Agenesis of the putamen and globus pallidus caused by recessive mutations in the homeobox gene GSX2

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Basal ganglia are subcortical grey nuclei that play essential roles in controlling voluntary movements, cognition and emotion. While basal ganglia dysfunction is observed in many neurodegenerative or metabolic disorders, congenital malformations are rare. In particular, dysplastic basal ganglia are part of the malformative spectrum of tubulinopathies and X-linked lissencephaly with abnormal genitalia, but neurodevelopmental syndromes characterized by basal ganglia agenesis are not known to date. We ascertained two unrelated children (both female) presenting with spastic tetraparesis, severe generalized dystonia and intellectual impairment, sharing a unique brain malformation characterized by agenesis of putamina and globi pallidi, dysgenesis of the caudate nuclei, olfactory bulbs hypoplasia, and anomaly of the diencephalic-mesencephalic junction with abnormal corticospinal tract course. Whole-exome sequencing identified two novel homozygous variants, c.26C>A; p.(S9*) and c.752A>G; p.(Q251R) in the GSX2 gene, a member of the family of homeobox transcription factors, which are key regulators of embryonic development. GSX2 is highly expressed in neural progenitors of the lateral and median ganglionic eminences, two protrusions of the ventral telencephalon from which the basal ganglia and olfactory tubercles originate, where it promotes neurogenesis while negatively regulating oligodendrogenesis. The truncating variant resulted in complete loss of protein expression, while the missense variant affected a highly conserved residue of the homeobox domain, was consistently predicted as pathogenic by bioinformatic tools, resulted in reduced protein expression and caused impaired structural stability of the homeobox domain and weaker interaction with DNA according to molecular dynamic simulations. Moreover, the nuclear localization of the mutant protein in transfected cells was significantly reduced compared to the wild-type protein. Expression studies on both patients’ fibroblasts demonstrated reduced expression of GSX2 itself, likely due to altered transcriptional self-regulation, as well as significant expression changes of related genes such as ASCL1 and PAX6. Whole transcriptome analysis revealed a global deregulation in genes implicated in apoptosis and immunity, two broad pathways known to be involved in brain development. This is the first report of the clinical phenotype and molecular basis associated to basal ganglia agenesis in humans.

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Received December 29, 2018. Revised June 6, 2019. Accepted June 18, 2019. Advance Access publication August 14, 2019
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Introduction

The basal ganglia are symmetrical subcortical grey nuclei at the core of the extrapyramidal system, composed by the striatum (which comprises the putamen, caudate nucleus, olfactory tubercle, and nucleus accumbens), the pallidum, the substantia nigra, and the subthalamic nucleus. These nuclei play essential roles in regulating many brain functions, including not only control of voluntary movements but also behaviour, cognition and emotion control (Obeso et al., 2014). Dysfunction of the basal ganglia is associated with a wide variety of disorders with either paediatric or adult onset. These include acquired conditions (such as hypoxic-ischaemic injury, infections and iatrogenic damage, tumours), neurodegenerative disorders (such as Parkinson’s, Huntington’s and Wilson’s diseases, as well as neurodegeneration with brain iron accumulation), and metabolic disorders (such as lysosomal storage disorders, mitochondrial disorders, organic acidurias, or gangliosidoses) (Zuccoli et al., 2015). In all these conditions, basal ganglia are normally formed and then undergo acute, subacute or chronic damage, often resulting in well-recognizable imaging patterns. Conversely, congenital malformations of the basal ganglia deriving from defects of embryogenesis are very rare. The basal ganglia embryologically derive from the prosencephalon (or forebrain), the most rostral of the three vesicles originating from early patterning of the neural tube along the anterior-posterior axis. Regional identity and pattern proliferation along this axis are carefully specified by the expression of molecules along two major signalling centres, the diencephalic-mesencephalic junction (DMJ) and the midbrain-hindbrain junction (Martinez et al., 1991; Nakamura et al., 2005). The prosencephalon divides into telencephalon and diencephalon, and the telencephalon then separates into two domains: the pallium, which gives rise to dorsal structures such as the cerebral cortex, and the subpallium, which generates ventral structures such as the basal ganglia. In particular, the latter arise from two protrusions of the ventral telencephalon termed the medial ganglionic eminence (MGE), from which the globus pallidus originates, and the lateral ganglionic eminence (LGE), which gives rise to the caudate, the putamen and the olfactory tubercle (Smart and McSherry, 1982; Smart, 1985; Deacon et al., 1994). The ganglionic eminences also represent the major source of interneurons which then migrate into the olfactory bulb and the cerebral cortex (Anderson et al., 1999; Parnavelas, 2000).

Dysmorphic basal ganglia, with lack of clear separation between the caudate, putamen and globus pallidus due to the absence of various parts of the internal capsule, represent a hallmark feature of tubulinopathies, a group of autosomal dominant disorders due to mutations in tubulin family genes. Dysmorphisms are often asymmetric and occur in association with a wide range of brain malformations, including commissural abnormalities, various cortical migration defects and hypo-dysplasia of the brainstem and cerebellum (Bahi-Buisson et al., 2014; Romaniello et al., 2017). Another rare disorder featuring poorly delineated basal ganglia is X-linked lissencephaly with abnormal genitalia (XLAG) due to mutations in the ARX gene. As in tubulinopathies, the malformative pattern in this condition is complex, featuring lissencephaly, agenesis of the corpus callosum, hypoplasia of the olfactory bulbs and of the mid-hindbrain, and corticospinal tract anomalies (Bonneau et al., 2002; Kato et al., 2004; Okazaki et al., 2008).

To our knowledge, neurodevelopmental disorders characterized by complete or partial agenesis of the basal ganglia, especially in the absence of cortical and/or commissural defects, are not known. A neuropathological finding of isolated basal ganglia agenesis was only reported in a 6-year-old male, with suggestion that downregulation of the ASCL1 gene could be responsible for this defect (Sarnat, 2000); however, clinical and genetic data were not available.

Here we describe two unrelated children presenting a highly distinctive congenital brain malformation characterized by agenesis of the putamen and globi pallidi and...
dysplasia of the caudate nuclei. This was associated with agenesis or hypoplasia of the olfactory bulbs and dysplasia of the DMJ, resulting in a severe phenotype of generalized dystonia, spastic tetraparesis and intellectual impairment. Both children carried homozygous pathogenic variants in the GSX2 gene, encoding a homeobox transcription factor essential for development of the LGE.

**Materials and methods**

**Patients and genetic studies**

The two probands and their unrelated parents underwent whole-exome sequencing (WES) as part of an ongoing research project to identify the genetic basis of developmental brain defects, approved by the Ethics Committee of University of Pavia. Written informed consent was obtained according to the Declaration of Helsinki by all participating families prior entering the study. Briefly, exome capture was performed using the SureSelectXT V4 S1mb Exome (Agilent Technologies) with 100-bp paired-end read sequences generated on a HiSeq2500 (Illumina). Sequences were aligned to hg19 and variants were identified and filtered as described previously (Roosing et al., 2016). Mean target coverage was 80×, and >80% of bases were sequenced with a quality score above Q30.

GSX2 variants were verified by bidirectional Sanger sequencing in available family members. Potential pathogenicity was predicted using the software tools SIFT, PolyPhen-2, PROVEAN, MutationAssessor, MutationTaster and CADD. Conservation of affected residues was assessed by the Clustal Omega software. Sanger sequencing was also used to bidirectionally sequence all exons and exon-intron junctions of the GSX2 gene in eight additional patients with DMJ dysplasia. Reference sequences were: GSX2 gene, NM_133267.2; and GSX2 protein, NP_573574.1.

**Molecular dynamics analysis**

The primary protein sequence of wild-type GSX2 was obtained from UniProt (ID: Q9BZM3). The protein structure resulted from multiple alignments between several templates and the query sequence, through MODELLER v9.16. (Webb and Sali, 2014). The obtained wild-type model was mutated in silico through UCSC Chimera (Pettersen et al., 2004), yielding a second model. The final models where embedded in boxes, extending up to 12 Å, and solvated using the TIP3P water model (Jorgensen and Madura, 1983). Additionally, *dnap* was used to add counter ions and thus to neutralize the overall charge of the models. Each model was first energy minimized and then equilibrated for ~5 ns, by a time step of 1 fs.

Molecular dynamics simulations were performed using AMBER 16 on the two equilibrated models for 30 ns by a time step of 2 fs, as reported (Case et al., 2016; Biagini et al., 2018).

The root-mean-square deviation of atomic positions (or simply root-mean-square deviation, RMSD) is the measure of the average distance between the atoms (usually the backbone atoms) of superimposed proteins. In this case, it was used to calculate the average distance between all heavy atoms (Cα atomic coordinates) with respect to the X-ray starting structure. Unfolded proteins usually exhibit increased RMSD values. Per-residue root-mean square fluctuation (RMSF) is a measure of the deviation over time between the positions of the Cα atomic coordinates of each residue with respect to the X-ray structure, and it was calculated to evaluate the local structure flexibility. The distance among amino acids forming the binding site for DNA and the DNA itself was calculated along the whole simulation process for the wild-type and mutant proteins. RMSF, hydrogen bonds and secondary structure content were determined with the Gromacs tools: *g_rmsf*, *g_bond* and *do_dssp*, which are interfaces to the DSSP program (Kabsch and Sander, 1983). 3D figures and motions were generated with UCSC Chimera.

**Cell cultures, plasmids, transfections and treatments**

Patients’ fibroblasts and HeLa cells were maintained in Dulbecco’s modified Eagle medium, supplemented with 2 mM l-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin and 10% heat inactivated foetal bovine serum at 37°C in 95% humidifier air and 5% CO2. pCMV6-AC-GSX2wt was obtained from OriGene Technologies. In situ mutagenesis was carried out at Eurofins Genomics to generate pCMV6-AC-GSX2Q251R and pCMV6-AC-GSX2Q251*.* HeLa cells were transfected with Lipofectamine® 2000 reagent (Thermo Fisher Scientific). For experiments of autophagy or proteasomal inhibition, fibroblasts were exposed to either 5 mM 3-methyladenine (3MA), 50 μM MG132, or the vehicle dimethylsulphoxide (DMSO) at the indicated times.

**Immunofluorescence**

Transfected HeLa cells were fixed in paraformaldehyde and coverslips were rinsed and blocked in phosphate-buffered saline with 10% bovine serum albumin prior to incubation with anti-GSX2 antibody (Novus Biologicals) overnight, followed by incubation with anti-rabbit Alexa Fluor® 555 (1:5000). Nuclei were stained with Hoechst (Thermo Fisher Scientific). Images were analysed with a confocal microscope (C2 Confocal Microscopy System). To evaluate subcellular localization of GSX2wt versus GSX2 Q251R, the fluorescence intensity of transfected proteins in the nucleus and cytoplasm were assessed, counting 10 fields in each of three independent experiments. In particular, for each cell it was evaluated whether nuclear fluorescence signal exceeded that of cytosol or vice versa. Cells with equivalent fluorescence signals in both compartments were not counted. The relative numbers of cells with predominant nuclear or cytoplasmic fluorescent signal per total number of examined cells were calculated and expressed as a percentage.

**Immunoblotting**

For total extracts, cells were lysed in RIPA buffer containing protease and phosphatase inhibitors and protein extracts were quantified by Bradford assay.

For nucleus/cytoplasm fractionation, transfected HeLa cells were first suspended in a buffer containing 10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl and protease inhibitors. After centrifugation (14 000 rpm), cytoplasmic protein extracts were
isolated and then pellets were suspended in a second buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA and protease inhibitors to obtain nuclear proteins. Equivalent amounts of lysates were resolved by electrophoresis through 4–20% Mini-PROTEAN TGX Gel (Bio-Rad) and probed with primary anti-GSX2 and secondary anti-rabbit-HRP antibody (Sigma Aldrich). Detection was performed by Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Experiments were normalized by α-Tubulin or GAPDH (Sigma Aldrich), or RB (BD Bioscience). Image contrast and brightness were optimized using Adobe Photoshop CS6 (Adobe Systems Incorporated). Densitometry measurements were calculated using ImageJ Software. Original blots of all experiments are shown in the Supplementary material (‘Expanded data’ section).

RT-PCR

Total RNA was extracted using the High Pure RNA Isolation Kit (Roche) and then reversely transcribed with SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). Resulting cDNAs were quantified by real-time PCR using SYBR® Green master mix on the HT-7900 platform (Thermo Fisher Scientific), to evaluate the expression levels of GSX2 target genes ASCL1, PAX6, DLX1, DLX2, SOX4 and MAP2, as well as GSX2 itself. Primers are listed in Supplementary Table 1. The relative expression was calculated using the ΔΔCT method.

Whole transcriptome analysis

Total RNAs extracted from fibroblasts of the two patients and three controls were quantified using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies) and a 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) to ensure a 260:280 ratio ≥ 1.5 and an RNA integrity number ≥ 8.

Sequencing libraries were prepared by SENSE Total RNA-Seq Library Prep Kit (Lexogen), assessed on a 2100 Bioanalyzer with a High Sensitivity assay, and sequenced on a NextSeq 500 Sequencer (Illumina). FastQ files were demultiplexed via illumina bcl2fastq2 (v.2.17.1.14) starting from binary base call files. Mapping and quantification of transcripts were computed by STAR/RSEM software using Gencode Release 19 (GRCh37.p13) as a reference and setting the ‘stranded’ option. Differential expression analysis was performed using R package EBSeq2 (Leng et al., 2013).

Pathway analysis

Gene set enrichment analysis was performed for coding genes. Gene Ontology (GO) enrichment analysis for biological processes, cellular components and molecular functions and a KEGG pathway analysis were conducted via enrichR web tool (Kuleshov et al., 2016). GO semantics classes and pathways were considered significant for P-values < 0.05.

Statistical analysis

Quantitative RT-PCR data and densitometric analysis are expressed as mean ± standard error. Statistical significance was evaluated by one tailed Student’s t-test. A P-value of < 0.05 was considered statistically significant.

Data availability

The raw data that support the findings of this study are available from the corresponding authors, upon request.

Results

Recessive variants of GSX2 cause a complex developmental brain defect with basal ganglia agenesis

We identified two unrelated females (Patient NG4690, aged 5 years and Patient NG4687, aged 14 years) who shared a unique and highly peculiar defect of the basal ganglia, characterized by bilateral agenesis of both the putamina and the globi pallidi with normal overlying insular cortex, hypoplasia of the thalami, and dysmorphic caudate nuclei with enlarged head and small nodules of periventricular grey matter in the body and tail (Fig. 1). In addition, both children presented agenesis or hypoplasia of the olfactory bulbs, dysplasia of the DMJ characterized by hypothalamic-mesencephalic fusion and bilateral oblique linear signal anomalies (hyperintense on T2- and hypointense on T1-weighted images) in the middle portions of the cerebral peduncles, and abnormal course of the corticospinal tracts in the brainstem (Fig. 2). No abnormalities in distribution or calibre of the cerebellothalamic tracts were found.

Both patients were born after uneventful pregnancies and appeared to be normal at birth. Clinical features manifested in the first weeks of life with developmental delay, feeding difficulties and axial hypotonia. They progressively developed spastic quadripareisis with superimposed severe dystonic movements and postures of the limbs and trunk. They maintained eye contact, relational smile and vocalizations but were unable to speak. Detailed clinical features of each patient are described in the Supplementary material.

After WES, variants identification, annotation and filtering in the two trios, both patients were found to carry homozygous variants in the GSX2 gene. Patient NG4690 carried the nonsense variant c.27G>A; p.(S9*), predicted to result in a truncated protein expressing only the first nine amino acids. Patient NG4687 carried the missense variant c.752A>G; p.(Q251R). This variant affected a highly conserved amino acid residue and was consistently predicted to be damaging or deleterious by all tested in silico predictors (Supplementary Fig. 1). Both variants segregated with the disease and were absent from all public reference databases (dbSNP, EVS, gnomAD) and from our combined in-house database including over 6000 individuals of different ethnicity.

Sanger sequencing of the coding region of GSX2 in additional eight probands with various types of DMJ dysplasia but without basal ganglia agenesis did not reveal pathogenic variants.
The missense variant p.(Q251R) alters dynamic properties and subcellular localization of GSX2 protein

GSX2 encodes a transcription factor with a highly conserved homeobox domain, consisting of three helical regions folded into a tight globular structure that recognize and bind to DNA. The p.(Q251R) variant falls within the third helix of the homeobox domain, which is the most important region for DNA recognition and binding. To assess the pathogenicity of this variant, we obtained a 3D model of the GSX2 protein and then performed several molecular dynamics analyses to compare structural stability and flexibility, long range interactions between residues, and distance between non-bonded entities in the contact region of the homeodomain-DNA complex in the wild-type versus the mutant protein. According to molecular dynamics simulations, the presence of the missense variant resulted in reduced stability and increased structural flexibility of the GSX2 homeodomain, particularly in helix III (Fig. 3A and B). In line with these findings, in the mutant protein the distances between non-bonded entities in the contact region of the homeodomain–DNA complex were largely floating and nearly always greater than those measured for the wild-type protein, in which conversely, the distance remained quite uniform throughout the simulation (Fig. 3C).

Previous molecular dynamics simulations performed on homologous proteins (Zhao et al., 2006) allowed identifying a number of water-mediated hydrogen bonds between residue Q50 (corresponding to Q251 in the GSX2 protein) and the DNA. Recent surveys of structural data on protein–protein and protein–DNA recognition sites have indicated that water is present in abundance at the interface, and the analysis of different high-resolution structures allowed the identification of at least as many water-mediated interactions as direct hydrogen bonds or salt bridges.
Thus, interfacial water may play an important role in mediating contact between protein residues and the corresponding DNA bases due to the presence of phosphate groups on the DNA side and of positively charged groups on the protein side. Interestingly, in wild-type GSX2 the side chain of Q251 allows water-mediated hydrogen bonds to establish, whereas these cannot be formed in the presence of the mutant arginine (Fig. 3D). Taken together, the results of molecular dynamics studies consistently suggest that the p.(Q251R) variant may significantly weaken the interaction between the homeobox domain and DNA.

To evaluate the disruptive effect of this missense variant on the protein function further, we overexpressed either wild-type or mutant GSX2 in HeLa cells and evaluated their subcellular localization by immunofluorescence. Wild-type GSX2 localized mainly in the cellular nuclei and to a much lesser extent in the cytoplasm, while nuclear localization was significantly reduced for mutant GSX2, which was more abundant in the cytoplasm (Fig. 4A). This observation was confirmed by nucleus-cytoplasm fractionation experiments, showing an inverted ratio of the mutant versus the wild-type protein (Fig. 4B).

Both variants impair transcriptional regulatory activity of the GSX2 protein

Next, we analysed by western blotting the expression levels of endogenous GSX2 protein in patients' fibroblasts. We failed to detect any protein expression in cells harbouring the nonsense variant p.(S9*), while GSX2 carrying the
p.(Q251R) missense variant was expressed, although at significantly lower levels than the wild-type protein in control cells (Supplementary Fig. 2A). To explore whether this could depend on enhanced degradation of the mutant protein, we evaluated protein expression levels upon treatment of fibroblasts with either MG132 or 3MA, to block the ubiquitin-proteasome or the autophagy pathway, respectively. Upon proteasomal blockage, the wild-type protein significantly increased, demonstrating that GSX2 is physiologically degraded through this pathway. Interestingly, the levels of GSX2Q251R remained unchanged after MG132 treatment, suggesting that either the mutant protein acquired conformational changes that made it less prone to be degraded by the proteasome, or that the protein levels were too low to trigger its degradation (Supplementary Fig. 2B). Conversely, both wild-type and mutant protein levels remained unchanged after 3MA treatment, indicating that GSX2 is not degraded through the autophagy pathway (Supplementary Fig. 2C). Taken together, these results indicate that the reduced levels of GSX2Q251R in patient’s fibroblasts do not depend on increased protein degradation.

We next measured by quantitative RT-PCR the levels of GSX2 mRNA in patients’ fibroblasts. Surprisingly, both mutant lines had GSX2 mRNA levels that were halved compared to control fibroblasts (Fig. 5). On one hand, this result excluded nonsense-mediated decay of the transcript carrying the nonsense variant c.27G>A. This mRNA is predicted to encode for a truncated protein of only nine amino acid residues, which likely could not be detected by the anti-GSX2 antibody. On the other hand, the interesting observation that GSX2 mRNA levels are significantly reduced in both patients suggests a mechanism of autoregulation of transcription by the GSX2 protein itself, as it was already proven for many transcription factors (Kielbasa and Vingron, 2008; Crews and Pearson, 2009).

In light of the results generated by dynamic modelling, subcellular localization and expression studies, we next sought to verify the impact of both variants on the transcriptional regulator activity of GSX2. To this aim, we assessed the expression levels of the two genes that are known to be positively (ASCL1) or negatively (PAX6) regulated by GSX2 (Toresson et al., 2000; Wang et al., 2009). Moreover, we also monitored the levels of two
additional genes (DLX1 and DLX2) that act downstream of ASCL1 in the same transcriptional network (Liu et al., 2017). As expected, in both patients’ fibroblasts, ASCL1 levels were significantly reduced (fold-change 0.2–0.4) while PAX6 levels were markedly elevated (fold-change /C24 8) compared to control fibroblasts. Interestingly, DLX1 and DLX2 levels were also upregulated, more evidently for DLX1 (fold-change /C24 10) (Fig. 5), confirming that GSX2 loss of function results in a global deregulation of the expression of downstream genes.

To confirm these findings and to obtain a global view of differentially expressed mRNA transcripts, we performed a whole transcriptome analysis from fibroblasts of the two patients and three healthy controls. Overall, we identified a small number of upregulated genes while many more genes were downregulated in the two patients (Supplementary Table 2). Data analysis showed a common profile of differentially expressed genes shared by the two patients compared to controls (Fig. 6A and B). Besides confirming previous results, whole transcriptome analysis showed a significant downregulation of other genes potentially related to GSX2, such as SOX4 and MAP2, which was confirmed by real-time PCR (Fig. 6C and D).

The complete list of significantly deregulated genes in patients’ versus control fibroblasts is presented in Supplementary material, File 2. Analysis of these genes and their related biological processes highlighted a downregulation of genes implicated in cellular senescence and apoptosis, and a global deregulation (with many upregulated transcripts) of genes implicated in inflammation and immunity (Supplementary Fig. 3).

Discussion

Homeobox genes represent an evolutionary conserved family of genes that play essential roles in regulating embryonic development. While Hox genes reside in clusters and their involvement in brain development is restricted to the hindbrain (Keynes and Krumlauf, 1994), several non-clustered homeobox genes are expressed in the developing forebrain and midbrain and are responsible for pattern formation of these regions (Rubenstein and Puelles, 1994). In mice, Gsx2 was first identified along with its closely related Gsx1, as two non-clustered homeobox genes homologues of the Drosophila ind gene,
et al. alone is much more prevalent in the developing LGE, caused by homozygous loss of function variants of the plex malformation of the basal ganglia and the DMJ, showed a deregulation of consequences, fibroblasts from both affected children properties which likely hamper its binding to DNA. As a result, the expression of several genes involved in GABAergic specification in the developing MGE and diencephalon, as well as in restricted areas of the midbrain and hindbrain (Hsieh-Li et al., 1995; Valerius et al., 1995), while Gsx2 alone is much more prevalent in the developing LGE, especially during early telencephalic development (Corbin et al., 2000). Within these regions, Gsx2 is highly expressed in neural progenitors, where it promotes neuronal differentiation while negatively regulating the formation of oligodendrocyte precursors (Chapman et al., 2013).

Here, we report two children presenting a unique complex malformation of the basal ganglia and the DMJ, caused by homozygous loss of function variants of the GSX2 homeobox gene. The nonsense variant results in lack of expression of the truncated protein, while the protein harbouring the missense variant is expressed at reduced levels, is retained in the cytosol, and shows altered dynamic properties which likely hamper its binding to DNA. As a consequence, fibroblasts from both affected children showed a deregulation of GSX2 downstream genes, with upregulation of PAX6, DLX1 and DLX2 and downregulation of ASCL1 and GSX2 itself.

Strikingly, the transcriptional defects as well as the neuroimaging phenotype observed in our patients closely mirror the developmental abnormalities seen in Gsx2 knock-out mice. In situ hybridization experiments in these animal models showed that Gsx2 is essential for the early expression of several genes involved in GABAergic specification and differentiation of the LGE (Szucsik et al., 1997; Corbin et al., 2000). In particular, the lack of Gsx2 was found to increase the expression of dorsal telencephalic genes such as Pax6, which is involved in the differentiation of both forebrain and midbrain, while impairing the expression of ventral regulators such as Ascl1 (also known as Mash1) within the LGE (Yun et al., 2001). Unexpectedly, DLX1 and DLX2 showed an opposite trend in our patients’ fibroblasts compared to the Gsx2 knock-out mice, in which their expression levels were selectively downregulated within the LGE while remaining unaltered in the MGE and other brain areas (Szucsik et al., 1997). Yet, it must be noted that these two genes are known to be negatively regulated by Ascl1, as they are both overexpressed in the ventricular zone of Ascl1/Mash1 knock-out mice (Casarosa et al., 1999). Thus, it can be speculated that in GSX2-mutated fibroblasts, the repressed ASCL1 levels may play a more relevant role than GSX2 itself in modulating DLX1 and DLX2, which therefore appear upregulated compared to controls, albeit their expression levels in the developing brain of GSX2-mutated patients remain unpredictable.

Whole transcriptome analysis in patients’ fibroblasts further disclosed the downregulation of genes more distantly related to GSX2, such as SOX4 and MAP2. SOX4, along with SOX11 and SOX12, form the group C of SRY-related (SOX) transcription factors, and play additive and redundant roles in multiple developmental pathways, including neurogenesis and skeletogenesis. In particular, during mouse cerebral cortex formation, Sox4 is highly expressed in intermediate progenitor cells where it regulates their survival and maintenance (Chen et al, 2015). MAP2 encodes a protein of the microtubule-associated protein family involved in microtubule assembly, which represents an essential step in neurogenesis. It is highly expressed in foetal brain, mainly in the cortex, amygdala, hypothalamus and basal ganglia. In mouse, Map2 expression is enriched in dendrites, where it likely stabilizes dendritic shape during neuronal development along with other MAP family proteins (Tucker, 1990). Several additional genes implicated in embryonic brain development were significantly downregulated in both patients’ fibroblasts. Among these are SPO11, SH3GL2, and CADM1, encoding for three proteins (spondin, endophilin A1 and cell adhesion molecule 1) involved in axonal and dendritic outgrowth and guidance, as well as SFRP2 and WISP2, encoding for two proteins (secreted frizzled related protein 2 and WNT1 inducible signalling pathway protein 2) related to the Wnt pathway.

**Figure 5** Impact of GSX2 mutations on the expression levels of downstream genes. mRNA levels of GSX2 and downstream genes ASCL1, PAX6, DLX1 and DLX2 in fibroblasts from patients and controls. GSX2 and ASCL1 levels are significantly reduced, while expression levels of PAX6, DLX1 and DLX2 are increased in mutated cells compared to control cells. Results are shown as mean ± standard error of at least three independent experiments; *P < 0.05.
Besides neurodevelopmental genes, whole transcriptome study and related pathway analysis in the two patients’ fibroblasts revealed a downregulation of genes related to cellular senescence and apoptosis and, more remarkably, a global deregulation of genes implicated in pathways related to immunity and inflammation, such as chemokine receptors and ligands, cytokine receptors, complement subunits and tumour necrosis factor-related proteins (Supplementary material, File 2). These findings are particularly intriguing in light of recent evidences pointing out to a central role for microglia, the specialized resident immune cells of the CNS, in orchestrating many of the complex processes taking place during embryonic brain development. These include regulation of neurogenesis both by

![Figure 6 Whole transcriptome analysis.](image)

(A) Heat map of log-normalized mRNA expression counts for GSX2 p.S9* (green) and p.Q251R (pink) versus controls (light blue), built using the top 60 differentially expressed mRNAs. The image shows a common deregulation between GSX2-mutated patients that clearly differ from healthy controls. (B) Common genes differentially expressed in both mutant cell lines share a very similar behaviour in terms of log2FC over controls. Black dots represent log2FC values in p.S9* (x-axis) and p.Q251R group (y-axis). Red solid line is the regression line, with a coefficient of determination $R^2 = 0.95$. (C) Real time PCR of SOX4 and MAP2 genes, confirming a significant downregulation in patients’ fibroblasts. (D) String analysis of GSX2-related pathways showing the interaction among proteins encoded by core deregulated genes.
direct phagocytosis of progenitor cells and by control of programmed cell death, regulation of brain wiring and modulation of synaptic formation and pruning (Frost et al., 2016). In particular, microglial control of neuronal death and survival is known to be mediated by the release of growth factors, interleukins and cytokines (Mosser et al., 2017; Thion et al., 2018); it can be speculated that the lack of functional GSX2 in the developing brain of our patients could have resulted in a global deregulation of microglia-related functioning, with a cascade effect eventually affecting not only the development of structures directly deriving from the LGE, but also of the CNS at large, thus contributing to the severe neurological phenotype observed in both children. This hypothesis, however, requires validation, as currently there are no specific studies on microglia or other immune-related cells in Gsx2 animal models.

Gsx2 knock-out mice are viable until birth but die within the first 24 h of postnatal life due to prolonged episodes of central apnoea. Upon histological analysis of the brain at developmental time points E12.5 to E17.5, the most striking abnormality is a significant reduction in size of the LGE, which persists at later time points (Szucsik et al., 1997). Although less severe, patterning defects are also found in the medial and caudal ganglionic eminences. Moreover, these mice show abnormal migration of interneurons in the olfactory bulb, while migration to the cerebral cortex seems to be largely unaffected (Corbin et al., 2000). In addition to the defects in LGE development, the area postrema and the adjacent solitary tract nucleus also failed to develop. These two hindbrain structures are relevant for the control of cardiorespiratory physiology and could be implicated in the prolonged apnoea experienced by these animals early in life (Szucsik et al., 1997).

Similar to the animal model, both Gsx2-mutated patients presented agenesis or dysgenesis of the structures derived from the lateral and median ganglionic eminences, including the putamina, the globi pallidi, the caudate nuclei and the olfactory bulbs. Conversely, while it is known that Gsx2 is expressed in the developing ventral thalamus of mice (Corbin et al., 2000), data on thalamic expression of this gene in humans are lacking, and it remains unclear if the thalamic hypoplasia observed in our patients is a primary process or the consequence of altered basal ganglia-thalamo-cortical connectivity. The observed complex malformation well explains the clinical picture, which is dominated by severe dyskinetic movements and generalized dystonic postures, albeit both patients failed to present the episodes of central apnoea which are lethal for the knock-out mice.

To date, congenital dysmorphisms of the basal ganglia have been reported in patients with either tubulopathies or XLAG (Bonneau et al., 2002; Kato et al., 2004; Okazaki et al., 2008; Bahi-Buisson et al., 2014). Tubulins represent a protein family essential for assembly and functioning of microtubules, dynamic cytoskeletal components that are crucial to sustain the extensive migration of neurons during brain development (Jaglin and Chelly, 2009). Despite the high degree of homology among distinct tubulins and their potential functional redundancy, distinct tubulin-mutant animal models consistently show robust and widespread neuronal migratory defects (Belvindrah et al., 2017). Of note, basal ganglia anomalies in TUBB4A mutations are related to an atrophic process in the context of a hypomyelination disorder (Simons et al., 2013), while in other tubulopathies they are secondary to the abnormal course of the white matter tracts in the anterior limbs of internal capsules (Mutch et al., 2016). In both cases, they are secondary processes rather than the result of an impaired basal ganglia formation. ARX encodes a transcription factor of the Aristaless-related paired-class homeobox family widely expressed in the forebrain, that is specifically involved in the proliferation, radial and tangential migration, and differentiation of GABAergic interneurons from the ganglionic eminences to the intermediate and subventricular zones and then to the cortical plate. Arx mutant mice showed periventricular accumulation of immature neurons in both the LGE and MGE, with strong impairment of both tangential migration towards the cortex and striatum and radial migration to the striatum and globus pallidus (Kitamura et al., 2002; Colombo et al., 2007). The widespread function of tubulins and ARX in the development of several embryonic brain structures explains the complex developmental defects associated with mutations in these genes, in which basal ganglia dysmorphisms are part of a wider, severe spectrum of cortical, callosal and midbrain-hindbrain anomalies.

In addition to the basal ganglia defects, Gsx2-mutated patients showed hypothalamic-mesencephalic fusion, representing a peculiar form of DMJ dysplasia. At difference from defects of the midbrain-hindbrain junction, dysplasia of the DMJ still remains poorly understood, and only few patients have been reported to date. Barkovich and coworkers first described three sporadic subjects presenting with midbrain enlargement, dorsoventral midline hyperintensity, and pontocerebellar hypoplasia likely due to a posterior to anterior transformation at the level of the DMJ (Barkovich et al., 2007, 2009). In 2012, Zaki and colleagues described six children from three consanguineous Egyptian families with a novel malformation termed ‘DMJ dysplasia’ characterized by a peculiar DMJ malformation with butterfly-like contour of the midbrain on MRI axial sections; additional features were supratentorial ventriculomegaly of variable degree, commissural abnormalities and lack of identifiable corticospinal tracts (Zaki et al., 2012). In 2016, we reviewed the MRI studies of 445 patients with brainstem malformations, and identified anomalies of the DMJ in 12, including the two patients reported in this study (Severino et al., 2016). In some cases, we observed a complete fusion between the hypothalamus and the midbrain, leading to dorsoventral enlargement and abnormal contour of the midbrain on axial planes (variably associated to midbrain ventral cleft, ventriculomegaly, pontocerebellar hypoplasia, and abnormalities of the corticospinal tracts, commissures, basal
ganglia, and olfactory bulbs), while others had a milder phenotype, with incomplete cleavage between the thalami and the mesencephalon on the sagittal plane, associated with midbrain and cerebellar vermis hypoplasia. These data suggest that the spectrum of DMJ patterning defects is wide and might be found in a heterogeneous group of genetic conditions that need to be further stratified according to clinical and other neuroimaging features.

Recently, biallelic loss of function variants in the protocadherin 12 gene (PCDH12), encoding a cell surface protein promoting cell adhesion and neurite outgrowth, have been identified in 14 patients with DMJ dysplasia from eight independent families. Patients presented a peculiar clinical-radiological phenotype characterized by progressive microcephaly, severe cognitive impairment, dysmorphic facies, variable spasticity and epilepsy, DMJ dysplasia with midbrain ventral cleft, callosal hypoplasia, and in most cases also calcifications (Guemez-Gamboa et al., 2018). Of note, PCDH12 mutations were found in two of the three families originally described (Zaki et al., 2012), while no pathogenic variants were identified in the third family with a different phenotype of massively dilated lateral ventricles, further supporting the hypothesis of different genetic mechanisms underlying DMJ disorders. In the present study, the two subjects carrying GSX2 mutations present a distinct anomaly of the DMJ, characterized by abnormal midbrain morphology on axial plane with bilateral oblique signal alterations at the level of the cerebral peduncles (Fig. 2). Sequencing of GSX2 in eight additional patients with other types of DMJ dysplasia and without basal ganglia agenesis failed to identify pathogenic variants, suggesting a relatively tight genotype-phenotype correlation. Moreover, we failed to identify biallelic GSX2 pathogenic variants by WES in our extended cohort of over 10,000 children with either spastic tetraparesis, movement disorders or intellectual disability, and additional mutated cases were not reported in online databases aimed at sharing phenotypic and genotypic data (such as Matchmaker), suggesting that GSX2 mutations are very rare and associate with a highly specific neuroradiological phenotype.

Interestingly, fibroblasts from both GSX2-mutated patients showed increased expression levels of PAX6, which is involved in the differentiation of both forebrain and midbrain (Yun et al., 2001; Spieler et al., 2004), and it is intriguing to speculate that disruption of the developmental cascade involving GSX2 and PAX6 may specifically interfere with DMJ positioning and functioning. Animal models have shown that the formation and location of the DMJ depend on a fine-tuned interplay among several genes (including Pax6, En1, and Pax2), orchestrated by Fgf8 expression (Nakamura et al., 2005). Finally, we noticed in both patients an abnormally dorsIALIZED course of the corticospinal tracts at the level of the pons (Fig. 2). Although the cause of this abnormality is not clear, we can speculate that the overexpression of PAX6 might eventually cause axon guidance defects of the corticospinal tracts, as this gene was found to play additional important roles in axonal pathfinding during CNS development (Mastick et al., 1997; Georgala et al., 2011).

In conclusion, we report the first genetic cause responsible for basal ganglia agenesis in humans. We demonstrate that loss of function of GSX2 may not be lethal but leads to an abnormal development of both LGE and MGE basal ganglia derivatives, associated with a severe hyperkinetic neurological phenotype.

Acknowledgements
We thank the patients and their family for their participation in this study.

Funding
This work was supported by grants from the European Research Council (ERC Starting Grant 260888 to E.M.V.), the Italian Ministry of Health (NET-2013-02356160 to E.M.V. and Ricerca Corrente 2018 RC18DSCOMU to T.M.), the Fondazione Mondino (5 per mille grant year 2016 to E.M.V. and C.C.), the Amber Project and NVIDIA Corporation (to T.M.), the National Institutes of Health (R01NS041537, R01NS048453, R01NS052455, P01HD070494, P30NS047101 to J.G.G.), the Howard Hughes Medical Institute and Simons Foundation (to J.G.G.). This research was also supported by a Grant of the Italian Ministry of Education, University and Research (MIUR) to the Department of Molecular Medicine of the University of Pavia under the initiative ‘Dipartimenti di Eccellenza (2018–2022)’. This publication is distributed under the terms of open access policies implemented by the the Italian Ministry of Education, University and Research (MIUR).

Competing interests
The authors report no competing interests.

Supplementary material
Supplementary material is available at Brain online.

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