanisms by which HGprt deficiency causes basal ganglia dopamine depletion remains elusive. Previous cell model studies have suggested that HGprt deficiency might affect the neural programming of early midbrain dopamine neurons. In this study, we aim to characterize the effects of HGprt loss on dopaminergic neuronal development in vivo in an HGprt knock-out mouse model. In E14.5 HGprt-deficient embryos, a cell-type specific, significant decrease in the total number of TH-positive (i.e. dopaminergic) cells was observed along the midline migration path, while they showed an increase in cell division. Furthermore, the cellular alignment was abnormal in the HGprt-deficient mice, and cells appeared to prematurely leave the midline migrational paths from ventral zone towards their final destinations. In addition, the distribution of specific subtypes of dopaminergic neurons was abnormal as well as the distribution of radial glia in the ventral midbrain. The abnormalities noted were not restricted to the dopaminergic population, as deeper cortical layering was also affected in the PFC, M1 and S1 of postnatal mice. These findings demonstrate that during embryogenesis HGprt deficiency in mice is associated with maldevelopment of the dopaminergic system that may involve abnormal proliferation, migration and neural programming. Moreover, the brain’s demand for HGprt function appears to increase markedly after birth, during the period that, in patients, the clinical symptoms arise.
Introduction

Lesch-Nyhan disease (LND) is an incapacitating disorder with a characteristic neurobehavioral syndrome, dominated by generalized dystonia, specific attentional and executive cognitive deficits and self-injurious behaviour (1-3). LND is caused by a mutation in HPRT1, the gene encoding the purine salvage enzyme hypoxanthine-guanine phosphoribosyl transferase (HGprt) (1, 2). The pathogenic mechanisms by which HGprt deficiency leads to the motor and behavioural abnormalities are incompletely understood. Available evidence from post-mortem analysis of human brain tissue (4, 5), in vivo PET imaging studies (6, 7) and experimental mouse (8-10) suggest that HGprt deficiency is associated with a rather selective decreased level of dopamine in the striatum. Dopamine is essential for proper functioning of many areas of the brain, including circuits that involve the basal ganglia and multiple cortical areas, contributing to functional circuits involved in motor, cognitive and motivational aspects of behaviour (11), all of which appear to be affected in LND (12).

Despite the striatal dopamine loss, no gross structural abnormalities can be observed in HGprt deficient human (3, 5) or mouse (10, 13) midbrain regions. The dopaminergic neurons are not altered in number by HGprt deficiency, but in humans they do show reduced melanisation and immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis (14). Microarray in vitro studies in HGprt-deficient cancer cell lines, have suggested that a disturbed expression of transcription factors involved in the development and differentiation of dopaminergic neurons may underlie the observed dopamine deficiency in patients (15). Results showed that HGprt deficiency or knock-down was associated with abnormal expression of transcription factors involved in dopaminergic specification (Lmx1a/b, Msx1), survival (En 1/2) as well as maturation and maintenance (Nurr1, Pitx3) of dopamine neurons. Moreover, these changes in intrinsic factors were accompanied by dysregulation of extrinsic factors encoding dopamine biosynthetic enzymes (TH, AADC, VMAT2) and abnormal neurite outgrowth (15, 16). Such disturbances of early developmental mechanisms due to HGprt-deficiency, i.e. where neurons do not fully develop their typical dopaminergic phenotype, without a morphological correlate may explain the apparent dopaminergic dysfunction, (13).

During normal development, the dopaminergic neurons in the ventral midbrain are organized into distinct and functionally characteristic cell groups including the ventral tegmental area (VTA) and substantia nigra (SN). This process involves proliferation, migration, and the molecular programming of the dopaminergic phenotype. Both VTA and SN dopaminergic neurons are derived from a common population of progenitor cells localized in the ventricular zone of the ventral midbrain. Proliferating progenitor cells will eventually exit the cell cycle, and migrate to their ultimate destinations according to the inverted fountain model. The entire population initially migrates in the midline from their birthplace to the mantle layer, following a route stipulated by the radial glial scaffold and guidance cues (17-20). Subsequently, the neurons forming the SN will undergo active tangential migration to settle in their more lateral position (17, 18, 21).

Despite their innovative value, the in vitro cell models used previously only partially recapitulate LND, as they are not exposed to the many other regulatory factors in the intact developing brain and lack the normal molecular programming displayed by innate dopaminergic neurons. In the present study, we evaluated the effects of HGprt deficiency in vivo on the proliferation and migration patterns of developing midbrain dopaminergic neurons in a mouse model of LND. In addition to increased numbers of cell divisions, we demonstrate that the radial glia scaffold that facilitates the migration as well as the distribution of a presumptive SN neuron marker is affected by HGprt deficiency. Together, these aberrations may underlie the abnormal organization of the dopaminergic system in LND.
Material and Methods

Animals and tissue preparation
HGprt+ and congenic HGprt- mutant mice, bred on a C57BL/6J background (C57BL/6JHPRT\textsuperscript{B6G}), were used in these studies. As the HGprt gene is X-linked, HGprt- male animals and HGprt+ littermate controls were generated by mating wildtype males with heterozygous females. All animal use, care and experimental procedures were performed in accordance with the institutional and national and European ethical guidelines and regulations and with the approval of the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands. The mice were housed in filter top macrolon cages in a temperature- and humidity-controlled room (21 ± 1 °C and 60% relative humidity, respectively) and had ad libitum access to food and water and a normal light-dark cycle was maintained. The day of the vaginal plug was considered embryonic day (E) 0.5 and the day of birth as postnatal day (P) 0. Timed pregnant mice were sacrificed by means of cervical dislocation. The embryos were dissected in ice-cold Leibovitz medium (L-15, PAA). Embryonic heads (E12-E14) and embryonic brains (E18.5) were fixed by submersion in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (wt/vol), pH 7.4, at 4°C for 1 - 1.5 hours (hrs) maximum. After a quick wash in PBS, brains were cryoprotected overnight in 30% sucrose (wt/vol) in PBS. Then, brains were frozen in M-1 embedding matrix (Thermo Fisher Scientific) in a plastic cup on dry ice and stored at -80°C. Cryostat sections were cut at 16 μm, mounted on SuperFrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -20°C. For qPCR analysis, brains and dissected brain areas were quickly frozen in liquid nitrogen and stored in -80°C before RNA was isolated.

Gender and HGprt genotype of the animals was identified by molecular confirmation of the mutation by polymerase chain reaction (PCR) applied to a DNA sample isolated from a tail clip (embryos) or ear punch (postnatal animals). Primer sequences can be found in Supplementary Table 1

Proliferation and migration assays
To assess proliferation and migration at multiple timepoints, timed pregnant mothers received one of two treatments. Treatment 1: the pregnant mothers received an intraperitoneal (i.p.) injection of 5-bromo-2'-deoxyuridine (BrdU, BD biosciences, 50ug/g) at E12.5, and were sacrificed at E14.5. Treatment 2: i.p. injection of 5-ethynyl-2'-deoxyuridine (EdU, Click-iT EdU Alexa Fluor 555 Molecular Probes, 30ug/g) at E16.5 and were sacrificed at E18.5. Embryonic heads or brains were dissected, fixed and cryosectioned as explained in ‘Animals and tissue preparation’.

Immunohistochemistry
Immunohistochemistry was performed as described before (22). Cryosections were rehydrated in PBS (0.08 M Na\textsubscript{2}HPO\textsubscript{4}, 1.36 M NaCl, 0.017 M KH\textsubscript{2}PO\textsubscript{4}, 0.026 M KCl). Sections were incubated in a blocking buffer (BB) solution (1.6% normal goat serum, 1.6% normal donkey serum, 1.6% normal horse serum, 1% BSA, 0.1% glycine, 0.1% lysine, 0.4% Triton X-100; BB) for 1hr at room temperature (RT). Primary antibodies, listed in Supplementary Table 2, were diluted in BB and incubated overnight at 4°C. Slides were washed three times in PBS for a total of 30 min at RT. Sections were incubated with species-specific Alexa-conjugated secondary antibodies (Molecular Probes, Thermofisher) diluted in BB for 1hr at RT. After three washes in PBS for a total of 30 min at RT, sections were incubated with blue fluorescent Nissl stain (NeuroTrace, Invitrogen; 1:500) for 15 min and washed three times in PBS; 10 min each at RT. Slides were mounted in 90% glycerol in PBS.

For a BrdU staining, cryosections were incubated with BB for 1hr at RT, and incubated with chicken anti-TH and rabbit anti-Ki67 diluted in BB overnight at 4°C. Cryosections were postfixed for 10 min in 4% PFA, followed by a quick wash step in PBS. Subsequently, sections were treated with 0.1% trypsin in 0.1% CaCl\textsubscript{2} in 0.1M Tris for 12 sec, incubated in 100% fetal bovine/calf serum for 10 min, and
rinsed in PBS. Sections were incubated in 2N HCl for 30 min at 37°C with agitation, the acid was neutralized in 0.1M sodium borate pH 8.5, and quickly washed in PBS. Standard immunohistochemistry using chicken anti-TH, rabbit anti-Ki67 and mouse anti-BrdU was then performed as described above. EdU staining was performed according to the manufacturer’s protocol. In short, cryosections were incubated with BB for 1hr at RT, followed by a incubation with the EdU reactionbuffer (4% CuSO₄, 0.25% Alexa Flaur Azide, 10% Reaction buffer additive in Click-iT reaction buffer) for 30 min at RT. After a quick rinse with PBS, standard immunohistochemistry using chicken anti-TH, rabbit anti-Ki67 was then performed as described above. Staining was visualized and images were captured using a Leica DMRA fluorescence microscope coupled with a DFC340FX digital camera and LASAF software.

**Quantitative (q)RT-PCR**
Total RNA from brains of different ages and individual brain areas was extracted with TRI reagent (Sigma Aldrich) and 1-2 µg total RNA was used for cDNA synthesis using the RevertAid H-minus first strand cDNA synthesis kit (Thermo scientific). A 1:15 dilution of the cDNA pool was used in a 10 µl reaction for quantitative PCR (qPCR) analysis using the Sensifast SYBR no ROX qPCR kit (Bioline) and a Rotor-Gene™ 6000 real-time analyzer (Qiagen, Hilden, Germany). The qPCR program used was (2 min. 95 °C, (5 sec 95 °C, 10 sec. 60 or 65 °C and 15 sec. 72 °C) x 40 cycles). The intron-spanning primers used were the following: FW1 GCCCTTGACTATAATGAGTACTTCAGG, RV1 AGGACTCCTCGTATTTGCAGATTC, FW2 TGATTGTGAAGATATAATTGACACTGG, and RV2 AACACTCGAGGTCCTTTTCAC. As reference transcripts, peptidyl prolyl isomerase (PPIA) and β-actin were used. QPCR data were analyzed by using comparative quantitation and the relative Q-values of HGprt calculated by equalizing the lowest Ct value to 1. The normalization factor for the reference genes was determined using the GeNORM program (medgen.ugent.be/genorm) and used to normalize the Q-values. Individual experiments were performed with three independent mouse brain/brain area RNA pools of various developmental ages.

**Data analysis**
All data quantification was performed blinded to the genotype. For quantification of the number of cells stained for TH, BrdU or EdU, coronal sections of the ventral midbrain of three (E18.5) to five (E14.5) embryos per genotype were divided in three regions: the radial migration path, the lateral flanking areas of the migration path, and the early ventral midbrain. The radial migration path was divided in four equal bins of 0.1 mm width along the dorsoventral axis, with bin 1 located at the VZ (Fig. 2F). The flanking area on each side was similarly divided (Fig. 3B). The ventral midbrain was divided in 10 equal bins along the medio-lateral axis; with bin 1 located at the midline of the VTA, and bin 10 at the most lateral edge of the SN. For these quantifications, cell numbers were averaged for the right and left side per section, and subsequently averaged over sections per genotype. For quantification of the Sox6 positive cells, sections capturing the entire span of the SN and VTA were segregated in a rostral and caudal group, and each section divided in the regions described above. All cells were counted per bin, using using Imagej software (NIH, Bethesda, USA) and Adobe Photoshop software.

To assess the structural appearance of the radial glia scaffold an overlay of 3 concentric circles, each subdivided in 4 zones (A-D), was placed over coronal sections of the ventral midbrain of E14.5 embryos. The number of intersections of radial glia fibers with the lines per zone of the overlay was counted and normalized to number of intersections per µm. Number of intersections per µm were averaged across right and left side, per section and subsequently averaged over sections.

Data were statistically analysed by one-way ANOVA analysis of variance (α = 5%) using Graphpad Prism software (La Jolla California USA) and expressed as means ± s.e.m. Differences between groups were considered significant for \( P < 0.05 \).
**Results**

*HGprt expression during development*

To explore the temporal aspects of HGprt expression during embryonic and postnatal development, we applied qPCR analysis to reveal HGprt mRNA expression in developing mouse brain from embryonic day 10.5 (E10.5) into adulthood (postnatal day P140). In whole brain homogenates, HGprt mRNA is expressed at relatively constant levels throughout embryonic brain development, with an increase in expression after birth, reaching a plateau around P36 (Supplementary Fig. 1A). When examining HGprt expression in the midbrain (E16.5 - P35) we found an increase in HGprt expression over time starting around P7 (Supplementary Fig. 1B).

*HGprt deficiency is associated with an increase in cell division and an abnormal cell alignment in the midline migrational path during early development*

As previously described, the dopamine neurons of the ventral midbrain are organized in distinct cell clusters including the VTA and the SN. Timed proliferation and migrational activities are necessary for the proper development of the cell clusters. The dopamine neurons are born in the VZ, after which they migrate according the inverted fountain model to their destinations in the ventral midbrain. This involves an initial radial migration, followed by a tangential migration by the presumptive SN neurons. To study whether HGprt has a role in the embryonic development of VTA and SN dopaminergic neurons, we examined the effect of HGprt loss on proliferation and migration of dopaminergic progenitors in the mouse ventral midbrain between E12.5 and E14.5. Proliferating cells were labeled by administering BrdU, a thymidine analog labeling the S-phase, to pregnant mice two days before sacrifice at E14.5. Visualization of the BrdU-labeled cells allowed us to distinguish between the proliferating progenitors and the post-mitotic population, while the location of BrdU labeled cells provides information about their migrational efforts. Dopaminergic neurons were identified by staining for TH; sections were counterstained with fluorescent Nissl.

![Figure 1](image-url)  
**Figure 1. Abnormal proliferation and migration in the dopaminergic ventral midbrain in HGprt deficient embryos.**  
(A) Coronal sections of the ventral midbrain showing the tangential migration path of TH-positive neurons of E14.5 HGprt+ and HGprt− embryos which received a BrdU pulse two days prior to sacrifice. Sections were stained for BrdU (red) and TH (green) and counterstained for fluorescent Nissl (blue). (B) Schematic representation of the area of interest and quantification approach with 1-10 designating the quantification bins. (C) Quantification of the number of BrdU+ cells. The area of interest was subdivided in 10 equal bins, bin 1 located at the midline of the VTA, and bin 10 at the lateral edge of the SN. The number of BrdU+ cells was counted per bin. Graphs show averages per bin ± S.E.M., n=5 HGprt+, n=5 HGprt−. One-way ANOVA (α = 0.05). *P<0.05, ***P< 0.001.
Comparing the ventral midbrain in HGprt- embryos at E14.5 with controls, an apparent disorganisation of the TH-positive and BrdU-positive cells (Fig. 1A) was observed. First, the BrdU-positive nuclei appeared rounded and not elongated in the direction of the migratory path, as was observed in the HGprt+ embryos. In addition, BrdU-positive cell clusters were seen in the HGprt-deficient ventral midbrain. To quantify the distribution and number of BrdU-positive cells, the TH-positive area was subdivided vertically in 10 equal bins, with bin 1 located at the midline of the VTA and bin 10 at the lateral most edge of the SN (Fig 1B). Quantification showed an overall increase in BrdU-positive cells, especially around the midline of the VTA.

VTA and SN dopaminergic neurons are initially generated in the VZ and from here commence their migration along the radial migrational path to the ventral regions of the midbrain (17, 23). Based on the differences found in the presumptive VTA and SN, we investigated whether these differences might be the result of abnormalities in the area containing the VZ and radial migration path. When examining the radial migration path, a similar disorganization was seen as in the presumptive VTA and SN area (Fig. 2A). Indeed, the number of BrdU-positive cells showed a significant increase in the area near the VZ in the HGprt- embryos (Fig. 2B). Oppositely, the number of TH-positive cells was decreased, particularly near the VZ, but this effect diminished further along the radial migration path (Fig. 2C). Interestingly, the number of BrdU and TH double positive cells remains the same or even increases more ventrally in the radial migration area (Fig. 2D). This significant increase became even more noticeable when the fraction of the TH cells that were also BrdU positive was quantified (Fig. 2E). Together, these results indicate the HGprt deficiency is associated with altered proliferation and migration patterns. In addition, the observed altered cell alignment in the migrational path which could be causative to the premature deviation of the migrating cells from this path.

**Apparent premature departure from midline migration path due to HGprt deficiency**

Besides deviations in cell numbers, an abnormal cell alignment was observed in the BrdU-positive cells in the radial migrational path. In the controls, the nuclei of a large portion of the BrdU-positive cells were visibly elongated, a hallmark of migrating cells (24, 25) and were oriented in the direction of the midline migrational path (Fig. 2A, 3A). In the HGprt- embryos however, elongated nuclei of the BrdU-positive cells were oriented in a visibly different angle, deviating from the direction of the migrational path (Fig. 2A,G, 3A). The difference in orientation was most pronounced at the start of the migrational path, close to the VZ (Fig. 2F,G). To explore if this altered progenitor alignment in HGprt-deficient embryos could indicate abnormal migrational paths away from the midline, we inspected the areas flanking the midline migrational path above the presumptive SN. We observed an overt increase in number of TH-positive neurons, presumably prematurely deviating from the radial migration path in the HGprt-deficient embryos (Fig. 3A). Quantification of the number of BrdU, TH and double-positive cells (Fig. 3B), showed a significant increase in number of TH-positive cells (Fig. 3C) and BrdU-positive cells (Fig. 3D), as well as double-positive cells (Fig. 3E). These results suggest that dopaminergic progenitors are prematurely deviating from the radial migration path in the absence of HGprt.

To investigate whether the abnormalities seen in the HGprt-deficient midbrain at E14.5 could be the result of a developmental delay causing proliferating cells to remain in cell cycle until a later developmental time point, we repeated the experiment labeling proliferating cells with EdU two days prior to sacrifice at E18.5 (Fig. 4A). Again, an increased number of EdU-positive cells could be found in the area close to the VZ and start of the former radial migration path (Fig. 4B, C). Moreover, a slight increase in EdU-positive cells could be found in the medial VTA (Fig. 4D-F). These data indicate that alterations in cell division and possible migration due to HGprt deficiency persist until later developmental stage.
Figure 2. HGprt deficiency causes an increase in dopaminergic cell division and an abnormal progenitor alignment in the midline migration path. (A) Coronal sections of the ventral midbrain midline migration path of E14.5 HGprt+ and HGprt- embryos which received a BrdU pulse two days prior to sacrifice. Sections were stained for BrdU (red) and TH (green). (B) Quantification of the number of BrdU+ cells. The midline migration path was divided in 4 equal bins (F) and the number of BrdU+ cells was counted per bin. (C) Quantification of TH+ neurons as in B. (D) Quantification of BrdU and TH double-positive neurons. (E) Quantification of the fraction of double-positive cells of the total number of TH+ neurons. (F) Schematic representation of the quantification method with 1-4 designating the quantification bins. (G) Quantification of the angle of the nucleus of BrdU+ cells. The angle was determined using the midline as the 0° line. Graphs and plot show averages per bin ± S.E.M., n=5 HGprt+, n=5 HGprt-. One-way ANOVA (α = 0.05) *p<0.05, **p<0.01, ***p<0.001.

HGprt deficiency is associated with changes in radial glia scaffolding

The changes in the proliferative profile and preliminary deviation from the midline migratory path due to HGprt deficiency led to the hypothesis that HGprt deficiency might affect the facilitating and guiding scaffold constructed by the fibers of the radial glial cells. With their long processes, radial glia calls span the entire width of the ventral midbrain, and provide directional and mechanical support to the migrating mDAs (19, 20). Using the radial glia marker RC2 (26, 27), we quantified the number and distribution of radial glia processes throughout the ventral midbrain. We placed an overlay of three concentric circles with a fixed distance, on the ventral midbrain and the number of intersections of radial glia fibers with these lines was quantified in 4 zones, A-D, and normalized to the number of intersections per µm (Fig. 5B). Originally, we aimed to quantify the number of intersections in sections capturing the entire span of the SN and VTA. However, on closer inspection the rostral sections appeared differentially affected by HGprt deficiency compared to the caudal sections. Subsequently, we quantified the number of intersections for the rostral and caudal ventral midbrain separately (Fig. 5A, B). In both the rostral and caudal sections, the number of radial glial fibers was affected only close to the
midline, i.e. at the location of the ventral part of radial migrational path (Fig. 5C). Of note, while in the rostral section, the number of fiber intersections was significantly reduced, they were increased in number in the caudal sections (Fig. 5D, E). No differences were found in the more lateral zones (Supplementary Fig. 2 A-F). In addition, in the E14.5 HGprt+ embryos the radial glial processes appeared as taut fibers while in the E14.5 HGprt- embryos the fibers appeared more sinuous. This was observed in both the rostral and caudal sections. These data indicate that the scaffold, formed by the processes of the radial glia cells of the ventral midbrain, is affected differentially in the rostral compared to the caudal ventral midbrain in HGprt deficient embryos. Remarkably, a rostral to caudal division in the number of BrdU-positive cells at E14.5 and EdU-positive cells at E18.5 could not be established (data not shown).

Effects of HGprt deficiency on dopaminergic subpopulation identity

Each of the stages in mdDA development is defined by the expression of a specific set of transcription factors and other molecular markers. Previous research has identified signaling and transcriptional networks that are important for neuronal subtype diversification during development of mdDA neurons, particularly those forming the SN and the VTA (17, 23). As such, the transcription factor SRY-box6 (Sox6) has been identified as a SN-specific marker, as it is a key factor for the early specification and development of SN dopaminergic neurons, and has been shown to be involved in suppressing VTA-specific characteristics (18). To analyze whether the altered proliferative patterns as observed affect mdDA neuronal subtype formation, or their distribution in the ventral midbrain during development, we

Figure 3. Premature departure of dopaminergic progenitors from midline migration path in absence of HGprt. (A) Coronal sections of the ventral midbrain showing the areas flanking the midline migration path of E14.5 HGprt+ and HGprt- embryos which received a BrdU pulse two days prior to sacrifice. Sections were stained for BrdU (red) and TH (green) and counterstained for fluorescent Nissl (blue). (B) Schematic representation of the area of interest and quantification approach with 1-4 designating the quantification bins. (C) Quantification of the number of TH+ neurons. The midline flanking areas were divided in 4 equal bins (B) and the number of TH+ neurons was counted (D) Quantification of the number of BrdU+ cells as described in (C). (E) Quantification of the TH and BrdU double-positive cells. Graphs show averages per bin ± S.E.M., n=5 HGprt+, n=5 HGprt-.

One-way ANOVA (α = 0.05) *P<0.05, **P<0.01, ***P<0.001
analysed the distribution of Sox6-positive cells in coronal sections of the ventral midbrain of E14.5 HGprt+ and HGprt- animals (Fig. 6A), in the same subareas as used before including the radial migration path (Fig. 6B), its flanking areas (Fig. 6E), and the ventral midbrain with the presumptive VTA and SN (Fig. 6H), and segregated the rostral and caudal sections. In the radial migration path, the number of Sox6-positive cells in the bin closest to the VZ was increased for both the rostral and caudal sections (Fig. 6A-D). However, in the caudal sections this transitioned to a decrease in the more ventral bins of the radial migration path (Fig. 6A,D). When we quantified the flanking areas, we once more found an opposite effect in the rostral sections compared to the caudal sections. A slight decrease in Sox6-positive cells was noted in the rostral sections of HGprt-deficient embryos (Fig. 6F), as opposed to the caudal sections where an increase in Sox6-positive cells was observed (Fig. 6G). In the rostral ventral
midbrain area, more Sox6-positive cells appeared to be located around the VTA midline in the HGprt, and less in the lateral bins (Fig. 6H). However in the caudal ventral midbrain area, we found an increased lateral distribution of the Sox6-positive cells (Fig. 6K). This could indicate that there is indeed altered migration activity of the mdDAs, causing the Sox6-positive cells to be differentially distributed at E14.5. However, the differentially distribution could also be a result of an altered molecular program run by the mdDAs.

HGprt deficiency is associated with abnormal cortical layering. It is well known that HGprt is expressed in virtually all cells in the brain (28). One could therefore expect that in addition to the dopamine system, other cell populations could be affected by the HGprt deficiency as well. Furthermore, research has shown that the innervation of the axons of the major neurotransmitter projection systems also contributes to the development of its target areas. The VTA is known to innervate cortical areas as well (29, 30). To explore the possibility of altered developmental
Figure 6. The expression of Sox6 in the rostral and caudal ventral midbrain is differentially affected by HGprt deficiency. (A) Coronal sections of the caudal midline migration path of E14.5 HGprt+ and HGprt- embryos. Sections were stained for Sox6 (Red) and TH (green). (B) Schematic representation of the midline migration path. (C) Quantification of the number of Sox6+ cells in the rostral midline migration path per bin (Fig. 1B). (D) Quantification of the number of Sox6+ cells in the caudal midline migration path per bin. (E) Schematic representation of the areas flanking the midline migration path. (F) Quantification of the number of Sox6+ cells in the rostral midline migration path flanking areas per bin. (G) Quantification of the number of Sox6+ cells in the caudal midline migration path flanking areas per bin. (H) Schematic representation of the tangential migration area. (I) Quantification of the number of Sox6+ cells in the rostral tangential migration area per bin. (J) Quantification of the number of Sox6+ cells in the caudal tangential migration area per bin. Graphs show averages per bin ± S.E.M., n=3 HGprt+, n=3 HGprt-.

One-way ANOVA (α = 0.05) *P<0.05.

Changes in dopaminergic target areas, we examined the cortical layer formation in the subareas of the medial prefrontal cortex (mPFC), primary motor cortex (M1), and the somatosensory cortex (S1) of P30 HGprt+ and HGprt- mice. Staining for Tbr1 was used to examine deeper layer formation (31). Visualizing Reelin-expressing neurons allowed the examination of a subgroup of interneurons (32, 33). In the PFC as well as the M1 and S1, an increase in the number of Tbr1-positive neurons was found along the entire cortical width (Fig. 7B-F). These data suggest that a global effect on cortical development was associated with HGprt deficiency, particularly affecting cortical layer development.

Discussion

**HGprt deficiency is associated with multiple neurodevelopmental abnormalities**

The present study is the first in vivo investigation of abnormal neurodevelopment in an animal model for LND. We show that HGprt deficiency is associated with multiple abnormalities in early brain
development in a knock-out mouse model for LND. First, we found a disorganization of the presumptive VTA and SN during embryogenesis due to HGprt deficiency, as well as altered proliferation and migration patterns of dopaminergic progenitors that are predominantly present around the ventral midline. Second, HGprt deficiency was shown to be associated with abnormal cell alignment in the midline migration trajectory, in combination with and increased number of TH-positive neurons in adjacent areas. These data suggest that HGprt deficiency causes a premature departure of migrating dopaminergic neurons from the midline migratory path. These alterations in dopaminergic proliferation and migration patterns associated with HGprt deficiency were found to be present across development. Third, the number of fibers formed by the radial glia cells, that form the facilitating and guiding scaffold for migrating dopaminergic neurons, was affected due to HGprt deficiency in a regionally restricted manner. More specifically, in the rostral part of the ventral midbrain the number of intersections of dopaminergic neurons with radial glial fibers was decreased, while in more caudal areas the number was increased compared to controls. Fourth, HGprt deficiency was found to affect the distribution of a specific subtype of dopaminergic midbrain neurons, i.e. neurons positive for Sox6 that mark early SN neurons. In addition to these multiple changes in early dopaminergic development, HGprt deficiency was also associated with effects on cortical organization, as cortical layering was affected in the PFC, M1 and S1 in postnatal mice.

Figure 7. Deep layer marker expression altered in the absence of HGprt during development. (A) Coronal sections of the prelimbic subarea of the mPFC of HGprt+ and HGprt- P30 mice. Sections were stained for Tbr1 (green) and counterstained with fluorescent DAPI (blue). (B) Quantification of the number of Tbr1+ cells in the infralimbic subarea of the mPFC. A cortical swatch was divided in a deeper layer region (Deep), superficial layer region (Sup) and the marginal zone (Marg). The number of Tbr1+ cells was counted and normalized to the total number of DAPI+ cells. (C) Quantification of the number of Tbr1+ cells in the prelimbic subarea of the mPFC as in (B). (D) Quantification of the number of Tbr1+ cells in the cingulate subarea of the mPFC. (E) Quantification of the number of Tbr1+ cells in the S1. (F) Quantification of the number of Tbr1+ cells in the M1. Graphs show averages per bin ± S.E.M., n=5 HGprt+, n=5 HGprt-. One-way ANOVA (α = 0.05). *P<0.05, **P<0.01. S1, somatosensory cortex. M1, primary motor cortex.
We provide *in vivo* evidence for the concept that LND is a neurodevelopmental disorder that involves the dopamine system. As opposed to the hyperuricemia in LND patients, explained by the lack of purine recycling that result in excess uric acid, the pathogenesis of the neurobehavioral phenotype is incompletely understood. Yet, it has been known for several decades that HGprt deficiency is associated with a relatively selective dopamine deficiency, and that the dystonia, executive dysfunction, and self-injurious behavior have pointed towards basal ganglia circuitry as the underlying neural substrate (12). However, the prominent decrease of dopamine in the striatum, and the absence of overt structural abnormalities in the SN in patients (34) and knock-out mice (13) have puzzled investigators for a long time. Only recently, the hypothesis emerged that HGprt deficiency might affect the maturation of the dopamine system. First, studies applying microarray techniques to HGprt-deficient cancer cell lines showed disturbed expression of transcription factors involved in dopaminergic neuronal development and differentiation (15, 16). The expression of both upstream regulators of dopaminergic specification (Lmx1a, Msx1, Lmx1b) and transcription factors that allow survival (En 1/2) and maturation (Pitx3, Nurr1) of dopamine neurons were affected. These transcriptional changes were accompanied by dysregulation of genes encoding dopamine biosynthetic enzymes and abnormal neurite outgrowth (15). Moreover, knockdown of HGprt in human fibroblasts and in neuroblastoma cells affected canonical Wnt/β-catenin and the presenilin signaling pathways, and downregulation of Lmx1a and En 1 (35). Other studies reported abnormal microRNA expression in neuroblastoma cell lines that may disturb pathways regulating the dopamine neurons and pathway development (36, 37). Of note, a decrease in exchange protein activated by cAMP (EPAC) has been reported in HGprt knocked-down human neuroblastoma cells and fibroblasts from LND patients as well as differences in cytoskeleton dynamics that lead to increased motility in the presence of an EPAC agonist (37). Although the exact relevance of these findings for human LND needs to be established, the present study provides the first notion that dopaminergic migratory processes might be affected.

**The effect of HGprt deficiency on the maturation of the midbrain dopamine system: defects in proliferation, migration or both?**

The present study demonstrates neurodevelopmental abnormalities in the developing dopamine system of the HGprt knockout mouse model. It is tempting to speculate that these abnormalities ultimately lead to the functional deficits in dopamine neurons, as demonstrated before (13-15).

Proliferation and migration patterns were assessed and revealed an increased number of cell divisions close to the VZ at the beginning of the radial migration path in HGprt-deficient embryos, i.e. in the area where the progenitors are generated and commence their migratory activities, could indicate an increase in proliferation in the progenitor pool or an earlier exit from the cell cycle. Of note, we found a decrease of TH-positive cells in this area at the specific time points measured, while previous reports show no change in the number of TH-positive cells in the midbrain of LND patients and HPRT-deficient mice (10, 14, 38) As we have seen in our results, the timing and position (either rostral or caudal) can be critical and can explain why it has not been observed before. A reduced TH expression has been demonstrated in the midbrain of post-mortem LND brains and adult HGprt-deficient mice (14). It could be speculated that the observed increase in cell divisions at specific time points during embryonic development is explained by a developmental delay resulting in a reduced number of TH-positive cells at this developmental time point. Alternatively, the reduction in TH-positive cells after birth could be the result of an improper development of the dopaminergic phenotype; either as a direct effect of the metabolic abnormalities due to HGprt deficiency, or due to abnormal migration patterns out of the sectioning plane and/or that this abnormal migration pattern has prevented the occurrence of proper spatiotemporally restricted signaling cues.

The latter hypothesis is fueled by the abnormalities seen in the ventral midline and adjacent areas i.e. increase in BrdU-labeled cells in the migrational path where the intermingled subpopulations of
dopaminergic neurons segregate to migrate to their destined locations in the ventral midbrain, and the suggestion of early deviation from the path of TH-positive cells. These findings could also indicate defects in migration, e.g. an impairment of the switch from radial migration to tangential migration by the presumptive SN neurons. Of note, using Sox6 as a specific marker for SN neurons (18), i.e. those that have the most extensive tangential migration towards lateral areas, we found an altered distribution in caudal to rostral direction of Sox6-positive cells in the ventral midbrain. Again, this supports the altered proliferation and migration patterns observed, and may suggest that the developing VTA and SN dopaminergic neurons are at an abnormal location at a specific time point in development, presumably resulting in an altered spatiotemporal restricted signaling cues perceived by the developing VTA and SN neurons from their environment, leading to abnormal development of the neurochemical phenotype.

The role of the radial glia
An important factor facilitating and steering migration of the mdDA neurons is the scaffold constructed by the fibers of the radial glial cells. With their long processes they span the entire width of the ventral midbrain, and provide directional and mechanical support to the migrating mdDA neurons (19, 20). In addition, the same floor plate radial glia cells have been shown to undergo neurogenic divisions, giving rise to dopaminergic progenitor cells (19, 39). This illustrates a dual role for the radial glia cells during the development of the VTA and SN dopaminergic neurons comparable to other developing systems. Markedly, in this study, alterations in the number of cell divisions as well as the number of radial glial fibers have been found in the HGprt-deficient embryos, indicating an HGprt dependency of the radial glia cells during early development. One could speculate that a differential development of the floor plate radial glia cells due to HGprt deficiency could contribute to the altered proliferation pattern and radial fiber scaffold organization. The concept of a dual function of the radial glia cells during development can also be seen in other brain regions. For example, the role of radial glia cells in cerebral cortical layer formation has been well established (40-43). It remains to be determined whether the differences observed in radial glia function due to HGprt deficiency are specific for the ventral midbrain, or that there is a more widespread radial glia defect due to loss of HGprt.

Effects of HGprt deficiency extend beyond the dopaminergic system
The SN and VTA project to multiple target areas. The SN dopaminergic neurons innervate the dorso-lateral striatum involved in controlling motor behavior (29), while those of the VTA extend to the mPFC, the ventral medial striatum and habenulae, involved in emotional behaviors and cognition (29, 30). Axonal outgrowth in order to make these connections is a highly spatiotemporally regulated process that involves axonal guidance and target selection. Again, disturbances in dopaminergic proliferation and migration patterns could result in altered spatiotemporal instructions to the maturing dopaminergic neurons, which could affect axonal outgrowth and targeting. Incoming dopaminergic axons from the VTA and SN contribute to the developmental processes that sculpt their target areas, and may contribute to the proper assembly of neuronal circuits (30, 44). In the present study, we found defects in deeper layer marker expression in multiple cortical areas in the HGprt deficient mice that might be therefore a result of an improper dopaminergic system development. Alternatively, this could indicate a more general cell autonomous effect of HGprt on brain development, being less dopamine-specific than previously thought. Indeed, recent imaging studies using voxel-based morphometry in LND patients reported profound abnormalities in white and gray matter in multiple brain areas, e.g. basal ganglia and PFC (45, 46). These findings support the concept that HGprt deficiency affects not only the dopaminergic midbrain, but – either via the dopaminergic defects or more directly – also other brain areas.
**Perspectives**

In the present study, the visualizations of BrdU- and EdU-positive cells two days after labeling provide ample information on alterations in dopaminergic proliferation and migration patterns, but the distinction between these processes is challenging. This could be resolved by extending the developmental timelines, e.g. combining the current approach with staining for cell cycle markers, such as Ki67, which will provide more acute information on the number and location of cells in the M-phase of cell cycle at the moment of sacrifice. Additionally, cell cycle exit experiments, and repeating the experiment at even earlier developmental time points may provide the further information that is necessary to better understand the effect of HGprt on the even earlier development of the VTA and SN. Although the HGprt knockout mouse has genetic, metabolic and dopaminergic disturbances that resemble those detected in LND patients, a frequently mentioned limitation of this mouse model is the lack of the classic LND neurobehavioral phenotype. Therefore, the model is probably best used for assessing early molecular and cellular aspects of the pathogenesis in LND.

As said, a more precise disentanglement of specific migration and proliferation patterns could be achieved by studying additional developmental time points and assessing additional subpopulation markers. In addition, the development of target areas should be examined. Furthermore, besides the number of radial glia fibers, their angle of position compared to the midline migration path should be further examined. There seems to be a curve in the fibers precisely in the area where we observed the deviations in migrational direction of the BrdU-positive cells. An altered radial fiber scaffold may underlie this observed phenomenon.

A number of hypotheses to account for the HGprt-dopamine relationship have been put forward. As many of these hypotheses have been disproven in the past, usually based on *in vitro* cell models, post-mortem human brains and adult HGprt knock-out mice, it remains possible that the mechanisms put forward in these rejected hypotheses contribute to LND pathogenesis during specific embryonic time points. Therefore, reconsideration of some of these theories in the light of neurodevelopmental processes could further contribute to our understanding of the link between HGprt enzyme deficiency and abnormal brain development.

**Conclusions**

Here we show, to our knowledge for the first time *in vivo*, that HGprt deficiency is associated with multiple abnormalities in early brain development. Most importantly, we demonstrate that HGprt deficiency causes abnormal proliferation and migration patterns in the developing dopaminergic midbrain. Additionally, the absence of HGprt affects the radial glia scaffold as well as dopamine neuron subtype marker expression. This is important since an impaired organization of the VTA and SN can contribute to an altered dopamine functioning in their target areas, a phenomenon observed in LND patients, and therefore highlights the need for further developmental research.

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**Conflicts of interests**

The authors declare no competing financial interests.
References


Supplementary Materials and Figures

**Supplementary Table 1**: PCR primer sequence for Gender and HGPRT genotyping.

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<td>Male Sry RV</td>
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**Supplementary Table 2**: Primary antibodies, dilution and their supplier

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Supplemental Figure 1. (A) HGprt mRNA expression levels in developing mouse brain at E10.5 and E13.5 heads and E14.5, E15.5, E16.5, E18.5, P0, P7, P14, P21 and adult brain (n = 3 series on non-related animals) measured with qPCR. Graph represents normalized mean transcript levels ± s.e.m. (B) HGprt mRNA expression levels in developing mouse midbrain at E16.5, E18.5, P7, P14, P21 and P35 measured with qPCR using two distinct primer sets. Graphs represent normalized transcript levels.
Supplemental Figure 2. The radial glial scaffold of the ventral midbrain is affected differentially in the rostral compared to the caudal ventral midbrain in HGprt-deficient embryos. (A-C) Quantification of the normalized number of intersections of radial glial fibers with the overlay in the rostral ventral midbrain in zone B-D (Fig. 5B). (D-F) Quantification of the normalized number of intersections of radial glial fibers with the overlay in the caudal ventral midbrain in zone B-D (Fig. 5B). Graphs show averages per bin ± S.E.M., n = 3 HGprt+, n = 3 HGprt-. One-way ANOVA (α = 0.05).
Supplemental Figure 3. Reelin expression is affected in the absence of HGprt during development. (A) Coronal sections of the prelimbic subarea of the mPFC of HGprt+ and HGprt- P30 mice. Sections were stained for Reelin (red) and counterstained with fluorescent DAPI (blue). (B) Quantification of the number of Reelin+ cells in the infralimbic subarea of the mPFC. A cortical swatch was divided in a deeper layer region (Deep), superficial layer region (Sup) and the marginal zone (Marg). The number of Reelin cells was counted and normalized to the total number of cells (DAPI). (C) Quantification of the number of Reelin+ cells in the prelimbic subarea of the mPFC as in (B). (D) Quantification of the number of Reelin+ cells in the cingulate subarea of the mPFC. (E) Quantification of the number of Reelin+ cells in the S1. (F) Quantification of the number of Reelin+ cells in the M1. Graphs show averages per bin ± S.E.M., n = 5 HGprt+, n = 5 HGprt-. One-way ANOVA (α = 0.05) *p<0.05, **p<0.01. S1, somatosensory cortex. M1 primary motor cortex.
Haploinsufficiency of MECP2-interacting transcriptional co-repressor SIN3A causes mild intellectual disability by affecting the development of cortical integrity

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Abstract

Numerous genes are associated with neurodevelopmental disorders such as intellectual disability and autism spectrum disorder (ASD), but their dysfunctioning is often poorly characterized. Here, we identified dominant mutations in the gene encoding the transcriptional repressor and MeCP2-interactor switch-insensitive 3 family member A (SIN3A; chromosome 15q24.2) in individuals who, in addition to mild intellectual disability and ASD, share striking features including facial dysmorphisms, microcephaly and short stature. This phenotype is highly related to that of individuals with atypical 15q24 microdeletions, linking SIN3A to this microdeletion syndrome. Brain MRIs revealed subtle abnormalities, including corpus callosum hypoplasia and ventriculomegaly. Intriguingly, in vivo functional knockdown of Sin3a led to reduced cortical neurogenesis, altered neuronal identity and aberrant cortico-cortical projections in the developing mouse brain. Together, our data establish that haploinsufficiency of SIN3A is associated with mild syndromic intellectual disability and that SIN3A can be considered a key transcriptional regulator of cortical brain development.
Introduction

The rapid advances in technologies to identify genetic causes of neurodevelopmental disorders, including intellectual disability, developmental delay and autism spectrum disorder (ASD), have led to the identification of numerous copy number variants (CNVs) and dominant gene mutations (1-7). However, the consequences of the associated protein disturbances mostly remain to be defined. The gene encoding the switch-insensitive 3 transcription regulator family member A (SIN3A) is one of the five genes located in the shortest region of overlap (SRO, ~260kb) of 15q24 microdeletions flanked by segmental duplication (SD) blocks C and D in individuals with intellectual disability and ASD (8-10). We now identified dominant loss-of-function mutations in SIN3A in nine individuals with intellectual disability/developmental delay and hypothesize that haploinsufficiency of SIN3A contributes substantially to the phenotype seen in individuals with these deletions. Brain MRIs performed in a subset of the individuals revealed persistent abnormalities, such as corpus callosum hypoplasia/dysgenesis and ventriculomegaly.

The development of dedicated cortical brain areas is a highly orchestrated process that involves proliferation of progenitors, migration of young neurons to final cortical layers, neuronal differentiation and network formation. Molecular control of cortical progenitor proliferation is directly linked to the eventual size of cortical areas and overall cerebral cortex functioning (11, 12). Furthermore, various intrinsic (e.g. transcriptional regulators) as well as extrinsic (e.g. guidance cues) factors are expressed during the early phases of corticogenesis (13-16), yet the precise roles of many of these factors remains to be elucidated.

Sin3a can bind to various members of a transcriptional regulatory complex (e.g. MeCP2, HDAC1/2, NCOR, CABIN1) to control a variety of developmental processes (17-21). Especially in cell cycle events and proliferation of embryonic stem cells, Sin3a seems to play an important role (17, 19, 22-24). Although Sin3a is known to be present in the rodent forebrain for over a decade (25-28), its exact role in brain developmental processes stays elusive.

In this report we investigated the consequences of SIN3A haploinsufficiency, by comparing clinical and MRI data of nine intellectual disability/developmental delay individuals in whom we identified SIN3A mutations and four so far unpublished cases with de novo atypical small 15q24 deletions (breakpoints outside SD blocks C and D) encompassing SIN3A (270kb-500kb; SRO 75.60-75.95 MB, Hg 19). Furthermore, to investigate the consequences of reduced Sin3a expression in brain development, we employed a select in vivo functional knockdown assay (29) and showed Sin3a to be a key regulator of cortical expansion and maturation. Overall, the observed human syndrome characterized by intellectual disability/developmental delay, seems to be a direct consequence of SIN3A downregulation by haploinsufficiency affecting correct cortical expansion.

Material and methods

Patient recruitment

All individuals except for individuals 7-9 were ascertained during a clinical genetic diagnostic work-up because of unexplained intellectual disability, at one of the involved genetic diagnostic services in Nijmegen, Groningen, Nantes, and Southampton. The total pool of recruitment consisted of over 12,000 individuals with unexplained intellectual disability/developmental delay. This number both comprises SNP-based microchromosomal array cases and whole exome sequencing cases. Regarding the sequencing cases (i.e. individuals 5, 6, 7, 10 and 11) these all concern cases from diagnostic whole exome sequencing. Individuals 5, 6, 10 and 11 were diagnosed out of ~2000 ID/DD cases in the Nijmegen lab and individual 7 out of 250 Bonn (Germany) laboratory. Thus, all together the frequency of single SIN3A mutations seems 1 in 250-500 individuals that underwent whole exome sequencing because of unexplained intellectual disability/developmental delay. Individuals 3 and 4 were recruited from the Decipher database. Individuals 7-9 were ascertained in a collaborative study.
Bonn-Essen-Munich of 250 individuals with intellectual disability of unknown origin. Inclusion into this study was in agreement with local medical ethics committee approval.

**Genome-wide chromosome micro-array analysis**

Different microchromosomal array platforms were used. The deletions of individuals 1 and 2 were identified using the Agilent 105K oligo array. The deletions of patient 3 and 4 were identified by the Agilent 8 x 60k platform (International Standards for Cytogenomic Arrays (ISCA), Oxford Gene Technology (OGT)). CNVs were mapped according to the UCSC genome browser build February 2009 (hg19).

**Whole exome sequencing**

In individuals 5, 6, 10, and 11, a family based whole exome sequencing (WES) analysis was performed essentially as previously described by (30). Briefly, capture of exons was done using an Agilent SureSelectXT Human All Exon 50Mb Kit. Sequencing was performed using a Life technologies 5500XL machine, or an Illumina Hiseq 2000. Read mapping and variant calling were done using LifeScope™ for the 5500XL data or BWA (mapping) and GATK (calling) for the Illumina data. In patient 7 and his father (patient 8) WES was performed on an Illumina HiSeq 2500 after enrichment with version 5 of the SureSelect XT Human All Exon 50Mb kit (Agilent Technologies) as published previously. De novo mutations in patients 5, 6 and 10, and inherited mutations in patients 7-9 and 11-13 were confirmed by Sanger sequencing.

**Animals and preparation of brain cryosections**

All animal use, care and experimental procedures were performed in accordance with the institutional and national ethical guidelines and regulations, and with the approval of the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands under protocol number RU-DEC 2011-190/2011-268/2014-059. The day of the vaginal plug was considered E0.5 and the day of birth as postnatal day (P)0. Timed-pregnant mice (normal C57BL/6JolaHSD background from Harlan laboratories B.V., The Netherlands) and pups were killed by means of cervical dislocation or decapitation, respectively. The embryos were dissected in ice-cold Leibovitz medium (L-15, PAA). Alternatively, postnatal mice were euthanized by decapitation and both embryonic as well as postnatal brains were quickly removed from their skulls in L-15. For the PCR experiment, brains and brain areas were quickly frozen in liquid nitrogen and stored in -80 °C before RNA was isolated. For immunohistochemistry, brains were fixed by submersion in 4% paraformaldehyde (PFA) in PBS (wt/vol), pH 7.4, for 4°C for 1 - 1.5 h maximum. After a quick wash in PBS, brains were cryoprotected overnight in 30% sucrose (wt/vol) in PBS. Then, brains were frozen in M-1 embedding matrix in a plastic cup (Thermo Fisher Scientific) on dry ice and stored at -80°C. Cryostat sections were cut at 16 μm, mounted on SuperFrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -20°C.

**RNA isolation and Real-Time quantitative RT-PCR (qPCR)**

Total RNA from brains of different ages, individual brain areas and mouse neuroblastoma (N2a) was extracted with TRileagent (Sigma Aldrich) and 1-2 μg total RNA was used for cDNA synthesis using the RevertAid H-minus first strand cDNA synthesis kit (Thermo scientific). A 1:15 dilution of the cDNA pool was used in a 10 μl reaction for qPCR analysis using the Sensifast SYBR no ROX qPCR kit (Bioline) and a Rotor-Gene™ 6000 real-time analyzer (Qiagen). qPCR program used was (2 min. 95 °C, (5 sec 95 °C, 10sec. 60 or 65 °C and 15 sec. 72 °C) x 40 cycles). The intron-spanning primers used are listed in Supplementary Table 1. As reference transcripts, peptidyl prolyl isomerase (PPIA) and β-actin were used. qPCR data were analyzed by using comparative quantitation and the relative Q-values of the genes of interest calculated by equalizing the lowest Ct value to 1. The normalization factor for the reference genes was determined using the GeNORM program and used to normalize the Q-values. Individual experiments were performed in triplicate with independent mouse brain/bra area RNA pools of various developmental ages derived from three non-related animals.
Plasmid construction
To downregulate the expression of Sin3a in cortical regions, two different shRNAs oligo’s were cloned via BglIII and HindIII restriction sites into pSUPER.GFP/Neo (a kind gift of Dr W. Hendriks): shSin3a-ex13 and shSin3a-ex16 (Supplementary Table 1d, Supplementary Fig. 4c-g). Both sequences target different regions of Sin3a respectively Exon 13 and Exon 16 based on the siRNA sequences as were described earlier (31). The reporter CMV-YFP-N1 plasmid and scrambled Sin3a sequences cloned into pSUPER.GFP/Neo (sc-shSin3a-ex13 and sc-shSin3a-ex16) were used as controls (Supplementary Table 1d, Supplementary Fig. 4c-g).

For construction of a mouse full length Sin3a short hairpin-insensitive construct (mSin3a*), total cDNA of PND 140 mouse brain was used to amplify two DNA fragments, 5’-MmSin3a and 3’-MmSin3a of respectively 2103bp and 1843bp by PCR using specific primers (MmSin3a-Ampli 5’part and MmSin3a-Ampli 3’part) as described in Supplementary Table 1d. Primer sequences were based on the NCBI Reference Sequence: NM_001110351.1. Both Sin3a cDNA fragments were cloned into the BamHI and SalI restriction sites of pGemzf3+ plasmid resulting in 5’-Sin3a_pGEMzf3+ and 3’Sin3a_pGEMzf3+. The 5’-Sin3a_pGEMzf3+ was cut via EcoRI and ApaLI and 3’-Sin3a_pGEMzf3+ was cut via MunI and BamHI. Both 5’-Sin3a_pGEMzf3+ and 3’-Sin3a_pGEMzf3+ fragments were isolated and purified from agarose gel.

A gBLOCK gene fragment of 1000 bp was ordered by IDT (NM_001110351.1: nt 1858-2857) containing the mutated target site of both the shSin3a-ex13 (GAG CAC ATC TAT CGA TGC G)(NM_001110351.1: nt 1986-2004) and shSin3a-ex16 (GT ATG GAC GAG GTA TAC AA)(NM_001110351.1: nt 2800-2818) (Supplementary Table 1c). The 3 fragments 5’-Sin3a_pGEMzf3+ (MunI/BamHI), 3’-Sin3A_pGEMzf3+ (EcoRI/ApaLI) and the gBLOCK fragment were assembled via the Gibson assembly (New England Biolabs) to generate shRNA-insensitive Sin3a (full length) _pGEMzf3+. Finally, the EcoRI/SalI shRNA-insensitive Sin3a full length fragment was isolated and cloned into the EcoRI/Xhol restriction sites of Chicken Actin (CAG) promoter containing mammalian expression plasmid pCAB, resulting in mSin3A*pCAB. The pCAB expression plasmid was derived from pCAB-EGFP (a kind gift of Dr W. Hendriks) by removing the EGFP using SmaI and Xhol (Supplementary Fig. 4g).

Cell culture and validation
Mouse N2a cells (below passage 20) were cultured in complete MEM medium (PAA) containing 10% fetal bovine serum (FBS, PAA) and COS-1 cells in complete DMEM (Gibco) containing 10% FCS and were maintained at 37°C and 5.5 % CO₂ atmosphere. One day prior to transfection, 1x10⁶ cells per well were seeded on 24-wells plates. Cells were transfected with either Sin3a siRNA (31) using 1.5 µl Lipofectamine 2000 (Invitrogen) and 33 nmol of siRNA or Sin3a shRNA constructs, using 2.0 µg plasmid DNA, 4.0 µl Lipofectamine LTX and 2 µl Lipofectamine LTX (Invitrogen) in Optimem (Gibco). siRNA- and shRNA-mediated mRNA degradation was allowed for 48 hours (hrs) and total RNA was extracted as described. For validation of the shRNA-insensitive Sin3a full length protein expression and intracellular localization, 4 x 10⁵ COS-1 cells were seeded in 12-wells plates and transiently transfected with 3.0 µg plasmid DNA using Lipofectamine LTX. Recombinant protein expression was allowed for 24 hrs. Both COS-1 as well as N2a cells were authenticated and screened for mycoplasma contamination monthly. Experiments were performed in triplicate.

Protein isolation and Western blotting
Cells were scraped in 1 ml PBS and collected by centrifugation (5 min, 500 g, 4 °C). Then, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% SDS, 50 mM TrisHCl pH 8.0, 2 mM EDTA and Complete protease inhibitors (Roche) and sonicated. Equal amounts of cell lysates were separated on 7% SDS-PAGE and subsequently transferred to PVDF membrane (Biorad). The membrane was incubated with an antibody against Sin3a (1:2000, Abcam) and secondary peroxidase-conjugated goat-anti-rabbit antibodies (1:5000, Nordic) followed by chemoluminescence (LumiLight Plus, Roche). Signals were detected using the LAS4000 imaging system (GE Healthcare). Experiments were performed in triplicate.
In utero electroporation (IUE)

In utero electroporation was performed as described previously with minor modifications (29, 32). Briefly, timed pregnant (E14.5) mice were anaesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine cocktail. Following laparotomy, a solution containing plasmid DNA (2 μg/μl of CMV-YFP-N1 or 2 μg/μl shSin3a-ex13, shSin3a-ex16, sc-shSin3a-ex16 or shSin3a-ex13 + 2 μg/μl or 4 μg/μl mSin3a* in Tris-buffered 0.02% Fast Green) was injected through the uterine wall into the lateral ventricle of each embryo using calibrated pulled glass capillaries (WPI) and a microinjector (BTX Microject 1000, Harvard Apparatus). A series of five unipolar square-wave current pulses (33 V max) from an electroporator (ECM 830, BTX, Harvard Apparatus) were delivered over the embryo’s head using tweezer-type electrodes (BTX). Embryos were kept hydrated during the procedure, placed back into the abdomen of the mother and gestation was allowed to proceed for 3 days. Only embryos showing effective transfection in the somatosensory cortex (S1) were included for analysis and brains were processed for cryosectioning as described earlier.

Immunofluorescent staining and fluorescent microscopy

Cryosections were rehydrated in PBS (0.08 M Na₂HPO₄, 1.36 M NaCl, 0.017 M KH₂PO₄, 0.026 M KCl). Sections were incubated in normal blocking solution (1.3% normal goat serum, 1.3% normal donkey serum, 1.3% normal horse serum, 1% BSA, 0.1% glycine, 0.1% lysine, 0.4% Triton X-100; NBS) for 1h at RT. Primary antibodies, which are listed in Supplementary Table 2, were diluted in NBS incubated overnight at 4°C. Slides were washed three times in PBS for a total of 30 min at room temperature (RT). Species-specific Alexa-conjugated secondary antibodies (Molecular Probes) diluted in NBS were incubated with sections for 1h at RT. After three washes in PBS for a total of 30 min at RT, sections were incubated with blue fluorescent Nissl stain (1:500, NeuroTrace, Invitrogen) for 15 min and washed four times in PBS; 10 min each, at RT. Slides were mounted in 90% glycerol in PBS. Staining was visualized and images were captured using a Leica DMRA fluorescence microscope coupled with a DFC340FX digital camera and LASAF software.

For immunofluorescent staining of transfected COS-1 cells, cells were cultured on cover slips.Twenty four hrs post transfection, cells were washed twice with PBS and fixed with 4% PFA/PBS for 1 hour (h) at 4 °C. Next, cells were washed with PBS/50mM NH₄Cl and permeabilized with PBS/0.1% Triton-X100 (PBS-T). Subsequently, cells were incubated with the Sin3a antibody (1: 500) in blocking buffer (PBS-T/1% BSA) for 16 h at 4 °C. Following PBS-T washing steps, cells were incubated for 1 h at RT with secondary antibody (goat anti-rabbit-Alexa 488 (Molecular Probes, 1:200) in blocking buffer. Finally, cells were washed with PBS-T, PBS, MilliQ water, dehydrated with methanol, air dried and embedded in Mowiol. Microscopic imaging was performed using an Olympus FV1000 laser scanning microscope.

Cell proliferation analysis

Pregnant mothers with electroporated embryos received an intraperitoneal injection (i.p.) of 5-bromo-2’-deoxyuridine (BrdU, BD biosciences, 50 μg/g) 1.5h before euthanasia to label cells in S-phase before going into mitosis. Embryonic brains were quickly dissected, fixed, frozen and sectioned. Cryosections were incubated with NBS for 1h at RT, and incubated with chicken anti-GFP and rabbit anti-Ki67 diluted in NBS overnight at 4°C as described in earlier (29). Cryosections were postfixed for 10 min in 4% PFA, followed by a quick wash step in PBS. Subsequently, sections were treated with 0.1% trypsin in 0.1% CaCl₂ in 0.1M Tris for 10-15 sec, incubated in 100% fetal bovine/calf serum for 10 min, and rinsed in PBS. Sections were incubated in 2N HCl for 30 min at 37°C with agitation, the acid was neutralized in 0.1M sodium borate pH 8.5, and quickly washed in PBS. Standard immunohistochemistry using chicken anti-GFP, rabbit anti-Ki67 (see also Supplementary Table 2) and mouse anti-BrdU was then performed as described above in ‘Immunofluorescent staining and fluorescent microscopy’. To calculate the rate of proliferation of the transfected cells, the number of double-positive cells was determined within the PZ.
Data analyses

For assessing the number of GFP\(^+\) neurons in the electroporated area, two to seven embryos/pups were analyzed and two to five well-spaced (128 µm) sections at the same neuroanatomical level were imaged of each embryo as was described earlier (33). A 0.15 mm-wide rectangle spanning the cerebral wall was placed over the centre of the transfected area within the somatosensory cortex (S1). The cortical swatch was divided into three zones within this rectangle: proliferative zone (PZ, including the VZ and SVZ), intermediate zone (IZ) and cortical plate (CP), which were identified on the basis of cell density and polarity visualized by fluorescent Nissl. GFP-positive cells, marker-positive cells and the total number of cells stained by fluorescent Nissl were counted using ImageJ software (NIH) and Adobe Photoshop software. GFP double- or triple-positive cells were quantified in the same way. Data were either normalized to the total number of GFP-positive cells, GFP/Ki67-positive cells or the surface area. All cell number quantifications were blinded and counted by two independent individual researchers (J.S.W. and T.C.C.D.). For quantification of neurite growth, three bins of equal size (50 µm) were drawn along the axon bundle 50 µm apart and average number of neurites in each bin was determined. The total length of each bundle was determined by tracking the 20 longest neurites using NeuronJ (ImageJ plugin). Data were statistically analyzed by one-way ANOVA analysis of variance (\(\alpha = 5\%\)) using Graphpad Prism software and expressed as means ± s.e.m. Differences between groups were considered significant for \(P < 0.05\).

Results

Haploinsufficiency of SIN3A causes a distinct syndrome

To better comprehend the consequences of absence of one copy of SIN3A, or SIN3A intragenic loss-of-function in human brain development, we collected clinical features of four individuals with small deletions in chromosomal region 15q24 overlapping the SIN3A gene (individuals 1-4) and nine individuals (individuals 5-13) with loss-of-function SIN3A mutations. The clinical features of both individuals with microdeletions and individuals with dominant mutations were related and could be classified as a syndrome characterized by mild intellectual disability (defined by TIQ between 50-69), a recognizable facial gestalt and in some individuals abnormalities in brain MRIs including ventricular dilatation (colpocephaly), corpus callosum dysgenesis and subtle signs of aberrant cortical development (Fig. 1 and Table 1, further clinical descriptions in Supplementary Note). In addition, a subset of individuals with microdeletions or frameshift mutations displayed ASD, hypermobile joints, seizures, microcephaly and short stature (Table 1 and Supplementary data).

The deletions in individuals 1-4 presented here were detected by genome-wide chromosomal array analysis using different platforms (see methods section). The shortest region of overlap is 350 kb (75.60-75.95 Mb) and comprises five genes, including SIN3A. None of these genes has been previously associated with human disease. The deleted regions of individuals 1-4 and two previously reported individuals with deletions between SD C and D in 15q24 (9) are schematically shown in Supplementary Fig. 1. The SIN3A intragenic mutations were loss-of-function mutations, including c.803dup (p.(Leu269fs), individual 5), c.1010_1013del (p.(Lys337fs), individual 6), c.1759_1759delT (p.(Ser587fs) individuals 7-9), c.2955_2956delCT (p.(Glu985fs), individual 10) and c.3310C>T (p.(Arg1104*), individuals 11-13), most likely leading to nonsense mediated decay of the mRNA product and haploinsufficiency.

Cortical progenitors and newborn neurons express Sin3a

Sin3a has previously been reported to be involved in various cellular processes (17, 19, 34) which could contribute to the neurological clinical symptoms when defective. To first investigate the mRNA and protein expression patterns of SIN3A specifically in the developing human brain, we analyzed its expression using the BrainSpan Atlas of the Developing Human Brain (Allen Institute for Brain Science)(35) and in developing mouse brain from embryonic day 10.5 (E10.5) into adulthood (postnatal day P140) employing real-time quantitative (q)PCR and immunohistochemistry.
Figure 1 Haploinsufficiency of SIN3A causes a distinct syndrome. (a-g') Clinical photographs of individuals 1-4 with a 15q24 microdeletion and individuals 5, 6 and 10 with a frameshift mutation in SIN3A. (h-l) Brain MRI scans of individuals 1, 5 and 10. (a,a') Individual 1 (7 and 26 years, respectively). (b) Individual 2 (2 years). (c) Individual 3 (4 years). (d) Individual 4 (3 years and 8 months). (e) Individual 5 (3 years). (f,f') Individual 6 (2 and 13 years, respectively). (g,g') Individual 10 (4 years and 7 months and 8 years, respectively). Note the overlap in facial appearance, including a broad and high forehead (in all), full eyelids (individuals 2, 4, 5 6 and 10), depressed nose bridge in younger individuals, mildly downslanted palpebral fissures (individuals 1, 2, 4, 5 and 6), a pointed/prominent chin (in all), a small mouth (individuals 3 and 4). Later, facial phenotypes evolved into longer faces (individuals 1 and 6). (h) Transversal STIR MRI of individual 1 (25 years) showing the frontal lobes seemingly underdeveloped (quality is suboptimal). The cortex seems to consist of several small gyri without clear delineation between white and grey matter. Myelination is delayed. (i,j) Transversal and sagittal MRIs of individual 5 (3 years). (i) Ventricles are enlarged (ventriculomegaly) and white matter volume decreased. (j) MRI showing a thin corpus callosum (arrow) and enlarged cerebellar tonsils (arrowhead). (k,l) Transversal and sagittal MRIs of individual 10 (10 years). (k) Ventriculomegaly. (l) Hypoplasia of the splenium of the corpus callosum (arrow).

Transcriptome and laser microdissection (LMD) microarray analyses showed the presence of human SIN3A mRNA prenatally with the highest levels in the ventricular zone (VZ) of various cortical regions, the place where progenitor proliferation occurs (Supplementary Fig. 2). Real time qPCR analysis of developing mouse brain from embryonic day 10.5 (E10.5) into adulthood (postnatal day P140) showed that Sin3a mRNA is expressed at relatively high levels throughout brain development with a slight decrease in expression during E16.5-P14 (Fig. 2a). Additionally, we analyzed expression of Sin3a
during development by qPCR in two cortical regions; the prefrontal cortex (PFC) and the somatosensory cortex (S1) at different developmental stages (E16.5-P60) and we found a relatively high Sin3a expression levels that decreased (E16.5-P14, PFC; E16.5-P7, S1) and subsequently increased (P14-adult, PFC; P7-adult, S1) over time (Fig. 2c). To obtain a better spatial resolution of Sin3a mRNA expression we analyzed in situ hybridization patterns in adult mouse brain (36-39)(Fig. 2b). Sin3a is expressed moderately throughout the brain with a somewhat higher expression in the neurogenic regions such as the subventricular zone (SVZ, Fig. 2b’ arrow), rostral migratory stream (RMS), olfactory bulb (OB) and dentate gyrus (DG). Within the primary S1, the expression was visible in virtually all neurons with low to moderate expression levels (Fig. 2b’).

Using a specific antibody against Sin3a, we examined Sin3a protein expression in the developing mouse cerebral cortex during embryogenesis (E14.5-E18.5) and postnatal development (P7-P23). Sin3a was found in various brain regions but was especially apparent in cortical regions such as the PFC and the S1 (Fig. 2d,e). Sin3a was localized to the nucleus in apical progenitors in the proliferative zone (PZ, including the VZ as well as the SVZ), and newborn neurons in the intermediate zone (IZ) and cortical plate (CP) partially colocalizing with layer 5 marker CTIP2 (Fig. 2d-j, Supplementary Fig. 3a). Although the intensity of Sin3a immunoreactivity in the S1 decreased during development, virtually all cells within the cerebral wall were positive albeit with a high degree of cellular heterogeneity (Fig. 2f-j, Supplementary Fig. 3a). Still, the highest level of Sin3a was detected in the PZ, with fainter staining being present in the IZ and a moderate staining within the CP (Fig. 2g-j). Furthermore, Sin3a was present in actively dividing cells in the PZ as shown by their co-localisation with the proliferation marker Ki67 (Supplementary Fig. 3b).

Thus, Sin3a is expressed in a large population of cortical progenitors and young neurons during corticogenesis. These expression patterns become more restricted over time, suggestive of an expression during the neurogenic phase.

***Reduced Sin3a leads to decrease in cortical progenitors***

Initially, there is lateral cortical expansion within the VZ since neuroepithelial progenitors - radial glial cells - divide symmetrically with their offspring going into another round of division (40, 41). As development proceeds, cell cycles are getting longer and radial expansion starts by asymmetric division of SVZ progenitors generating both intermediate progenitors and postmitotic neurons (11, 41, 42). Both Sin3a expression patterns as well as the neurological indications of individuals with a Sin3a microdeletion/mutation (dilated ventricles/colpocephaly) hinted towards a role in neurogenesis. To determine the potential role of Sin3a in early corticogenesis, we employed a loss-of-function approach. Toward this aim, we used two different small hairpin RNA (shRNA) constructs targeting either exon 13 or 16 of the mouse Sin3a mRNA in an in vivo knockdown setup of Sin3a. The constructs were introduced by in utero electroporation at E14.5 and the embryos were euthanized at E17.5 (Fig. 3b). First, to validate the effectiveness of the Sin3a knockdown, we used two small interfering RNAs (siRNAs) which target on exon 13 and exon 16 of Sin3a mRNA, in a mouse neuroblastoma cell line (N2a). Real-time qPCR, using two primer pairs, showed that endogenous levels of Sin3a expression were comparable between N2a cells and mouse P35 brains, indicating a sufficiently high level to allow knockdown by siRNA transfection (Supplementary Fig. 4a). qPCR analysis using two primer pairs showed that Sin3a expression levels were significantly decreased after 48 hrs after transfection with the Sin3a siRNAs in N2a cells (Supplementary Fig. 4b). A reduction in Sin3a mRNA expression of about 40% was observed for both siRNA constructs. The sequences of the siRNAs were used to design Sin3a shRNAs that were cloned into pSUPER.GFPNeo expressing both the shRNA and GFP (Supplementary Fig. 4c). Validating these constructs by N2a cell transfection followed by measurement of endogenous Sin3a mRNA levels by qPCR showed a 50% decrease of Sin3a mRNA levels by the shRNA targeting exon 13 and a 60% decrease by the shRNA construct targeting exon 16 (Fig. 3a, *P < 0.05, ***P < 0.001).

Next, we electroporated both Sin3a shRNA constructs into the developing mouse cortex at E14.5 to study the effect of Sin3a downregulation on cortical development. Three days following electroporation (E17.5) we found that Sin3a protein levels were reduced in the shRNA-
Figure 2 Sin3a is expressed by cortical progenitors. (a) Sin3a mRNA expression levels in developing mouse brain at E10.5 and E13.5 heads and E14.5, E15.5, E16.5, E18.5, P0, P7, P14, P21 and adult brain (n = 3 series of non-related animals) measured with qPCR. Graph represents normalized mean transcript levels ± s.e.m. (b) In situ hybridization of antisense Sin3a (sagittal section of adult mouse brain). Sin3a is expressed in neurogenic regions of the subventricular zone (SVZ, arrow in b’), rostral migratory stream (RMS), olfactory bulb (OB) and dentate gyrus (DG) of the hippocampus. Scale bars, 300 µm (b) and 50 µm (b’). (c) Sin3a mRNA expression levels in developing prefrontal cortex (PFC) and primary somatosensory cortex (S1) at E16.5, E18.5, P7, P14, P21 and adult brain (n = 3 series of non-related animals) measured with qPCR. Graph represents normalized mean transcript levels ± s.e.m. (d,e) Immunostaining for Sin3a(green) and CTIP2 (red) counterstained with fluorescent Nissl (blue) in PFC and S1, respectively, of E14.5 embryos. (d’,e’) Enlargement of the boxed area in (d, e) showing Sin3a staining in grey shades. Scale bar, 50 µm for (d) and (e). (f) Quantification of the Sin3a staining (in arbitrary units, a.u.) in S1 of three E14.5 brains. (g–j) Immunostaining for Sin3a (green) and CTIP2 (red) counterstained with fluorescent Nissl (blue) in the S1 of E16.5, E18.5, P7 and P23 brains. Cb, cerebellum. Scale bars, 100 µm (d), (e), (g) and (h), 50 µm (i) and 200 µm (j).

electroporated cortices (Figure 3b, Supplementary Fig. 5a) and that reduction of Sin3a was accompanied by a significant decrease in the proportion of GFP cells within the PZ (Fig. 3c,d, Ctrl vs shSin3a-ex13, ***P = 0.000161; Ctrl vs shSin3a-ex16, **P = 0.001164; sc-shSin3a vs shSin3a-ex13, **P = 0.004362; sc-shSin3a vs shSin3a-ex16, **P = 0.001262). This reduction in cell number was confirmed by Nissl staining showing a significantly lower cell number in the Sin3a knockdown embryos as compared to control ones (Fig. 3e, Supplementary Fig. 3c, Ctrl vs shSin3a-ex13, ***P = 4.6E-05; Ctrl vs shSin3a-ex16, ***P = 0.000201; sc-shSin3a vs shSin3a-ex13, **P = 0.006892; sc-shSin3a vs shSin3a-ex16, **P = 0.002783).

The observed decrease in electroporated cell number and total cell number within the proliferative area after reduction of Sin3a could be caused by a decrease of actively dividing cells. First, to determine whether the decrease in cortical progenitors in absence of Sin3a was due to an increase in cell death, we performed a colabeling of electroporated cells with cleaved Caspase 3
Figure 3 Sin3a downregulation decreases number of cortical progenitors. (a) Normalized Sin3a expression levels in N2a cells transfected with shRNAs targeting Sin3a mRNA (shSin3a-ex13, shSin3a-ex16), a scrambled shRNA (sc-shSin3a) or a control construct expressing GFP (Ctrl). Graph represents normalized mean (n = 3) transcript levels ± s.e.m; student’s t-test (in duplicate with two distinct primer pairs). (b) Schematic of Sin3a downregulation via in utero electroporation (IUE) in cortical regions at E14.5 and sacrificed (sac) three days later. Adapted from (29). (c) Mouse cortex electroporated with Ctrl, shSin3a-ex13, shSin3a-ex16 or sc-shSin3a at E14.5 and analyzed at E17.5. Sections are immunostained for GFP (green) and counterstained with fluorescent Nissl (blue). Asterisks: cell scarcity within the proliferative zone (PZ) of Sin3a downregulated cortex. Scale bar, 50 µm for (c), (f) and (g). (d,e) Quantification of GFP+ (d) or Nissl+ cells (e) within cortical zones (150 µm width); n = 7 for Ctrl, n = 7 for shSin3a-ex13, n = 4 for shSin3a-ex16 and n = 2 for sc-shSin3a. Graph represents number of cells per mm² per embryonic zone ± s.e.m. (f,g) Cortical swatch as in (c) electroporated with either Ctrl (f) or Sin3a shRNAs (g) immunostained for GFP (green), BrdU (red), Ki67 (cyan) and counterstained with fluorescent Nissl (blue). Dashed PZ areas (f,g) are enlarged on the right (Ki67: f’,g’; BrdU: f”,g”; merged channels: f”’g”’ with colocalization in white. (h) Quantification of (percentage of total cells ± s.e.m.) GFP+/BrdU+/Ki67+ cells within cortical zones (150 µm width); n = 2 for Ctrl, n = 3 for shSin3a. (i) Quantification of GFP+/Ki67+ cells (number of cells per mm² per embryonic zone ± s.e.m.) within cortical zones (150 µm width); n = 2 for Ctrl, n = 3 for shSin3a. (j) Quantification of GFP+/BrdU+ cells (number of cells per mm² per embryonic zone ± s.e.m.) within cortical zones (150 µm width); n = 2 for Ctrl, n = 3 for shSin3a. All statistical tests on cell numbers were performed using one-way ANOVA (α = 0.05).
(CC3), a marker for caspase-mediated apoptosis. No elevated number of dead cells was observed in either control or Sin3a-deficient cortical swatches that were either GFP+ or in the vicinity of GFP+ cells to rule out the autonomous and non-autonomous effects of the transfection (Supplementary Fig. 5b). We furthermore examined the proliferation state of electroporated progenitors results indicated a clear decrease in actively dividing cells within the PZ after knockdown of Sin3a (Fig. 3f-h, Ctrl vs shSin3a, **P = 0.004197 within the PZ). To mark all cells in S-phase, we administered a BrdU pulse 1.5 hrs prior sacrifice and counted all BrdU/Ki67/GFP+ cells within the pool of Ki67/GFP+ cells. Consistent with this observation, Ki67/GFP+ (Ctrl vs shSin3a, **P = 0.002926 within the PZ, *P = 0.011132 within the IZ and **P = 0.005380 within the CP) and BrdU/GFP+ (Ctrl vs shSin3a, **P = 0.004305 within the PZ and **P = 0.009149 within the IZ) cells within the PZ become more sparse when Sin3a levels were diminished (Fig. 3i,j).

Knockdown of Sin3a in S1 cortical regions resulted in a decrease of neuroprogenitor proliferation. To further assess the role of Sin3a in generation of cortical neurons and in order to rescue the observed phenotype, we introduced a full-length shRNA-insensitive mSin3a and colabeled the electroporated cortices with Pax6, which labels apical neural stem cells, and phosphohistone H3 (pH3), a marker for mitotically active cells. The results showed a clear reduction of GFP+/Pax6+ (Ctrl vs shSin3a-ex13, **P = 0.000419; Ctrl vs shSin3a-ex16, **P = 0.001656; sc-shSin3a vs shSin3a-ex13, **P = 0.006748; sc-shSin3a vs shSin3a-ex16, *P = 0.030691; shSin3a + mSin3a* vs shSin3a-ex13, **P = 0.008304; shSin3a + mSin3a* vs shSin3a-ex16, *P = 0.032958) and GFP+/pH3+ double-labeled cells (Ctrl vs shSin3a, **P = 0.000996; shSin3a + mSin3a* vs shSin3a, **P = 0.004114) after knockdown of Sin3a which was rescued by the co-electroporation of the shSin3a-insensitive Sin3a (Fig. 4d-g).

Taken together, these results suggest that haploinsufficiency of Sin3a causes a decrease in the amount of cortical progenitors in the PZ at the peak of neurogenesis and that Sin3a is essential for early cell division and the production of neurons within the cerebral cortex.

Reduced Sin3a results in altered cortical neuronal identity

During development, neuronal progenitors will migrate along radial glia cells to the correct cortical layer in an inside-out fashion (43). Distinct transcription factors mark layer-specific cortical neurons which control their identity (44-46). To further assess whether the reduction of Sin3a within cortical proliferative zones had an effect on migration or identity of cortical progenitors, we immunostained for CTIP2, a layer 5 marker, and Brn2, a marker controlling the identity of upper-layer neurons (layer 2/3)(Fig. 5a,b). At E17.5, number of GFP+ neurons expressing CTIP2 was comparable between control and shSin3a-electroporated cortex in IZ and CP (Fig. 5a,c), although there seemed to be less GFP+/CTIP2+ cells within the IZ when Sin3a was downregulated (Fig. 5c). The number of GFP+ cells expressing Brn2 however was significantly lower in PZ of the cortex electroporated with shRNAs for Sin3a and seemed to be slightly higher in IZ (Fig. 5b,d, Ctrl vs shSin3a-ex13, **P = 0.00225; Ctrl vs shSin3a-ex16, **P = 0.003335; sc-shSin3a vs shSin3a-ex13, *P = 0.031708; sc-shSin3a vs shSin3a-ex16). The GFP+/Brn2+ number was restored to relatively normal levels when shSin3a-insensitive Sin3a was co-electroporated (Fig. 5d, *P = 0.039242; shSin3a + mSin3a* vs shSin3a-ex13, *P = 0.02463; shSin3a + mSin3a* vs shSin3a-ex16, *P = 0.035403 within the PZ). These data suggest that neurons with lower Sin3a levels have changed their identity which implies that Sin3a is required for differentiation of cortical progenitors.

Sin3a affects cortical differentiation and axon elongation

Within their target cortical layers, postmitotic neurons will start extending their leading and trailing processes to become functional dendrites and axons, respectively (47, 48). Trailing processes within the IZ will navigate toward the corpus callosum by extension (49). We explored characteristics of electroporated neurons within the CP in their ability to connect to the contralateral cortex by axon elongation when Sin3a was downregulated. We first checked at E17.5 whether the neuronal polarity (multipolar-to-bipolar transition) was affected. Categorizing the newly generated neurons in the IZ of either control or shSin3a-electroporated S1 on the basis of their morphology (multipolar, bipolar, unipolar) and assessing their percentages, showed no significant difference in various types of
Figure 4 Proliferation phenotype can be rescued by introduction of shRNA-insensitive Sin3a. (a) Schematic representation of the pCAB shRNA-insensitive mSin3a showing site-directed mutagenesis of the two shRNA target areas (yellow). (b) Western blot showing overexpression of the mSin3a* protein after transfection in COS-1 cells (black arrow). (c) COS-1 cells showing endogenous nuclear Sin3a staining (upper panel) or upregulation of Sin3a expression within the nucleus after overexpression (white arrows, lower panel). Scale bar, 10 µm. (d,e) Representative images are shown of the S1 PZ electroporated with either Ctrl, shSin3a-ex13, shSin3a-ex13, sc-shSin3a and shRNA for Sin3a together with the rescue construct (shSin3a + mSin3a*). Sections are immunostained for GFP (green), Pax6 (red, d) or pH3 (red, e) and counterstained with fluorescent Nissl (blue). Scale bar, 50 µm for (d) and (e). (f) Quantification of the number of GFP/Pax6-positive cells within the PZ; n = 5 for Ctrl, n = 5 for shSin3a-ex13, n = 3 for shSin3a-ex16, n = 2 for sc-shSin3a and n = 2 for shSin3a + mSin3a*. Graph represents number of GFP/Pax6+ cells per mm² ± s.e.m. One-way ANOVA (α = 0.05). (g) Quantification of the number of GFP/PH3+ cells within the PZ; n = 4 for Ctrl, n = 5 for shSin3a and n = 2 for shSin3a + mSin3a*. Graph represents number of GFP/PH3+ cells per mm² ± s.e.m. One-way ANOVA (α = 0.05).

neurons within the IZ in control compared to shSin3a-electroporated animals (data not shown). However, inspection of callosal axons emerging from transfected neurons indicated that downregulation of Sin3a increased both the length (Ctrl vs shSin3a-ex13, **P = 1.88E-06; Ctrl vs
Reduced Sin3a affects Sin3a-partner and -target expression

Given its expression in cortical progenitors and abnormal proliferation and cell fate patterns in Sin3a knockdown mouse embryos, we asked whether knockdown of Sin3a in N2a cells would affect expression levels of well-known Sin3a binding partners MeCP2a and MeCP2b (50, 51) or downstream targets of Sin3a that are known to play a role in proliferation/differentiation such as Nanog (19, 52, 53), CyclinD1 (17, 54), Cdkn1a (55, 56) and E2f1 (31, 57, 58). N2a cells were transfected with either shSin3a-ex16, ***P = 0.000802; shSin3a + mSin3a* vs shSin3a-ex13, ***P = 0.000876; shSin3a + mSin3a* vs shSin3a-ex16, *P = 0.040822 for bin 0-50 µm; Ctrl vs shSin3a-ex13, ***P = 4.18E-05; Ctrl vs shSin3a-ex16, **P = 0.001018; shSin3a + mSin3a* vs shSin3a-ex13, **P = 0.003374; shSin3a + mSin3a* vs shSin3a-ex16, *P = 0.017581 for bin 100-250 µm; Ctrl vs shSin3a-ex13, **P = 6.4E-05; Ctrl vs shSin3a-ex16, *P = 0.027764 for bin 200-250 µm) as well as the number (Ctrl vs shSin3a-ex13, ***P = 1.35E-05; Ctrl vs shSin3a-ex16, ***P = 2.8E-05; shSin3a + mSin3a* vs shSin3a-ex13, *P = 0.025997; shSin3a + mSin3a* vs shSin3a-ex16, *P = 0.026939) of axons following the callosal path and crossing the midline, with some axons deviating from the original tract (Fig. 6a-f). These results strongly support a role for Sin3a in cortical neuron differentiation and callosal axon elongation in vivo.

Figure 5 Diminished Sin3a results in an altered layer-specific identity of cortical progenitors. (a) Mouse cortex electroporated in utero (IUE) at E14.5 with Ctrl or shSin3a-ex13, shSin3a-ex16, sc-shSin3a and shSin3a + mSin3a* and analyzed at E17.5. Sections were double-stained with CTIP2 (a, red) and Brn2 (b, red) and counterstained with fluorescent Nissl (blue). Scale bar, 50 µm for (a) and (b). (c) Quantification of the GFP and CTIP2 double-positive cells within the embryonic zones; n = 4 for Ctrl, n = 4 for shSin3a-ex13, n = 3 for shSin3a-ex16, n = 2 for sc-shSin3a and n = 2 for shSin3a + mSin3a*. Graph represents number of GFP/CTIP2+ cells per mm² within the embryonic zones ± s.e.m. One-way ANOVA (α = 0.05). (d) Quantification of the GFP and Brn2 double-stained cells within the embryonic zones; n = 4 for Ctrl, n = 4 for shSin3a-ex13, n = 3 for shSin3a-ex16, n = 2 for sc-shSin3a and n = 2 for shSin3a + mSin3a*. Graph represents number of GFP/Brn2+ cells per mm² within the embryonic zones ± s.e.m. One-way ANOVA (α = 0.05).

Reduced Sin3a affects Sin3a-partner and -target expression

Given its expression in cortical progenitors and abnormal proliferation and cell fate patterns in Sin3a knockdown mouse embryos, we asked whether knockdown of Sin3a in N2a cells would affect expression levels of well-known Sin3a binding partners MeCP2a and MeCP2b (50, 51) or downstream targets of Sin3a that are known to play a role in proliferation/differentiation such as Nanog (19, 52, 53), CyclinD1 (17, 54), Cdkn1a (55, 56) and E2f1 (31, 57, 58). N2a cells were transfected with either...
shRNAs of Sin3a or control constructs (sc-shSin3a or Ctrl) for 48 hrs. We then analyzed transcript levels of MeCP2a, MeCP2b, Nanog, CyclinD1, Cdkn1a and E2f1 by qPCR. Upon downregulation of Sin3a (Fig. 3a), the expression levels of the binding partners in the repressor complex, MeCP2a and MeCP2b, were comparable to that of control levels (Fig. 7a and Supplementary Fig. 4i). In contrast to this, we found that expression levels of the target Nanog was reduced by 50-60% (Fig. 7b), i.e. in a similar fashion with Sin3a. We found no significant change in transcript levels of CyclinD1 or Cdkn1a (Supplementary Fig. 4h,j). Of note, the knockdown of Sin3a significantly de-repressed the levels of E2f1 by approximately 30% (Fig. 7c). These findings suggest that Sin3a is a key member in the complex regulating the expression levels of transcription factors involved in proliferation/differentiation (Fig. 7d).

Figure 6 Knockdown of Sin3a leads to a neurite outgrowth defect of cortico-cortical projections. (a) A Ctrl electroporated cortex showing early neurite outgrowth in the intermediate zone (IZ). Schematic on the right shows the electroporated area in the somatosensory cortex (S1) (green) with the extending neurites and their projection path towards the corpus callosum (CC; grey dotted line). Scale bar, 100 µm for (a–d). (a') Enlargement showing extending neurites as indicated in (a). (b) A shSin3a-ex13 electroporated cortex showing early neurite outgrowth in the IZ. (b') Enlargement showing aberrant extending neurites as indicated in (b). (c) A shSin3a-ex16 electroporated cortex showing early neurite outgrowth in the IZ. (c') Enlargement showing aberrant extending neurites (c). (d) A Sin3A shSin3a-ex13 together with the rescue construct (shSin3a + mSin3a*) electroporated cortex showing early neurite outgrowth in the IZ. (d') Enlargement showing extending neurites as indicated in (d). (e) Quantification of neurite number within each of the three bins (dotted box in a; n = 7 for Ctrl, n = 7 for shSin3a-ex13, n = 3 for shSin3a-ex16 and n = 2 for shSin3a + mSin3a*). Graph represents mean number of extending neurites ± s.e.m. One-way ANOVA (α = 0.05). (f) Quantification of the length of the 10 longest neurites; n = 7 for Ctrl, n = 7 for shSin3a-ex13, n = 3 for shSin3a-ex16 and n = 2 for shSin3a + mSin3a*). Graph represents the mean length of 10 longest neurites ± s.e.m. One-way ANOVA (α = 0.05).
Figure 7 Knockdown of Sin3a mRNA in N2a cells by shRNAs affects Nanog and E2f1 expression. (a) Normalized mRNA expression levels of co-repressor MeCP2a did not change 48 hrs after knockdown of Sin3a (n = 3). (b) Normalized mRNA expression levels of Nanog decreased 48 hrs after knockdown of Sin3a (n = 3). (c) Normalized mRNA expression levels of E2f1 increased 48 hrs after knockdown of Sin3a (n = 3). Graphs represent normalized mean transcript levels ± s.e.m. Student’s t-test, 0.1 > P > 0.05,*P < 0.05,**P < 0.01, ***P < 0.001. (d) Schematic model representing the function of Sin3a in neuronal progenitors.

Discussion

In this study we show that loss-of-function of the SIN3A gene in humans is associated with a distinct intellectual disability/developmental delay syndrome and identify SIN3A as a key factor in corticogenesis. The considerable number of affected individuals allows us to draw firm conclusions relating SIN3A variations to clinical symptoms of the syndrome. Together with the complementary data generated by mouse in utero electroporation studies, we establish Sin3a as an important regulator of mammalian cerebral cortex development.

SIN3A adds to a number of genes encoding epigenetic factors that are implicated in intellectual disability and ASD (59). Moreover, SIN3A interacts with various proteins that have already been shown to contribute to intellectual disability phenotypes and display a neuronal function, such as MECP2, HDAC and MLL (25, 60). Interestingly, one of the best-studied and closest interactor is the MeCP2 protein which is associated with Rett syndrome (OMIM #312750) (60, 61). Loss of MeCP2 in mice causes synaptic defects in neural circuit development by dysregulating GABAergic transmission and cortical excitability (62, 63). This impaired cortical circuit functioning is thought to underlie the loss of motor and cognitive abilities and the impaired social interactions seen in individuals with Rett syndrome (63-67).

The phenotype we observed in the individuals with loss-of-function mutations in SIN3A is highly similar to the phenotype in individuals with microdeletions, strongly supporting a causal role for haploinsufficiency of SIN3A in the reported intellectual disability/developmental delay phenotypes of the individuals with a 15q24 microdeletion between SD blocks C and D. The level of intellectual disability/developmental delay is in the mild to low normal range which explains the presence of inherited changes in two unrelated families with a mildly affected parent and more severely affected children. This finding is in agreement with a recent report on a familial 15q24 deletion encompassing SIN3A segregating in twins and their father (68). Besides intellectual
disability/developmental delay, ASD features were present in six individuals. One potential loss-of-function variant in SIN3A was found to be present in the ExAC population database. However, this variant is classified as “low confidence”, as this was derived from a study with low sequencing coverage. Furthermore, the variant is not localized within the coding sequence of any SIN3A transcript, and therefore it is likely not of functional significance.

Other shared clinical features include a marked overlap in facial gestalt, microcephaly, a (relatively) small head circumference, (relatively) short stature, hypermobile joints, hearing loss and ectodermal symptoms such as thin hair. In addition to ASD, obsessive compulsive behaviors and attention and concentration problems were observed in several individuals. We also noticed a history of mild seizures in three individuals. Cerebral imaging (MRI) was performed in eight out of ten indexed individuals. Dilated ventricles/calocephaly and corpus callosum dysgenesis were the most consistent abnormalities observed. Of note, four individuals showed some irregularities of the cortex (Fig. 1h-j), though these abnormalities were subtle and evaluation of the MRI scans was hampered by suboptimal quality. Altogether, the observed human phenotypes prompted us to study the role of Sin3a in brain development.

Mouse Sin3a protein closely resembles its human orthologue (69). The in utero-mediated gene transfer to knockdown Sin3a in mice produced a clear cortical phenotype that resembles the human symptoms. Although there are differences between human and mouse manifestations, the observed phenotype is reminiscent of disturbances in early cell proliferation. Of note, in a significant number of the human cases (6/13, 46%), microcephaly is observed which seems in line with impaired proliferation. The observed enhanced neurite outgrowth in Sin3a knockdown mice can be a cause of premature neuronal cell cycle exit normally migrating towards layer II/III of the CP. By becoming postmitotic at the wrong time and place, they start to extend their neurites too early which might be the prerequisite of the longitudinal callosal projections or Probst bundles often observed in callosal dysgenesis (49, 70).

During the last couple of years, genetic studies, fueled by the emergence of whole-exome/genome sequencing, have identified a number of genes involved in cortical malformations (64, 66, 71, 72). Some of these genes are involved in early proliferation of progenitors, resulting in primary microcephaly and intellectual disability when mutated. For example, mutations in WDR62 cause severe cortical malformations including microcephaly, pachygyria with cortical thickening as well as hypoplasia of the corpus callosum (73). Due to spindle instability, cortical progenitors lacking WDR62 will undergo premature differentiation eventually leading to mitotic arrest and cell death that underlie the malformations (72, 74-76). Another transcription factor, Tbr1, regulates axonal projections from the amygdala (77) and regional and laminar fate of the developing cortex (78, 79). Next Generation Sequencing (NGS) revealed that de novo TBR1 mutations cause ASD as well as intellectual disability (30, 77, 80, 81).

The observed phenotype in absence of Sin3a raises furthermore the possibility that the diminished number of Brn2-positive neurons in absence of Sin3a is a result of increased differentiation of cortical progenitors. This is in line with the neurite outgrowth results showing premature outgrowth when Sin3a levels are downregulated. This will most likely result in aberrant projections leading to compensatory mechanisms such as pruning of the projections as they are hampered in getting to the correct target area (33). More experiments using BrdU in a cell-cycle exit and cell fate paradigm will shed light on the exact functioning of Sin3a.

As a transcriptional regulator, the SIN3/HDAC/MECP2 co-repressor complex is involved in diverse functions during various phases of life including embryonic development (17, 34, 82). For example, in embryonic stem cells, expression of Nanog is upregulated via the SIN3/HDAC complex and downregulated during differentiation (17, 52, 83). Interestingly, in our studies, reduced Sin3a expression within N2a cells led to lower Nanog expression levels. E2f1, as a Sin3a downstream target, is inversely correlated with the proliferation rate of cerebellar progenitors and is upregulated postnatally (57). A phenomenon we can clearly correlate with the proliferation rate of cortical progenitors that was diminished following Sin3a knockdown. Yet, how these downstream targets relate to the transcription factor Brn2 or any other marker for layer-specific cortical identity needs to
be elucidated. Knockdown studies in mice have demonstrated that Sin3a is involved in embryogenesis (22) and synaptic plasticity in the rodent forebrain (25, 26). However, we found for the first time that Sin3a is crucial for the early steps in cortical development such as proliferation, cell fate and axon outgrowth.

Development of the human cerebral cortex is a tightly orchestrated process that is unique among all vertebrates. The timely events of cortical proliferation, neuronal migration, differentiation, axonal guidance and connectivity are a prerequisite for the higher-order functioning of human beings. The clinical observations together with the results of our functional studies in mouse brain revealed a crucial role for Sin3a in these processes. The present era of high-throughput genome sequencing in combination with brain imaging in intellectual disability/developmental delay and/or ASD alongside preclinical cellular and animal studies will allow us to unveil other important players in the development of cortical integrity.


Acknowledgments
We are grateful to the families and subjects participating in this study for their involvement. This work was supported by funding from Science without Borders, CAPES-Brasil (BEX 12044/13-0) to T.C.D.D complemented with extra support by the Educational Institute Biosciences Radboud University Nijmegen, by grants from The Netherlands Organization for Health Research and Development, ZonMw (grant 907-00-365) to T.K., the Dutch Brain Foundation (HsN F2014(1)-16) to J.E.V. and the German Ministry of Research and Education (grant numbers 01GS08164, 01GS08167, 01GS08163 German Mental Retardation Network) to H.E. and T.M.S., as part of the National Genome Research Network. We thank lab members, colleagues and the reviewers for critically reading this manuscript and members of the various labs for helpful editing and discussions. We express thanks to Dr. W. Hendriks (Dept. Cell Biology, Radboud University Nijmegen) for sharing plasmids and Dr. N. Nadif Kasri (Dept. Human Genetics, Radboud University Medical Centre, Nijmegen) for kindly providing mouse anti Ki67. We are grateful for the mouse Sin3a cDNA clone from Dr Robin Floyd and Dr Brian D. Hendrich (Wellcome Trust Centre for Stem Cell Research and MRC Centre for Stem Cell Biology and Regenerative Medicine, University of Cambridge). We thank the RIMLS microscopy platform (for URL, see above) for excellent support and maintenance of the equipment.

Author Contributions

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.
References


Table 1. Clinical features of six patients with 15q24 microdeletions comprising SIN3A and nine patients with a loss-of-function mutation in SIN3A.

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**Table 1.** Clinical features of six patients with 15q24 microdeletions comprising SIN3A and nine patients with a loss-of-function mutation in SIN3A.
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dn: *de novo*; DD: developmental delay; ID: intellectual disability; NR: not reported; NP: not performed; P: percentile; +: mild ID; +/-: low-normal IQ; CD: cortical dysgenesis; CC: corpus callosum dysgenesis; WM: white matter abnormalities; VD: ventricle dilatation; H: hypermobile joints; D: delayed bone age; TH: thin hair; N: brittle nails T: teeth anomalies. *Mb positions of the breakpoints of the deleted regions on chromosome 15 are indicated (UCSC genome browser, version Hg 19) # SIN3A NM_001145357.1
Supplementary Note

Clinical phenotype description

Individual 1
The pregnancy was complicated by intra-uterine growth retardation since 20 weeks of pregnancy. She was born preterm at 36 weeks of pregnancy duration with a low for gestational age birth weight of 1,140 grams (<P2.3). Birth was complicated by neonatal hypoxia and an intracranial hemorrhage was suspected. She was admitted to a neonatal care unit for six weeks. Cerebral ultrasound examination revealed wide cerebral ventricles. Her motor development was delayed. This was partly attributed to joint hyperlaxity. She learned to walk at the age of 2 years with the help of a physiotherapist. Her cognitive development was mildly delayed as well. A formal intelligence test was not performed. At the age of 29 years she lived in sheltered housing. She had psychiatric problems, including a conversion disorder with psychogenic seizures, and an autism spectrum disorder. Medical problems included diabetes mellitus type 2 since the age of 15 years with insulin treatment since the age of 26 years, progressive weight gain and she had complaints of fatigue and dizziness. She had severe constipation with distention of the rectum and distal colon and had suffered from bladder retention for which catheterization was necessary. Vision was impaired by bilateral cataract for which she was operated at the age of 26 years and she had hypermetropia (+2/+2.75 Dioptry). Since the age of 1 year she was wearing hearing aids because of bilateral sensorineuronal hearing loss of 50 dB. She underwent a hysterectomy because of excessive menstrual bleedings and has a very narrow introitus. At the age of 17 years brain MRI scan showed an irregular aspect of the cortex with irregular gyri and sulci, ventricle dilatation, corpus callosum dysgenesis and a low volume of white matter. Because of weakness of her leg musculature she went to a physiotherapist. Upon physical examination at the age of 26 years she had a height of 158 cm (P2), weight of 75 kg (P99) and a head circumference of 51.5 cm (0.6th to 2nd centile). Facial dysmorphism included full eyelids, slightly downsloped palpebral fissures, deeply set eyes, high nasal bridge, flat philtrum, thin upper lip and pointed chin (Fig. 1a,a’). In addition, she had small hands and very small feet, clinodactyly of the 5th fingers and flexions contractures of the distal interphalangeal joints of the 4th fingers with hypoplastic creases. A 105K oligo array analysis revealed a de novo 500 kb deletion of chromosomal region 15q24.2 (75.6-76.1 Mb, Hg 19).

Individual 2
The pregnancy was complicated by maternal hypertension at 38 weeks of pregnancy. She was born at 39 +6 weeks of pregnancy after induced but uncomplicated birth with a birth weight of 3,250 grams (25th centile), and head circumference of cm (5th-10th centile). Her motor development was delayed; she could walk with support at 18 months. Her cognitive development was mildly delayed as well. A formal intelligence test was not performed. In her first year she developed seizures for which she was successfully treated with valproic acid. Brain MRI showed an irregular aspect of the cortex in the frontal regions, corpus callosum dysgenesis and delayed myelinisation. Physical examination at the age of 2 years and one month revealed a height of 86.5 cm (25th centile), weight of 11.16 kg (10th centile) and a head circumference of 45.3 cm (3rd centile). She had downsloped palpebral fissures, deeply set eyes, a small mouth and a broad, overhanging nasal tip (Fig. 1b). In addition, her 2nd toes were positioned crossing the 3rd toes. A 180K Agilent micro array analysis revealed a de novo ~500 kb deletion of chromosomal region 15q24.2 (75.6-76.1 Mb, Hg 19).

Individual 3
This individual is one of naturally conceived, non-identical female twins. She and her sister were born at 35 week gestation following spontaneous rupture of membranes. Her birth weight was 1950g (<P16)(normal twin 2070g). On day four she developed jaundice and was admitted for phototherapy, requiring a 5 day hospital stay. Early developmental milestones were a little delayed, having sat at 8 months and walked at 18 months. Expressive speech was also delayed, but felt to have caught up to age appropriate by 4 years. She attends mainstream school, with significant fine motor problems.
resulting in a poor pencil grip, immature writing and difficulties holding cutlery resulting in very messy eating. Assessment at 8yrs shows her to be 2 years behind her peers for academic subjects, but she is felt to have age-appropriate social skills. Additional issues have been an episode of anxiety at 4 yrs, resulting in her pulling on her hair and causing a bald patch and the need for supportive orthotic boots for ankle hypermobility until the age of 8 years. Her medical history has included recurrent glue ear and tonsillitis with sleep apnoea necessitating a tonsillectomy, and frequent urinary tract infections. She has mild hypermetropia with a prescription of +2 diopters bilaterally. Growth parameters at 4 years were height 96.8cm (5th centile), weight 15.4kg (50th centile) and head circumference 48cm (10th). She has dysmorphic facial features with a broad and tall forehead, short palpebral fissures with shallow orbits, a very low nasal bridge with a small nose and mouth and pointed chin (Fig.1c). She has generalized joint hypermobility. 60-Mer oligonucleotide array (ISCA) revealed a ~400 kb de novo deletion of chromosomal region 15q24.2 (75,60-76,02 Mb, Hg 19).

**Individual 4**
This male was born at term with a normal birthweight (3280 gram, P50). During the first months he had feeding difficulties due to gastroesophageal reflux. A slight motor delay was noticed with sitting at the age of 9 months and walking at the age of 21 months. Speech development was mildly delayed as well. At the age of 2 years he spoke his first words. At the age of 3 years and 8 months he started to use short simple sentences. Comprehension of spoken language was better than expression. A formal developmental test was not performed. Behaviour was unremarkable. He had no congenital anomalies and hearing and vision were normal. She had difficult bowel movements. At the age of 3 years and 8 months he had a height of 98 cm (P16), weight of 13.8 kg (16th centile) and a head circumference of 49.4 cm (25th centile). Facial dysmorphism included a triangular face, broad and high forehead, downslanted palpebral fissures, epicanthic folds, a small mouth and pointed chin (Fig. 1d). A 60K array CGH analysis revealed a ~350 kb microdeletion in chromosomal region 15q24.2 (75.60-75.95, Hg 19). The deletion was confirmed by metaphase FISH. Segregation analysis in the parents with FISH showed that the deletion had occurred de novo.

**Individual 5**
This male was born after a complicated pregnancy. At 18 weeks of pregnancy his mother underwent a cervical cerclage. At 20 weeks, moderate dilatation of the cerebral ventricles was noticed. He was born by Caesarean section at 38 weeks of pregnancy. His birth weight was 2410 gram (2nd centile) The neonatal period was complicated by respiratory problems for which he needed 4 days assisted ventilation with continuous positive airway pressure (CPAP). He was treated with antibiotics because of a Streptococcus group B in his mother. After 2 weeks at a neonatal care unit he was discharged. The early motor development was unremarkable. He learned to walk at the age of 14 months. His muscle tone was relatively low and he had hypermobile joints. Speech development was delayed. At the age of 7 years he was able to speak simple sentences, but did not always understand the content. Formal intelligence tests measured an intelligence quotient of 55. His behavior was characterized by an autism spectrum disorder with temper tantrums, compulsive behaviours and sleeping problems. At the age of 4 years he was diagnosed with frontal lobe epilepsy, which was under good control with Depakine and Clobazam. His brain MRI showed dilated ventricles, bilateral polymicrogyria of the insula corpus callosum hypoplasia, dilated ventricles and enlarged cerebellar tonsils (Fig. 1j). He was diagnosed with a bilateral conductive hearing loss of 30-35 dB on the left and 40-45 dB on the right. Upon physical examination at the age of 3 years he had a height of 85 cm (4 cm 0.6th centile) and a head circumference of 48.8 cm (16th centile). At the age of 7 years he had a height of 112 cm (0.6th centile). Facial dysmorphism included a high and broad forehead, downslanted palpebral fissures, bilateral telecanthic/epicanthic folds, long and smooth philtrum, thick helices of the ears, upturned ear lobes and a pointed chin (Fig. 1e). He had small and convex nails, eczema and small and narrow bluish coloured teeth. His hair was thin and curly. He had hypermobile joints and his muscle tone was low-normal. His phenotype showed similarities to cardio-facio-cutaneous syndrome. Though, DNA analysis of BRAF, MAP2K1 and MAP2K2 revealed no abnormalities. 250 K SNP array analysis showed
a normal male karyotype. Subsequently family based exome sequencing analysis was performed. A de novo frameshift mutation in SIN3A (NM_001145357.1;c.803dup (p.(Leu269fs))) was detected.

**Individual 6**
This female was the 4th child born to a non-consanguineous healthy couple after a normal pregnancy at 41+4 weeks gestational age with a birth weight of 3730 gram (75th centile). The neonatal period was slightly complicated by respiratory problems for which she needed 1 day assisted ventilation. The congenital hearing test was repeatedly abnormal and it appeared that she had a congenital mixed hearing loss predominantly left and additional to that, a right-sided choroidal scar and a lateral neck fistula. At the age of 2 years she was diagnosed with absences for which she received treatment for few years, but after that age, seizures never reoccurred. EEG tests were repeatedly performed but never revealed abnormalities. Both her language/speech and motor development were delayed and she had dyspraxia. She walked independently at the age of 24 months. Several IQ tests have been conducted and revealed different results related to the ages of testing: SON-IQ 90 at the age of 32 months, WISC-IQ 62 (verbal 72; nonverbal 57) at the age of 10 years. So there was a large discrepancy between verbal and performal capacities. Her muscle tone was relatively low and she had hypermobile joints. Her behavior was characterized by friendly and quiet behavior with adequate contact making, though there were significant problems with attention and concentration.

At the age of 15 months, a cerebral MRI showed enlarged intra- and extracerebral spaces, but the MRI was not optimal because of movement effects.

Upon the most recent physical examination at the age of 13 years she had a slender built with a height of 165 cm (50th centile) and a head circumference of 55.6 cm (75th centile). Facial dysmorphism included a high and broad forehead, downsloaning palpebral fissures, bilateral telecanthic/epicanthic folds, long and smooth philtrum, thick helices of the ears, upturned ear lobes and a pointed chin (Figure 1f,f’). She had small hands and feet. She had hypermobile joints and her muscle tone was low-normal. Because of the neck fistula and hearing loss, DNA analysis of EYA1 was performed but revealed no abnormalities. 250 K SNP array analysis showed a normal female karyotype. Subsequently family based exome sequencing analysis was performed. A de novo frameshift mutation in SIN3A was detected: c.1010_1013del (p.(Lys337Serfs)).

**Family 1 (individuals 7, 8 and 9)**
This individual is the 4th child of healthy non-consanguineous Ukrainian and German parents. The individual’s father (individual 8) had difficulties in learning but finished school in Russia. He had no professional training and works as a sawyer. The older sister (individual 9) of the individual shows a more severe intellectual disability, suffers from hearing impairment for which she wears hearing aids. She attended special school and works in a sheltered workshop now. A paternal cousin is said to have Down’s syndrome (no reports available).

Individual 7 was born after a pregnancy complicated by vaginal bleeding at a gestational age of 42 weeks by spontaneous vaginal delivery with low birth weight of 2,750 grams (<3rd centile), normal length of 50 cm (10th centile), and normal head circumference of 36 cm (50th centile). Especially in the first seven months of life, muscular weakness and only little active movement were described by the mother. He was able to crawl at the age of 9 months and to walk without support at the age of 14 months. Development of speech was described as normal. Because of difficulties in learning he left primary school in his third year and started attending special school. A formal intelligence test measured an intelligence quotient of 60. In childhood he was treated with growth hormones because of short stature. At the age of 10 years a retarded bone age was diagnosed. Electroencephalography (EEG) gave normal results. He wears glasses because of myopia. Besides a phimosis no other malformations were present. Upon physical examination at the age of 16 years, all growth parameters were below the normal range with a height of 161 cm (<3rd centile), weigh of 40 kg (<3rd centile), and head circumference of 52 cm (<3rd centile). Height and head circumference of the parents were in normal ranges. He had facial dysmorphisms similar to his father, including a prominent forehead, frontal bossing, prominent nose, short philtrum, pointed chin and slightly large
ears. Conventional karyotyping, Multiplex Ligation-dependent Probe Amplification (MLPA, MRC Holland, probe sets P036E1, P070 and P245) and chromosomal microarray analysis gave normal results. Subsequently exome sequencing was performed and revealed a paternally inherited frameshift mutation in SIN3A in the index and his affected sister: c. 1759_1759 delT (p.Ser587fs).

Individual 10
This individual was born after an uneventful pregnancy after 41 weeks of gestation with a birth weight of 3070 grams (5th centile). After birth, hypospadias and phimosis were seen, which were surgically corrected. He was diagnosed with celiac disease after introduction of solid food. His development was delayed, with speech delay more prominent then motor delay. He could walk without support after 15 months. He attended special education. A formal intelligence test showed an total intelligence quotient of 64 with discrepancies between verbal (IQ 70) and performal (IQ 54). At the age of 9 years, he was diagnosed with autism spectrum disorder. His growth parameters were all within the normal range, at the age of last investigation (9.5 years) his height was 141.5 cm (50th-75th centile), weight was 37.6 kg (84th-98th centile) and head circumference was 54 cm (50th centile). Facial dysmorphic features included a broad forehead, slightly downslanting palpebral fissures, a flat nasal bridge with an upturned nasal tip, large fleshy and upturned earlobes and a small mouth with a high palate. His joints were hypermobile. His fifth fingers were short with clinodactyly and the nails of his halluces were upturned. His skin was hyperkeratotic. His hair was normal. Conventional karyotyping, chromosomal microarray analysis, PCR analysis of the FMR1-gene, direct sequencing of exons 21 and 22 of the MED12-gene (FG-syndrome), all exons of the UPF3B-gene (Lujan-Fryns syndrome), and direct sequencing and MLPA of the ZFHX1B-gene (Mowat-Wilson syndrome) all were normal. Exome sequencing revealed a de novo mutation in SIN3A: c.2955_2956delCT (p.(Glu985fs).

Family 2 (Individuals 11, 12 and 13)
Individual 11 was born by caesarean section at 40 weeks of pregnancy with a birth weight of 2950 gram (P5-P10) and no neonatal complications. At the age of 2 years a delay in speech development was noted. His motor development was normal, with sitting at the age of 6 months and walking at the age of 14 months. He has a tendency for walking on his toes and his movements are characterized by his parents as clumsy and he falls easily. He has a hyperactive behavior and is treated with melatonin because of sleeping problems. He has a mild hypermetropia (+1.5/+2 Dioptry) and normal hearing. Because of recurrent glue ears a tonsillectomy was performed. He attends a normal primary school but receives individual support for his language problems. A formal intelligence test has not been performed. No epilepsy or seizures occurred. He has constipation and feeding problems; he eats little and needs stimulation to increase his intake. Physical examination at 4 years and 10 months of age revealed a height of 98.4 cm (< 0.6th centile), weight of 13 kg (< 0.6th centile) and a head circumference of 48.4 cm (between 2nd and 16th centile). He had prominent ears, short down slanting palpebral fissures, a high broad forehead with a pointed chin and mild fifth finger clinodactyly. A brain MRI showed no abnormalities. A clonidine test showed no evidence for a growth hormone deficiency. A metabolic screening test in urine was normal. 180K Agilent microarray analysis revealed a paternally inherited 567 kb 5q14.1 duplication (80,425,911-80,993,522 Mb, hg19) with no known clinical relevance. Exome sequencing was performed and a heterozygous nonsense mutation in SIN3A (NM_001145357.1;c.3310C>T (p.Arg1104*)) was identified. The older brother (individual 12) of the index individual was born by caesarean section at 42 weeks gestation with a birth weight of 3750 gram (P50-P75). There were no neonatal complications. He has a developmental delay, clumsy movements and behavioral problems consisting of an attention deficit hyperactivity disorder, temper tantrums and an autism spectrum disorder. A formal intelligence test measured a total intelligence quotient of 65, and from the age of 4 years he attended special schools for children with learning disorders. At the age of 9 years, he was treated for a urethral meatus stenosis because of obstructive urinary flows. For several years during childhood he kept having problems of nocturnal enuresis and incontinence for urine. He had no visual or hearing problems and no epilepsy or
seizures. On physical examination at 11 years of age his height was 134 cm (between 0.6th and 2nd centile), his weight 33.5 kg (between 2nd and 16th centile) and his head circumference 49.1 cm (<0.6th centile). He had slightly prominent ears, down slanting palpebral fissures, epicanthic folds, a prominent maxilla, fifth finger clinodactyly and mild 2-3 syndactyly of the toes. His penis was small (3.3 cm, <P10) and he had cryptorchid testes. On a brain MRI a deep sulcus reaching the falk bilaterally in the superior frontal gyrus was seen, which was considered as a possible form of dysgyria. An extensive screen for metabolic disorders in blood and urine was normal. DNA analyses for fragile-X syndrome (FMR1), Ohdo syndrome (KAT6B), Opitz-Kaveggia syndrome (MED12) and Borjeson-Forssmann-Lehman syndrome (PHF6) showed no pathogenic mutations. As in his younger brother, a paternally inherited 567 kb 5q14.1 duplication with no known clinical relevance was found with microarray analysis. At the age of 14 years exome sequencing revealed a heterozygous mutation in SIN3A (NM_001145357.1;c.3310C>T (p.(Arg1104*)). The same mutation was identified in the mother of these boys (individual 13). She has a short stature (153 cm, <0.6th centile) and had some learning difficulties as a child. She attended a regular primary school, a lower level occupational secondary school and now works as a shop assistant. No formal intelligence test was performed. Her facial appearance resembles the appearances of her sons.
Supplementary figures.

Supplementary Figure 1 Schematic representation of the deleted regions in chromosome 15q24 in individuals 1-4 and two previously reported individuals with a 15q24 microdeletion. The chromosomal 15q24 region contains several segmental duplication blocks, including breakpoints A, B, C, D and E (breakpoints C-D are indicated), that are thought to predispose to the occurrence of deletions and duplications in this region by non-allelic homologous recombination (NAHR) during meiosis (74-76). Different deletions mediated by different combinations of SD blocks were previously reported. In addition, some individuals have an atypical deletion of which one or both of the breakpoints are not located in a segmental duplication block. Consequently, the clinical features of the 15q24 microdeletion syndrome are heterogeneous (6, 74, 77-79). Genotype-phenotype studies of individuals with typical overlapping deletions have suggested the 1.1 Mb sized region between segmental duplication block B and C (72.2-73.3 Mb, Hg18/74.4-75.5 Hg19) as critical region for the core phenotype. (77). However, Mefford et al. reported two small de novo deletions only involving the region between breakpoints C and D (78). These two individuals shared only five genes in the deleted region, PTPN9, SINC3A, MAN2C, NEIL1, and COMMD4. Their phenotype was reported to be milder with less pronounced speech delay compared to the larger deletions between B and C.
Supplementary Figure 2 Developmental transcriptome and LMD microarray analysis reveals high expression levels of SIN3A in proliferative regions of human developing cortex. (a) Overview of the online database of Allen Institute for Brain Science showing RNA sequencing data across developmental stages (4-7 weeks post conception, wpc, into adulthood) of human brain development showing a low to moderate expression of SIN3A across development in various brain structures. (b) Detail of the expression levels of SIN3A in cortical regions 9 wpc with a higher expression level in temporal neocortex (TGx). (c) Detail of the expression levels of SIN3A in cortical regions 9 wpc with a higher expression level in primary motor-sensory cortex (M1C-S1C). (d) Overview of the online database of Allen Institute for Brain Science showing LMD microarray data across developmental stages (4-7 wpc into adulthood) of human brain development showing a low to moderate expression of SIN3A 21 wpc in the ventricular zone (VZ) of the posterior frontal cortex (motor cortex). (e) Comparison of the expression levels of SIN3A in deeper cortical regions (e.g. V2) compared with more superficial cortical regions (e.g. marginal zone, MZ) 21 wpc. Details on the complete SIN3A transcriptome profiling can be found on www.brainspan.org.
Supplementary Figure 3 Protein expression of Sin3a over time and Nissl validation. (a) Immunostaining for Sin3a (green) at E14.5, E16.5, E18.5, P7, P14 and P21 counterstained with fluorescent Nissl (blue). (b) Coronal sections showing the somatosensory cortical area (S1) of E14.5 (left) and E16.5 (right) mouse brains showing Sin3a (green) and Ki67 (red) and counterstained with fluorescent Nissl (blue). Arrows and insets show colocalization (yellow). (c) Representative images of the Nissl staining of a control (Ctrl) electroporated cortical area (left) and a shSin3a electroporated cortical area (right) immunostained for GFP (green) and counterstained for fluorescent Nissl (blue) flanked by an black and white image of the Nissl staining (asterisks in the cell sparser area).
Supplementary Figure 4 Validation of Sin3a knockdown at the mRNA level. (a) Relative expression levels (percentage) of Sin3a (tested with two primer pairs (PP1 and PP2, two shades of grey) compared to β-actin (black) in mouse brain P35 and N2a cells. (b) Normalized expression levels of Sin3a mRNA in N2a cells transfected with two siRNAs targeting Sin3a mRNA (siSin3a-ex13 and siSin3a-ex16), two scrambled siRNAs (sc-siSin3a-ex13 and sc-siSin3a-ex16) compared to mock (no construct) as a control determined by qPCR using two primer pairs (PP1, black and PP2, grey). (c-g) Schematic representations of the constructs containing the shRNA for Sin3a exon 13 and exon 16 with accompanying scrambled constructs and the pCAB expression vector with the shRNA-insensitive mSin3a*. (h-j) Normalized mRNA expression levels of Cdkn1a, MeCP2 and CyclinD1 (n = 3) did not change 48 hours after knockdown of Sin3a. Graphs represent normalized mean transcript levels ± s.e.m. Student’s t-test.
Supplementary Figure 5 Validation of in vivo Sin3a knockdown at the protein level. (a) Representative images are shown of the electroporated (shSin3a-ex13, green) area double-labeled with Sin3a (red) and counterstained with fluorescent Nissl (blue, left). At the site of electroporation with shRNA, Sin3a protein levels are downregulated (right, white arrows). (b) Representative image of an electroporated (shSin3a-ex13, green) area double-labelled with cleaved Caspase 3 (CC3) showing an apoptotic cell double-labelled with GFP (arrowhead) and in the vicinity of GFP-labelled cells (arrow). (c) Positive control showing an area (septal area E17.5) positive for CC3. (d) Quantification of the number of CC3-positive cells within the electroporated area; n = 4 for Ctrl, n = 5 for shSin3a and n = 2 for shSin3a + mSin3a*. Graph represent number of cells per mm² ± s.e.m. One-way ANOVA (α = 0.05). (e) Quantification of the number of GFP/CC3-positive cells within the electroporated area as an indication for cell-autonomous effects; n = 4 for Ctrl, n = 5 for shSin3a and n = 2 for shSin3a + mSin3a*. Graph represent number of cells per mm² ± s.e.m. One-way ANOVA (α = 0.05).
### Supplementary Table 1a. *Mus musculus* (Mm) sequences of primers used for qPCR amplification.

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### Supplementary Table 1b. Single stranded oligo sequences for cloning *Mus musculus* shRNA constructs

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### Supplementary Table 1c. *Mus musculus* (Mm) sequence (gBlock; NM 001110351.1) used for cloning shRNA-insensitive Sin3a (capital letters indicate mutated shRNA target sites).

1858-2857 bp Sin3a

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**Supplementary Table 1d.** *Mus musculus* (*Mm*) sequences of primers used for cloning shRNA-insensitive *Sin3a*.

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**Supplementary Table 2.** Sources, specificity and working dilutions of primary antibodies used for immunohistochemistry and Western blotting.

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</table>
General Discussion
The human brain is an immensely complex structure composed of a magnitude of neurons that make connections through synapses and form intricate networks. The emergence of this incredible structure during development depends on a tight regulation of multiple molecular mechanisms and their proper cellular orchestration. A disturbance of these complicated processes may be the origin of neurodevelopmental disorders (1-3). However, the specific molecular and cellular mechanisms underlying neurodevelopmental disorders are poorly understood. The aim of this thesis was to provide a contribution to a better understanding of the neurodevelopmental role of a number of genes associated with neurodevelopmental disorders and/or a brain area that is part of the etiology of a neurodevelopmental disorder.

Studies on the genetic basis of neurodevelopmental disorders have been greatly supported by the establishment of relevant animal models. The animal models have been essential for obtaining fundamental knowledge of brain development as well as for our understanding of the functions of genes linked to neurodevelopmental disorders. The preferred animal for studying central nervous system development is often a rodent. The rodent central nervous system bears notable similarities to the human central nervous system, including its development, allowing the study of normal and affected neurological states in vivo in ways that are inaccessible in human (4-7). Genetic animal models, often created by establishing a loss-of-function of the gene of interest, are powerful tools to study the molecular basis of neurodevelopmental disorders. In general, the ultimate usefulness of a genetic animal model depends on the ability to translate the various aspects of the studied neurodevelopmental systems from the animal model to human brain development. However, the human brain exceeds the rodent in its tremendous complexity, for example higher absolute cell numbers, and more diversity, connectivity and functional areas (4, 8, 9). Also, the mechanisms underlying neurodevelopmental disorders are thought to be multifactorial and complex, with many causal - genetic and environmental - factors involved. It is therefore unclear whether the broad spectrum of symptoms and pathologies of a specific human neurodevelopmental disorder can be fully recapitulated in a rodent model. Nevertheless, the significant efforts made to generate and analyse rodent models have provided more insights into the etiology of neurodevelopmental disorders (2).

In this thesis, four genetically manipulated rodent models were used to explore the functions of four neurodevelopment-related genes. In chapter 2, the serotonin transporter (5-HTT) mutant rat model was used to study the role of 5-HTT and the subsequent alterations in 5-HT levels in rostral raphe-prefrontal network formation. Chapter 3 describes a knock-in mouse line of the transcription factor bicoid-related paired-like homeodomain 3 (Pitx3), a well-established model of Parkinson’s disease (PD) (10, 11), which demonstrated a role for Pitx3 in ventral tegmental area (VTA) development and its mesocortical projections. In chapter 4, a mouse line deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGprt) was used to examine early development of the dopamine system as a model of Lesch-Nyhan disease (LND). Lastly, in chapter 5 a knockdown of the switch-insensitive 3 transcription regulator family member A (Sin3a) was generated through in utero electroporation (IUE)-mediated gene transfer, allowing us to study its function in relation to a new distinct intellectual disability/developmental delay syndrome. In this final chapter, the main findings of this thesis will be discussed and put into a broader perspective.
The role of the 5-HTT in rostral raphe-prefrontal network formation and its link with autism spectrum disorder (ASD), schizophrenia and intellectual disability

- **The trophic nature of the interaction between the origin (rostral raphe cluster) and a target (mPFC) of the 5-HT projection system depends on the presence of 5-HTT during development.**

- **The 5-HT projections from the MnR are normally repelled by the mPFC but become strongly attracted by the mPFC in the absence of 5-HTT during development.**

- **5-HT innervation of the mPFC was significantly increased in the absence of the 5-HTT during development. Additionally, the number of Satb2-positive callosal projection neurons is reduced in the mPFC.**

The 5-HT projection system is one of the earliest systems to develop during brain formation (12, 13). Interestingly, prior to this, the 5-HT machinery – such as receptors, transporters and enzymes – is already transiently expressed (14). This indicates that there may be a developmental dependency on an exogenous source of 5-HT during the first stages of development. The exogenous source of 5-HT is of maternal origin. In fact, the essential amino acid tryptophan originates from the pregnant mother, and is converted to 5-HT in the placenta which contains the necessary machinery, and subsequently delivered to the fetal circulation (15-18). The dependency on this placental source of 5-HT declines as development progresses and eventually the endogenous production by the fetus takes over. Most of the 5-HT neurons develop in specific cell clusters called Raphe nuclei in the midline of the rhombencephalon. The cell clusters can be divided into caudal groups, including the Raphe Pallidus, Magnus, Obscurus and Pontis, and rostral groups consisting of the dorsal and median Raphe (19, 20). In mouse, one day after their generation, 5-HT neurons are able to synthesize 5-HT and start to extend their axons (19, 21). The caudal cell groups form descending axon tracts towards the spinal cord, while the rostral cell groups form profound axon tracts to the forebrain (20, 22). One of the distant targets of the ascending 5-HT projections is the medial prefrontal cortex (mPFC) (23, 24). In chapter 2, the role of the 5-HTT in network formation between the rostral raphe cluster and the mPFC was studied. This is of relevance since improper 5-HTT functioning and alterations in 5-HT innervations of the mPFC have been implicated in the etiology of several neurodevelopmental disorders, including autism spectrum disorder (ASD), schizophrenia and intellectual disability (12, 25-27).

The 5-HTT is a presynaptically located transporter and a key modulator of the 5-HT signal by its reuptake of 5-HT for recycling and degradation. The 5-HTTLPR polymorphism in the human 5-HTT gene, consisting of a short (s) and a long (l) allelic variant, influences 5-HTT expression and function. The s-allele has been associated with reduced 5-HTT functionality, and clear and robust neurodevelopmental changes in corticolimbic structures (25). Additionally, human s-carriers show anxiety-related traits (28), and an increased risk for depression in the context of stress (25, 29). It is thought that the 5-HTTLPR-related changes reflect neurodevelopmental changes due to an altered 5-HT signal. To study 5-HTT function and to model the 5-HTTLPR, the unique homozygous (5-HTT\(^+/+\)) and heterozygous (5-HTT\(^+/−\)) rats represent a highly valuable animal model. The 5-HTT rodent model shares many behavioral aspects with those seen in human 5-HTTLPR s-allele carriers such as increased anxiety and depression-like behavior (30, 31). Additionally, in both the 5-HTT\(^+/−\) rodent as well as the human s-variant carriers, extracellular levels of 5-HT are increased throughout the brain (31-33).
The 5-HT<sup>−/−</sup> rat model has also been exploited to study cortical integrity of the PFC during development, as emotional and cognitive disturbances in neurodevelopmental disorders can be partly attributed to an altered architecture of this cortical region (34, 35). In fact, the PFC is the seat of our highest cognitive abilities and known to be involved in attentional processes, behavioral flexibility and working memory (36, 37). Studies have shown that, in addition to increased extracellular 5-HT levels, there is a differential expression of various 5-HT receptors in the 5-HT<sup>−/−</sup> rat model (31-33). The cortical layer I Cajal Retzius cells express a number of these receptors, and are contacted by the 5-HT projections (38, 39). Cajal Retzius cells are well-known for the release of Reelin which is crucial for correct cortical layer formation (40, 41). As 5-HT binds to these receptors, it is hypothesized that cortical development could be affected by altered 5-HT synaptic input on the Cajal Retzius cells by alterations in Reelin release. In addition, altered 5-HT innervations of the PFC have been implicated in the etiology of neurodevelopmental disorders such as schizophrenia, ASD and intellectual disability (25, 42-45). Together, this portrays the relevance of studying 5-HT functioning and developing 5-HT innervations of the PFC in the rat to understand neurodevelopmental disorders.

As pointed out in chapter 3, using three dimensional collagen explant assays, we studied whether the trophic nature of the interaction between the rostral raphe cluster and the PFC is dependent on the presence of 5-HT to modulate 5-HT levels during development. Explant assays are an excellent tool to study the behavior of outgrowing neurites as a reaction to the released chemotrophic signals of the co-cultured tissue while leaving the cells in their extracellular matrix (46, 47). Our most prominent observation was that the 5-HT projections of the median raphe were repelled when co-cultured with the mPFC in wild type, whereas in the absence of the 5-HT this chemotrophic interaction switched to a strong attraction of the outgrowing neurites. This could be the result of increased 5-HT levels due to the absence of 5-HTT that modulated the guidance of the outgrowing neurites, or that the characteristics of the rostral raphe cluster 5-HT neurons were changed due to the altered 5-HTT expression – or a combination of both. Moreover, the lack of the 5-HTT may have intrinsically affected mPFC development as well, which in turn could result in abnormally released chemotrophic signals.

In parallel, we showed <i>in vivo</i> that the absence of 5-HTT during development results in increased 5-HT innervations in the mPFC. 5-HT has previously been shown to modulate outgrowing axons by affecting their response to classical guidance cues (48-50), and may therefore contribute to the maturation of a variety of neuronal projection systems, including its own (20, 51). The altered levels of 5-HT, due to lack of the 5-HTT, could therefore affect the observed responses of the outgrowing neurites from the rostral raphe cluster, but other developing projection systems could be affected as well.

As mentioned, the 5-HT innervations are thought to make synaptic contacts with the Cajal Retzius cells that generate the Reelin gradient that is crucial for proper cortical development. The gradient of Reelin provides the developing cortical neurons with positional information during migration across the cortical layers. Interestingly, we showed that the number of Satb2 positive callosal projection neurons in all layers of the mPFC is reduced in mutant rats, indicating abnormal cortical integrity in the absence of 5-HTT during development. Based on this altered integrity and in combination with the changes in 5-HT innervations, we speculated that a disturbed 5-HT synaptic input on the Cajal Retzius cells contributes to impairment of cortical integrity. Whether this is directly due to changes in 5-HT innervations, or that the altered neuron identity caused by the increased 5-HT levels in absence of 5-HTT, remain intriguing questions.

The placental source as well as the endogenous source of 5-HT levels can be modulated by genetic modifications. However, environmental disturbances could also have a significant effect on
5-HT levels. This is especially interesting in light of recent deliberations on the effect of 5-HT reuptake inhibitors (SSRIs) during development. SSRIs given to the pregnant mother as a treatment for depression have been shown to pass the placenta and increase 5-HT levels in the brain of her unborn child (52, 53). The increased 5-HT levels in the fetal brain could have an effect on its development, as we showed in this chapter in a rat model. These children acquire an increased risk to develop reduced somatosensory responses and/or psychomotor control (54). Additionally, they appear to have higher risk to develop autism-like symptoms (55, 56). There are indications that SSRIs have an effect on the anatomy of the corpus callosum (57-59), which is interesting since we report a decrease in Satb2 positive callosal projection neurons in the 5-HTT mutant rat.

In summary, our studies on the 5-HTT contribute to the understanding of the role of 5-HT levels during brain development, specifically in axonal outgrowth, targeting and target innervation of the mPFC. By putting the results into a broader perspective, we help to better understand the onset and etiology of neurodevelopmental disorders such as ASD, schizophrenia, and intellectual disability associated with altered 5-HT levels during development.

The role of Pitx3 in mesoprefrontal network formation and function, and its link with PD

- Pitx3 deficiency caused a defect in the migratory paths of dopaminergic (DA) neurons in the ventral tegmental area (VTA) during development.
- Fasciculation of the DA axons of the medial forebrain bundle (MFB) is affected in the absence of Pitx3. Additionally, Pitx3 deficiency caused a decrease in DA innervation of the mPFC.
- Prefrontal-steered behavior was affected in Pitx3-deficient adult mice, showing increased anxiety and impairment in social behavior.

As described in Chapter 3, PD is characterized by a selective loss of substantia nigra (SNc) neurons and a subset of the VTA neurons (60, 61). As a consequence, there is a reduction in the DA innervation of the dorsolateral striatum, which contributes to the characteristic deficit in motor-controlled behaviour including hypokinesia and rigidity (62, 63). While the pathological changes and motor dysfunction are well characterized, the mechanisms responsible for the differential vulnerability for degeneration of the SN DA neurons versus the preserved gross of the VTA DA neurons, are not well understood. Differences in the development of the DA neurons of the two cell clusters, such as differences in progenitor domains, migrational timing and trajectories, and differential dependency on specific factors may contribute to this selective vulnerability.

The differential development of SN vs VTA starts as early as the induction of the progenitor pool. DA progenitors arise from a progenitor domain in the floor plate of the midbrain that is defined by factors including sonic hedgehog (SHH), that – together with the SHH-target gene Gli1 – is expressed dynamically to generate a spatiotemporal genetic fate map of the DA precursors (64, 65). As such, early SHH expression, in the medial progenitor domain, gives rise to DA neurons that prefentially contribute to the SN. The subsequent switch to exclusion of SHH from the medial domain and a restricted SHH expression in the lateral domain results in descendants that have a VTA-biased distribution (64, 65). This suggests that medial and lateral progenitor domains give rise to different subsets of DA neurons.
In addition to differences in progenitor domains SN and VTA dopamine neurons follow different migratory trajectories to establish the complex organization of the ventral midbrain. After cell cycle exit, the newborn neurons of the future SN and VTA will become postmitotic migratory DA precursor cells. First, these cells migrate radially towards the mantle layer (66, 67), after which the DA neurons destined to form the SN will continue migrating in a tangential orientation, while the presumptive VTA neurons remain in the central domain (64, 68, 69). This requires a differential intrinsic gene expression to react differentially on extrinsic cues, such as guidance cues, of the surroundings.

Furthermore, the expression profiles of a set of transcription factors during development contribute to the DA SN vs VTA subtype generation. VTA identity is controlled by the expression of the transcription factor orthodenticle homeobox 2 (Otx2), which is first expressed in the progenitor cells but is only maintained at high levels in a subset of differentiating and mature VTA neurons (68, 70, 71). Zincfinger protein 503 (Zfp503, Nolz1) expression additionally adds to VTA dopamine neuron identity (68). The transcription factor SRY-box6 (Sox6) has been identified as a key factor for the early specification and development of SN DA neurons, and has been shown to be involved in suppressing VTA-specific characteristics (68). In addition, bicoid-related paired-like homeodomain 3 (Pitx3), a transcription factor expressed in both SN and VTA DA neurons, has been proven crucial for the maintenance of the SN neurons (72, 73). Remarkably, the VTA DA neurons display a differential dependency on Pitx3 during development. In the absence of Pitx3, all SN neurons are lost during early development, while only a fraction of VTA DA neurons degenerate postnatally. The similarities of this pattern of degeneration compared with the degeneration seen in PD makes Pitx3 a valuable and relevant candidate gene to study the differential DA vulnerability in PD.

To study the role of Pitx3 in VTA DA neuron development, we used the Pitx3-GFP knock-in mouse, a genetic mouse model of PD (10, 11). In these Pitx3-deficient mice, the DA neurons of the SN are specifically lost during embryonic development, resulting in a loss of motor control due to decreased DA levels in the dorsal striatum that may resemble some of the symptoms of individuals with PD. Using immunohistochemistry, we documented an aberrant development of the VTA DA cell cluster in the Pitx3-deficient mice, characterized by defects in the migratory paths. The observed halted migration could result in incorrect positional information as these neurons do not migrate the stipulated trajectory in the required time frame. This could have results in alteration in the maturation of these neurons, possibly affecting their axon outgrowth and targeting of their distant destinations.

The DA neurons of the VTA form profound projections to several frontal brain areas including the ventromedial striatum, habenula and PFC, forming the mesocorticlimbic pathway. We showed that the mesocortical targeting was impaired, when Pitx3 is lacking, which resulted in a decrease of DA innervations in the PFC during development. Interestingly, preliminary evidence suggests that in PD, but also in many neuropsychiatric disorders, this mesocorticlimbic connectivity is perturbed in addition to the well-described DA depletion of striatal areas (61, 74). This could contribute to the multiple non-motor clinical signs associated with PD, such as depression, anxiety and cognitive decline (75, 76). Of note, when we examined a number of behavioral aspects in Pitx3 heterozygous mice, we found alterations in behavior, that suggested increased anxiety levels, impaired risk assessment, increased compulsivity and altered social behavior—that all resemble non-motor clinical signs associated with PD. Together, our results support the idea that early developmental events contribute to the fate of DA neurons throughout life.

Thus, our studies on Pitx3 contribute to the understanding of its role in the development of the VTA DA neurons, specifically their axon targeting, innervation, and network function in relation to
behavior. Putting the results into a broader perspective elucidates an aspect of the differential development of the VTA and SN DA neurons, which may explain the selective vulnerability of DA neurons in PD and the mechanisms that contribute to the non-motor symptoms associated with PD.

The role of HGprt during early DA neuron development and its link with LND

- **HGprt deficiency results in disturbed proliferation and migration patterns in the DA ventral midbrain at several developmental time points in a mouse model of LND.**
- The observed abnormal cell alignment coincides with a premature departure from the midline migration path found in HGprt-deficient embryos.
- The facilitating and steering scaffold formed by the fibers of radial glia cells is affected due to HGprt deficiency.
- HGprt deficiency is associated with abnormal cortical layering at a later developmental stage.

An example of a neurodevelopmental disorder generated by a mutation in a single gene is LND, caused by mutations in the *HPRT1* gene. The resulting deficiency in HGprt, an enzyme involved in purine metabolism, gives rise to a syndrome that includes hyperuricemia and a characteristic neurobehavioral phenotype (77-79). The pathophysiology leading to the hyperuricemia has been well defined. In the absence of HGprt purine bases cannot be salvaged and are degraded and excreted as uric acid instead. Simultaneously, there is an acceleration of the rate of purine synthesis, presumably to compensate for the defects in the salvage process. Together, these two processes are responsible for the overproduction of uric acid, leading to the hyperuricemia (80, 81). The mechanisms underlying the neurological and behavioral signs however, are not well understood. However, neuroimaging and neurochemical studies have demonstrated significant aberrations in dopamine content and function in the basal ganglia – presumably contributing to the neurobehavioral phenotype (82-85).

The mechanisms by which deficiency in HGprt could affect the basal ganglia, and more specifically the DA systems, remain unknown. It has recently been suggested that a dysregulation of neurodevelopmental processes may underlie the DA defect. In vitro studies have shown that transcription factors that are essential during development for the differentiation, maturation and maintenance of the DA cells were differentially expressed HGprt deficient cancer cell lines (86, 87). Furthermore, no clear signs of a degenerative process were found in post-mortem human patients and HGprt-deficient mice (88-90). This prompted us to study the role of HGprt in early development of the DA neurons in vivo (Chapter 4).

In this study, we used an HGprt knock-out mouse line as a model of LND. The HGprt-deficient mice are of historical value as they provided the first indication that a genetically engineered mouse line could be obtained as a direct model for a specific human disorder (91, 92). However, some phenotypic limitations are linked to the use of this mouse model: HGprt deficiency does not lead to hyperuricemia, due to the presence of uricase that catalyzes the oxidation of uric acid and is absent in humans, nor do they exhibit the LND neurobehavioral syndrome. Nevertheless, HGprt-deficient mice exhibit many of the metabolic disturbances that are strikingly comparable to those detected in LND patients, including failure of purine recycling, an accelerated synthesis of purines (93, 94) and decreased levels of dopamine. These findings coincide with microstructural anatomical
abnormalities in the basal ganglia (86, 87). Using this animal model, we studied the embryonic development of the DA neurons in the ventral midbrain. As mentioned above, the main DA neurons are located in the ventral midbrain in cell clusters including the SN and VTA. Proliferation of radial glia-like cells in the ventricular zone (VZ) generates a progenitor pool that will initially migrate radially, following the midline migration path, toward the mantle layer (66, 67). Here, the DA neurons destined to form the SN continue migrating in a tangential orientation to their lateral positions, while the presumptive VTA neurons remain in the medial domain (64, 68, 69). In the studies described in chapter 4, we first showed that the absence of HGprt causes an aberrant proliferation and migration pattern at several developmental time points. As DA neurons are assisted and guided in their migratory activities by a scaffold constructed by the radial glia cells, we examined the radial glia scaffold. The results suggested an abnormal density of radial glia due to HGprt deficiency, where the rostral presumptive VTA and SN area was oppositely affected compared to the caudal regions of the ventral midbrain. This may be highly relevant as, for example, the rostral VTA is known to innervate the habenula, while the more caudal VTA forms projections towards the corticolimbic structures including the medial PFC (95, 96). Both areas may contribute to the disinhibited phenotype of LND patients.

In previous research, the number of DA neurons in the ventral midbrain of adult HGprt-deficient mice appears not to be affected; however they do show a reduced expression of TH (88-90). We could therefore speculate that the altered proliferation and migration patterns during early development do not affect the eventual gross cells numbers, but may contribute to an altered organization of the dopamine cells within the cell clusters. Proliferation and migration are tightly regulated and timed processes, and any alternations in positional information could cause these neurons to deviate from their stipulated trajectory. Indeed, when staining for a presumptive SN-specific dopamine neuron marker, we observed an altered distribution of these cells. This could indicate that the organization of the neurons, with their specific neuronal identities may be abnormal – in the presence of overall normal DA neurons. Whether this is due altered migration, altered developmental cues from surrounding areas (i.e. the neurons being either at the wrong time, the wrong place, or both) resulting in altered expression of intrinsic factors, remains to be determined.

Since HGprt expression is not unique to DA neurons, other cells could also be affected by the lack of HGprt during development. The abnormal migration patterns observed in HGprt-deficient embryos inspired us to study radial glia. The radial glia cells of the ventral midbrain have complicated functions, as they generate DA progenitors by asymmetric division, and additionally form the scaffold that is used during migration of these developing neurons. Since we found alterations in both, HGprt may be essential for radial glia functioning during early development. It remains an intriguing question whether the radial glia cells of the ventral midbrain are specifically affected, or that HGprt deficiency causes a more general radial glia defect, which would also affect the development of other brain areas. However, when we examined cortical layer integrity in cortical targets of the DA system, we found an altered deeper layer marker distribution; the labeled cells were spread throughout the entire cortical width and not restricted to the deeper layers. This demonstrates that cortical layer formation and thus cortical integrity is dependent on HGprt during development. Whether this is a direct dependency of the developing cortex, or an indirect effect of aberration of for example incoming – perhaps specific DA – projections due to an impaired development of the VTA and SN remains subject for further study. Of note, the The concept that LND is associated with widespread effects in the brain DA is supported by recent imaging studies using voxel-based morphometry in LND patients reporting profound abnormalities in white and gray matter in multiple brain areas, e.g. prefrontal cortex (97, 98).
Thus, HGprt deficiency causes aberrant DA midbrain embryonic development involving proliferation and migration patterns, radial glia functioning, and cortical layering. As mentioned, LND is an inherited metabolic disorder associated with hyperuricemia and a characteristic neurobehavioral phenotype, including dystonia, specific attentional and executive cognitive deficits and self-injurious behavior. It is a rare disease with an estimated incidence of 1 in 380000 births. However, the incapacitating nature of the disorder – for which no effective therapies exist – warrants the elucidation of its pathogenic mechanisms as a prelude to novel treatment development. Moreover, the main finding of our studies so far, i.e. the vast dependency of proper HGprt function during the development of the DA neurons of the ventral midbrain, would probably not have been easily discovered in more general studies of development of the DA system. Research on HGprt function may thus have broader implications for understanding the development and functioning of the DA system. As such, findings may even be relevant to other, more common DA diseases, such as PD, Tourette syndrome and schizophrenia (62, 63, 99, 100).

The role of Sin3A in cortical expansion and maturation and its link with a new distinct intellectual disability/developmental delay syndrome

- **Loss-of-function of the SIN3A gene in humans is associated with a distinct intellectual disability/developmental delay syndrome.**

- **Haploinsufficiency of Sin3a causes a decrease in the number of cortical progenitors in the proliferative zone (PZ) at the peak of neurogenesis. Additionally, Sin3a is essential for early cell division and the production of neurons within the cerebral cortex.**

- **The decrease in the expression of a marker controlling the identity of upper-layer neurons in cells with Sin3a knockdown implies that Sin3a is required for the differentiation of cortical progenitors.**

- **Downregulation of Sin3a causes an increase in callosal axon outgrowth in both length and number, indicating that Sin3a has a role in cortical neuron differentiation and callosal axon elongation in vivo.**

During the last couple of years, genetic studies, supported by the emergence of whole-exome and whole-genome sequencing, have resulted in the identification of genetic components underlying neurodevelopmental disorders such as intellectual disability and ASD (101-105). Yet, the consequences of the disturbances in function of the associated protein mostly still need to be defined. In Chapter 5, we identified SIN3A mutations and four so far unpublished cases with de novo atypical small deletions encompassing SIN3A in individuals presenting intellectual disability/developmental delay. To better comprehend the consequences of haploinsufficiency of SIN3A, we compared clinical data and found that these individuals share striking features, such as facial dysmorphisms, microcephaly and a short stature. Magnetic Resonance Imaging (MRI) data revealed subtle abnormalities including corpus callosum dysgenesis and ventriculomegaly.

Through our study we can add SIN3A to a growing number of epigenetic factors that are implicated in intellectual disability and ASD (106). SIN3A has been shown to have the ability to bind to various members of a transcriptional regulatory complex to control several developmental processes, such as cell cycle events, proliferation of embryonic stem cells (107-114). Our studies indicate that the haploinsufficiency of SIN3A is linked to alterations in brain development in affected individuals. Mouse Sin3a protein closely resembles its human orthologue (115). We showed that in
wild-type mice Sin3a is expressed in a large population of cortical progenitors and young neurons during the period of corticogenesis. Since this expression pattern becomes more restricted in later developmental time points, we speculated that Sin3a is expressed specifically during the neurogenic phase of corticogenesis. Together this illustrates the relevance of studying the role of Sin3a in early cortical development.

In contrast to the research described in the other chapters, we did not use a Sin3a mutant rodent model for our experiments but instead opted for a technique with which the regionality and time of gene modulation can be restricted: IUE-mediated gene transfer. Through the use of small-hairpin (sh)RNAs we were able to knockdown Sin3a in specific developing cortical regions.

During cortical development, there is an initial lateral expansion within the VZ since neuroepithelial progenitors divide symmetrically with their offspring going in another round of division. Subsequently, cell cycles get longer and radial expansion starts by asymmetric division of subVZ (SVZ) progenitors generating both intermediate progenitors and post mitotic neurons (116-118). IUE-mediated gene transfer takes advantage of the fact that these cell divisions occur close to the ventricular lining. Progenitor cells lining the ventricular zone of the target area, in our study the primary somatosensory cortex (S1), incorporate plasmids directing the knockdown of Sin3a after electroporation. The additional expression of a fluorescent reporter allowed us to monitor the developing cortical progenitors and thus aberrant mechanisms caused by the knockdown of Sin3a.

We showed that a knockdown of Sin3a in S1 cortical regions results in a decrease of neuroprogenitor proliferation. Since we could rescue this proliferative phenotype by introducing an shRNA-insensitive Sin3a construct, unequivocally demonstrated that Sin3a is essential for early cell divisions and the production of neurons within the cerebral cortex. Interestingly, these results are in line with the microcephaly and ventriculomegaly that is observed in a significant number of the studied individuals.

During normal development, neuronal progenitors will migrate along a scaffold of radial glia fibers to their correct position in the cortex to form the well-known six-layered organization in an inside-out fashion (119). Distinct labelled transcription factors can be used to mark layer-specific cortical neurons. We showed that in neurons with lower Sin3a levels the expression of the upper layer marker Brn2 had decreased, indicating that Sin3a is additionally required for the differentiation of the cortical progenitors. After the neurons have reached their correct position in the cortex, they will start to extend their axons to their projection targets. A subset of projection neurons elongates their axons to the contralateral cortex, forming callosal projections. We observed that knockdown of Sin3a caused an increase in the number and length of callosal projections emerging from the transfected neurons. The increase in axon outgrowth will most likely result in aberrant projections that fail to properly innervate their target area. This strongly supports a role for Sin3a in cortical neuron differentiation and callosal elongation which again seems in line with results from MRI studies revealing corpus callosum dysgenesis in eight out of ten indexed individuals. We speculate that the decrease in proliferation can be the result of premature departure from the cell cycle. The neurons become postmitotic at the wrong time and place, which affects their differentiation, identity and axon elongation.

The development of the human cerebral cortex is a highly complex process and includes timely events such as cortical proliferation, neuronal migration, differentiation, axonal outgrowth and connectivity. By linking clinical observations with the results of molecular functional assays in mice we were able to establish that Sin3a is crucial for these cortical developmental processes. As such, our results elucidate another important player in cortical development, and demonstrate that a bridge between clinical observations and fundamental research contributes to the unveiling of the molecular mechanisms underlying genetic components of neurodevelopmental disorders that are more and more being discovered.
Molecular mechanisms underlying neurodevelopmental disorders

For many neurodevelopmental disorders, the accompanying symptoms cannot be treated effectively yet. The identification of the genetic defect alone is insufficient for the development of effective treatments. Although tremendously informative, additional levels of complexity have demonstrated that supplementary detailed knowledge and characterization of the molecular and cellular mechanisms that are dysregulated by the genetic defect are necessary, and knowledge of this information is essential for the development of new treatment strategies. The research discussed in this thesis sheds new light on the dysregulation of neurodevelopmental mechanisms in the absence of each of four distinct genes, all in the context of their associated disorder (Fig. 1).

First, we show that adequate 5-HT levels are essential for proper rostral-raphe prefrontal cortex network formation with a link to the etiology of ASD. Second, we show how Pitx3 affects the development of the VTA and its derived DA projections, contributing to the growing pool of knowledge about the differential vulnerability of the SN and VTA neurons during maturation of the DA system, and suggesting the need for investigating the mesocorticostriatal projections in the context of PD. Third, studying HGprt revealed that LND may be associated with an impaired early development of the midbrain DA system. This may be the causative factor for the DA defects observed later in life in the affected individuals, and demonstrates the need for fundamental developmental research in relation to LND. Finally, we demonstrate both clinically and with fundamental research that Sin3a is essential for proper cortical development, and that dysregulation of the mechanisms involved in this process may contribute to the clinical signs observed in individuals with mutation in SIN3A such as intellectual disability/developmental delay, striking similar features including facial dysmorphisms, microcephaly and short stature, and more subtle abnormalities, including corpus callosum hypoplasia and ventriculomegaly.


eurodevelopmental mechanisms underlying brain disorders

Future prospects

The ultimate goal of identifying the mechanisms that underlie the specific defects accompanying a neurodevelopmental disorder is to translate this information to human neurodevelopment and create new treatment strategies. For example, the primary molecular and cellular deficits could be reversed or compensated, e.g. by gene therapy with replacement of the defective gene. The timing
of the pathogenic defects underlying neurodevelopmental disorders adds another level of complexity to these approaches. Corrective efforts in adults are more likely to be effective when there is a continued dependency on the mutated gene throughout life, and not only during early brain development. For many disorders, it may be too late to correct them after birth, since the abnormalities occurred during early critical developmental periods when cell migration, axon guidance, synaptogenesis or even the plasticity of the developing networks took place. On the other hand, for some neurodevelopmental disorders postnatal developmental processes (or compensatory mechanisms) may contribute to the development of symptoms. For example, individuals with PD or LND appear to be born normal, but develop their full phenotype usually later in life. Better knowledge of the specific time frame in which the developmental aberrations occur may therefore also help the correct timing of therapeutic intervention. For now, the reality is that in general treatment of neurodevelopmental disorders only involves management of the symptoms rather than repairing the underlying mechanism causative to the symptoms. For a better understanding of the symptoms and possibly new treatment design, a detailed knowledge of the underlying developmental mechanisms is required. We hope to have contributed to such knowledge with the studies described in this thesis. The fact that neurodevelopmental disorders affect a large portion of the population reinforces the importance of fundamental research focused on the molecular and cellular neurodevelopmental mechanisms underlying these disorders.
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