

Helios Transcription Factor Expression Depends on *Gsx2* and *Dlx1&2* Function in Developing Striatal Matrix Neurons

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Development of the nervous system is finely regulated by consecutive expression of cell-specific transcription factors. Here we show that *Helios*, a member of the *Ikaros* transcription factor family, is expressed in ectodermal and neuroectodermal-derived tissues. During embryonic development, *Helios* is expressed by several brain structures including the lateral ganglionic eminence (LGE, the striatal anlage); the cingulate, insular and retrosplenial cortex; the hippocampus; and the accessory olfactory bulb. Moreover, *Helios* is also expressed by Purkinje neurons during postnatal cerebellar development. Within the LGE, *Helios* expression follows a dynamic spatio-temporal pattern starting at embryonic stages (E14.5), peaking at E18.5, and completely disappearing during postnatal development. *Helios* is expressed by a small population of nestin-positive neural progenitor cells located in the subventricular zone as well as by a larger population of immature neurons distributed throughout the mantle zone. In the later, *Helios* is preferentially expressed in the matrix compartment, where it colocalizes with *Bcl11b* and *Foxp1*, well-known markers of striatal projection neurons. In addition, we observed that *Helios* expression is not detected in *Dlx1/2* and *Gsx2* null mutants, while its expression is maintained in *Ascl1* mutants. These findings allow us to introduce a new transcription factor in the cascade of events that take part of striatal development postulating the existence of at least 4 different neural progenitors in the LGE. An *Ascl1*-independent but *Gsx2*- & *Dlx1/2*-dependent precursor will express *Helios* defining a new lineage for a subset of matrix striatal neurons.

Introduction

DURING EMBRYONIC DEVELOPMENT, neural lineages are derived from neural progenitor cells (NPCs) localized in specific brain areas, which consecutively generate both neurons and glial cells [1,2,3]. Within the developing telencephalon, neural stem cells from the wall of the lateral ventricle, the ventricular zone (VZ) [4], undergo successive divisions to expand

the pool of NPCs, thereby increasing the volume of the subventricular zone (SVZ). A number of signaling pathways controlled by different factors, such as Notch, Wnt, or FGFs, have been implicated in the self-renewal, proliferation and/or maintenance of the undifferentiated state of NPCs [5]. At certain developmental stages depending on the telencephalic area, NPCs divide for the last time and become specific immature neurons that migrate to postmitotic regions [4,6,7]. Multiple transcription factors finely regulate this neurogenic process [8,9].

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The striatum is one of the main telencephalic constituents of the basal ganglia; its embryonic anlage is the lateral ganglionic eminence (LGE) [10,11]. The progenitor zone of the LGE is the germinal zone (GZ) a source of striatal projection neurons and olfactory bulb interneurons [10,12,13]. Within the LGE, separate waves of neurogenesis are coupled to the production of different compartments known as the patches, or striosomes, and the matrix [14]. Neurogenesis between E12 and E13 produces clusters of substance P (SP)-expressing neurons that form the patches or striosomal compartment. The second wave of neurogenesis taking place at E14–E16 in mice will give rise to the matrix compartment, which contains both enkephalin (ENK)- and SP-positive neurons [15,16].

Several transcription factors such as *Gsx1 & 2* (formerly named *Gsh1 & 2*), *Ascl1* (formerly named *Mash1*) and *Dlx*-family members have distinct but overlapping patterns of expression in the VZ, SVZ, and mantle zone (MZ), and regulate distinct steps in LGE patterning and/or differentiation [17,18,19,20]. In addition to these transcription factors, *Ebf-1*, *Bcl11b* (formerly named *Ctip2*) and *Ikaros* are exclusively expressed in the striatal MZ where they regulate differentiation of striatal projection neurons [21,22,23,24].

Ikaros-family members are a Krüppel-type zing-finger transcription factors that play essential roles during lymphocyte development [25,26]. *Ikaros* is the founder member of this small family of DNA-binding proteins which consists of *Ikaros*, *Helios*, *Aiolos*, *Eos*, and *Pegasus* [27,28]. Besides the well characterized role of this family of transcription factors during hematopoietic development, *Ikaros* has also been recently involved in LGE development [24,29]. However, little is known about the expression of *Helios* in the central nervous system (CNS). In this work on the developing mouse, we present the first description of *Helios* expression during brain development. We focused on its transient expression in the developing striatum, providing evidence that *Helios* is a marker of a subpopulation of immature matrix striatal neurons. Further, we defined *Helios*' expression in several transcription factor mutants, thereby placing it within the known transcriptional hierarchy that drives striatal development.

Material and Methods

Animal Subjects

Mice were maintained in standard conditions with food and water ad libitum. All animal procedures were approved by local committees [Universitat de Barcelona, CEEA (133/10) and Generalitat de Catalunya (DAAM 5712)], in accordance with the European Communities Council Directive (86/609/EU). B6CBA wild-type (wt) mice (from Charles River Laboratories, Les Oncins, France), *Dlx-5/6-Cre-IRES-EGFP* transgenic mice [12], and *Ikaros* [30], *Gsx2* [31], *Ascl1* [32], *Dlx-1/2* [33], *Helios* [34], and *Ebf-1* [35] knockout mice ($^{-/-}$) were used. E18.5 *Dlx5/6-cre-IRES-EGFP/gtROSA* double transgenic brains were obtained by crossing heterozygous B6;129-Gtosa26^{tm1Sho}(*gtROSA*) mice [36] with hemizygous *Dlx5/6-cre-IRES-EGFP* mice.

All strains were maintained by backcrossing to C57BL/6 mice. Genotypes were determined by polymerase chain reaction (PCR) as described elsewhere [12,30,31,32,33,34,35]. The day of pregnancy, determined by the first detection of a

vaginal sperm plug in daily inspection, was considered embryonic day (E)0.5.

Histology

All immunostainings were performed with the following antibodies: anti-Helios (1:1,000; polyclonal; a generous gift of Dr. Stephen T. Smale (University of California Los Angeles (UCLA) School of Medicine [37]), anti-Helios (1:50; polyclonal, Santa Cruz), anti-Ikaros (1:2,000; monoclonal; [24]), anti-*nestin* (Rat 401; 1:50; polyclonal; Developmental Studies Hybridoma Bank; The University of Iowa), anti-GFAP (1:200; monoclonal; Sigma Chemical Co.), anti- β -III-tubulin (Tuj1; 1:200; monoclonal; Sigma Chemical Co.), anti-MAP2 (1:200; monoclonal; Sternberger Monoclonals), anti-NeuN (1:100; monoclonal; Millipore), anti-dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32; 1:500; polyclonal; Chemicon International, Inc.), anti-Ctip2 (1:200; polyclonal; Abcam), anti-calbindin (1:1000; monoclonal; Sigma Chemical Co.) and anti-Foxp-1 (1:200; polyclonal; Abcam).

For histological preparations, brains were removed at specific developmental stages and frozen in dry-ice cooled isopentane. Serial coronal cryostat sections (14 μ m) were cut on a cryostat and collected on silane-coated slides and frozen at -20°C . Fluorescent immunolabeling was performed according to the protocol described in [38]. Briefly, tissue sections were blocked for 1 h in PBS containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA) to avoid unspecific binding. Thereafter, they were incubated overnight at 4°C in PBS containing 0.3% Triton X-100 and 1% BSA with the corresponding primary antibodies. After three PBS washes, sections were incubated for 2 h at room temperature with the following secondary antibodies: Cy3-conjugated donkey anti-rabbit IgG (1:500) or Cy2-conjugated donkey anti-mouse or Cy3 donkey anti goat (1:200; all from Jackson ImmunoResearch Laboratories Inc.). At the end, tissue sections were counterstained with 4,6'-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) and mounted in Mowiol (Calbiochem). Fluorescent photomicrographs were taken on a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH). All pictures acquisitions were done as tiff files and adjustments of brightness and contrast were done with Adobe Photoshop 6.0 program (Adobe Systems Incorporated). No signal was detected in control preparations from which the primary antibody was omitted.

Nonisotopic in situ hybridization was performed using a riboprobe for *Helios* gene (Gene accession No. NM_011770). To obtain the riboprobe, an EcoRI digested fragment of the MSCV plasmid encoding *Helios* (kindly provided by Dr. Christopher A. Klug, University of Alabama at Birmingham [39]) was cloned into Bluescript SK II vector (Stratagene). To obtain the antisense probe, the plasmid was linearized with SacI, and transcribed with T7 RNA polymerase using the Digoxigenin labeling kit (Roche). Digoxigenin labeled sense probe, linearized with KpnI and transcribed with T3 RNA polymerase, was used as a control. In brief, brains were removed at E18.5 and frozen in dry-ice cooled isopentane. Serial coronal cryostat sections (14 μ m) were cut on a cryostat, collected on silane-coated slides, and frozen at -20°C . Frozen tissue sections were air

dried, fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, and processed for in situ hybridization as described elsewhere [40].

Production of viral particles and cell transduction

To overexpress *Helios*, we used the pLV-*Helios*-IRES-EGFP plasmid or the pLV-IRES-eGFP plasmid, which encode for human *Helios* and the green fluorescent protein (eGFP) or for eGFP alone, respectively. The pLV-IRES eGFP plasmid was generated as described elsewhere [41]. Briefly, MCS-IRES-eGFP was cloned from the PRV-IRES-eGFP (Genetrix SL, Tres Cantos) using the *Bam*HI and the *Sall* restriction sites into the pRRLsinPPT plasmid (pRRL) constructed by the Miami Project to Cure Paralysis Viral Vector Core Lab based on the lentiviral transducing plasmid developed by Naldini et al. [42]. To construct the pLV-*Helios*-IRES-eGFP, the human *Helios* gene from the SPORT6-*Helios* plasmid (Invitrogen S.A.) was cloned into pLV-IRES-eGFP between *Bam*HI and *Xho*I sites.

To overexpress *Gsx2*, the human *Gsx2* gene from the pcDNA-h*Gsx2* plasmid kindly provided by Dr. Peter Marynen (Université de Leuven, Belgium) was PCR-cloned into the retroviral vector pRV-IRES-eGFP using the MCS *Bam*HI and *Xho*I sites as described elsewhere [41].

For retroviral or lentiviral production, 293T cells were plated at a density of approximately 6×10^4 cells per cm^2 . The following day, cells were transfected by either a 3-plasmid system (pRV-*Gsx2*-IRES-eGFP or pRV-IRES-eGFP plasmid, the plasmid that expresses *gag* and *pol* genes, and the plasmid that expresses vesicular stomatitis virus G) or a 4-plasmid system (pLV-IRES-eGFP or pLV-*Helios*-IRES-eGFP plasmid, the plasmid that expresses HIV-1 Rev, the plasmid that expresses *gag* and *pol* genes, and the plasmid that expresses vesicular stomatitis virus G) using the calcium phosphate/DNA coprecipitation method. Cells were transfected for 7 h and afterwards the medium was replaced with fresh one. The supernatant from vector-producing 293T cells was collected every 22 h during 3 days. Then, the supernatant was passed through a 0.45- μm -pore-size filter to remove producer cells and subjected to 2 centrifugations at 4°C and 25000 g for 120 min to concentrate the virus. The virus-containing pellet was dissolved in BSA 1%. To determine the viral titer, a total of 2×10^5 3T3 or 293T cells were inoculated with serial dilutions of concentrated retrovirus or lentivirus. Forty-eight hours after infection, eGFP titers (IU/mL) were determined by using a fluorescence-activated cell scanner (FACS).

Neurosphere assay and overexpression

LGE from E14.5 wt mice were dissected out and mechanically disaggregated as previously described [24]. Briefly, LGE-derived neurosphere cultures were obtained by seeding 50,000 cells/ cm^2 in basal medium [Dubelcco's modified Eagle's medium (DMEM; Sigma Chemical Co.):F12 (Invitrogen S.A.) (1:1), 0.3% glucose (Sigma Chemical Co.), 0.3 mg/mL glutamine (Invitrogen S.A.), 5 mM HEPES (Invitrogen S.A.), 100 IU/mL penicillin, 100 mg/mL streptomycin (Invitrogen S.A.), 4 μg /mL heparin (Sigma Chemical Co.), 4 mg/mL BSA (Sigma Chemical Co.), $1 \times \text{N}2$ supplement (Invitrogen S.A.)], supplemented with 20 ng/mL fibroblast growth factor (Sigma Chemical Co.) and 10 ng/mL

epidermal growth factor (Invitrogen S.A.). Every 5 days, neurospheres were collected, dissociated by pipetting approximately 40 times with a P100 micropipette, and plated down in fresh media at a density of 10,000 cells/ cm^2 .

To overexpress mouse *Dlx-2*, we used the pCAGGS-*Dlx-2* vector as described previously Studhmer and colleagues [43]. To this end, neurospheres were disaggregated mechanically and 5×10^6 cells were transfected by nucleofection following the manufacturer's protocol (A33 Nucleofector Program; Amara Biosystems). Viable cells were counted by trypan blue exclusion and plated down at a cell density of 50,000 cells/ mm^2 .

To overexpress *Helios* or *Gsx2*, neurospheres were transduced with the pLV-*Helios*-IRES-eGFP or the pRV-*Gsx2*-IRES-eGFP lentivirus, or the control pLV-IRES-eGFP as described elsewhere [41].

Cell pellets were collected 3 or 5 days after transfection or transduction and frozen at -80°C for mRNA analysis.

Q-PCR assay

Gene expression was evaluated by quantitative PCR (Q-PCR) assays as previously described by Martin-Ibanez and coworkers [44]. The following TaqMan® Gene expression assays (Applied Biosystems) were used: 18s, Hs99999901_s1; *Helios*, Mm00496108_m1; vimentin, Mm00449208_m1; nestin, Mm00450205_m1; β -tubulin III, Mm00727586_s1; GFAP, Mm00546086_m1; olig1, Mm00497537_s1; olig2, Mm01210556_m1; *Dlx2*, Mm00438427_m1; and *Gsx2*, Mm446650_m1. To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and the samples were subjected to the PCR reaction with each TaqMan® gene expression assay.

Analysis and quantification was performed with the Comparative Quantitation Analysis program of the MxPro™ Q-PCR analysis software version 3.0 (Stratagene), using the 18S gene expression as internal loading control. All Q-PCR assays were performed in duplicate and repeated for at least three independent experiments. The results were expressed as relative levels with respect to the expression of the same gene in E14.5 for developmental studies or the control vector overexpressing cells considered as 100%.

Statistical analysis

All results are expressed as the mean of independent experiments \pm standard error of the mean. Results were analyzed using the Student's *t*-test or one-way analysis of variance, followed by the Bonferroni post-hoc test.

Results

Helios is expressed in specific brain and sensitive areas during mouse development

To study the expression and distribution of *Helios* during mouse brain development, we performed immunohistochemistry analysis at different developmental stages. At E16.5, *Helios* was expressed in the granule cell layer of the accessory olfactory bulb (Fig. 1A), the LGE, the cingulate (Fig. 1B), insular (Fig. 1B), and retrosplenial (Fig. 1C) cortices, and hippocampal CA1/2 (Fig. 1C). At early postnatal ages, *Helios* expression was also observed in the Purkinje cell layer of the cerebellum (Fig. 1D), while its expression decreased in

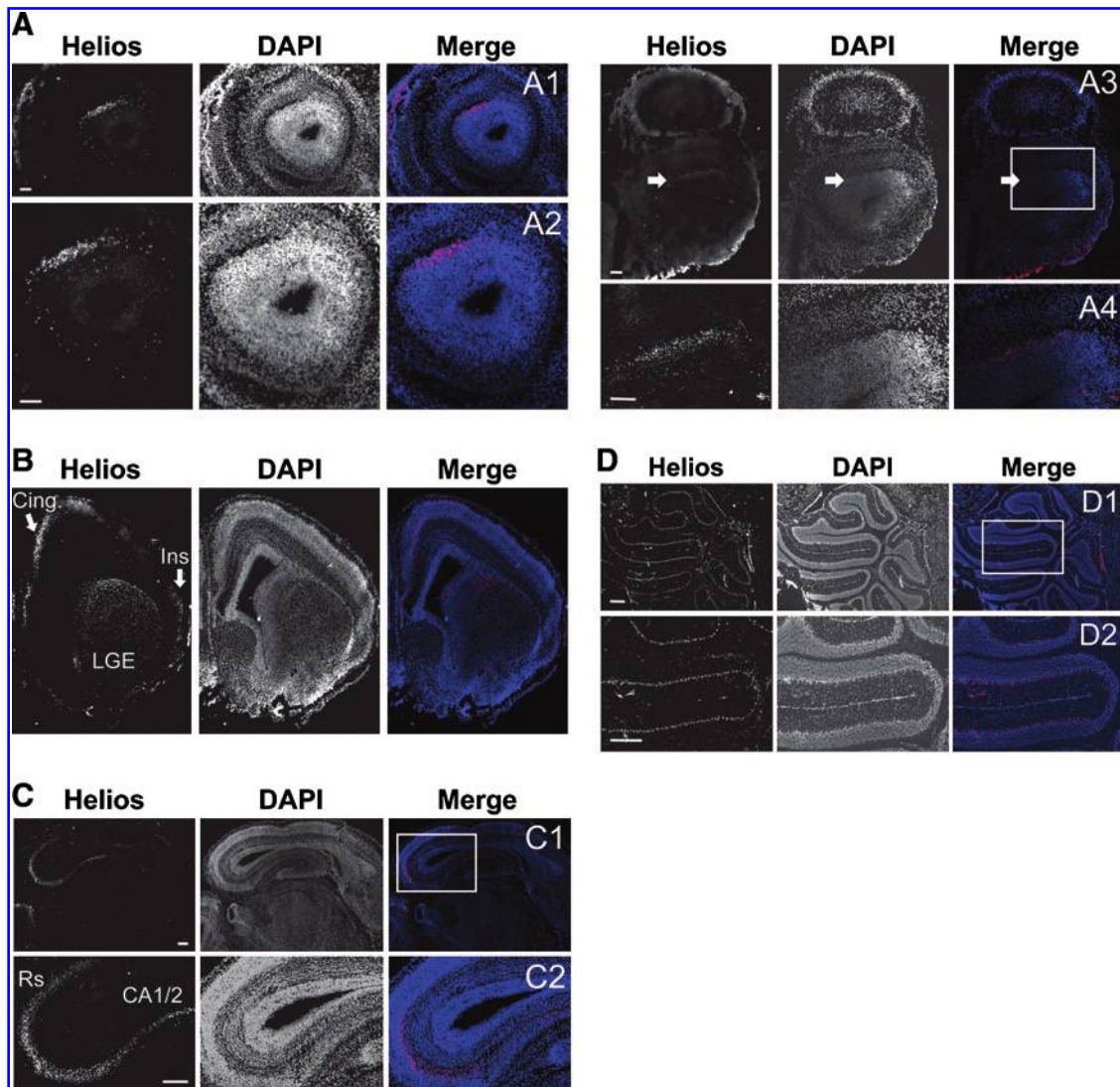


FIG. 1. *Helios* is expressed in specific brain areas during central nervous system development. *Helios* immunohistochemistry was performed in mouse brain coronal sections at E16.5 and P15. Representative fluorescent photomicrographs corresponding to anterior and posterior sections at E16.5 show *Helios* expression in: (A) the granular layer of accessory olfactory bulb from the anterior (A1, A2; note that A2 is a high magnification of A1) to the posterior axis (A3, A4; A4 represents the inset in A3); (B) the lateral ganglionic eminence (LGE), the cingulate (Cing) and insular (Ins) cortex; and (C) the retrosplenial cortex (Rs) and CA1/2 layer (CA1/2) of the hippocampus (C1, C2; C2 represents the inset in C1). (D) *Helios* is expressed by Purkinje neurons in the cerebellum at P15 (D1; D2 represents the inset in D1). Scale bars: (A) 1 mm; and (C, D) 2 mm.

the above-mentioned brain areas. In the adult brain, *Helios* expression was not detected (data not shown).

Helios is also expressed outside of the CNS during development (Supplementary Fig. S1; Supplementary materials are available online at <http://www.liebertpub.com/>). At E16.5, *Helios* expression was detected in the nasal and oral cavities as well as in the optic vesicles. Within the nasal cavity, this zinc finger transcription factor was detected in the epithelial tissue (Supplementary Fig. S1A). Similarly, in the oral cavity, *Helios* was also detected in the epithelium of the palate (Supplementary Fig. S1B). Both epithelia showed *Helios* expression located in the intermediate layers but not in basal cells, which constitute the stem cells of the epithelium. In addition, *Helios* was also detected in the taste buds of the tongue (Supplementary Fig. S1B).

Within the optic vesicles, this transcription factor was detected in the neuro-epithelial layers such as the pigmented epithelium and the neuronal layer of the retina (Supplementary Fig. S1C).

Helios shows a dynamic expression pattern during LGE development

Here we focused our study on *Helios* expression in the LGE and its derivative the striatum, because of the role of other Ikaros-family members in striatal development [29,24]. Immunohistochemistry studies performed at different LGE developmental stages showed that *Helios* expression was initially detected at E14.5; its levels increased at later stages, peaking between E18.5 and P3 (Fig. 2A). Subsequently, the

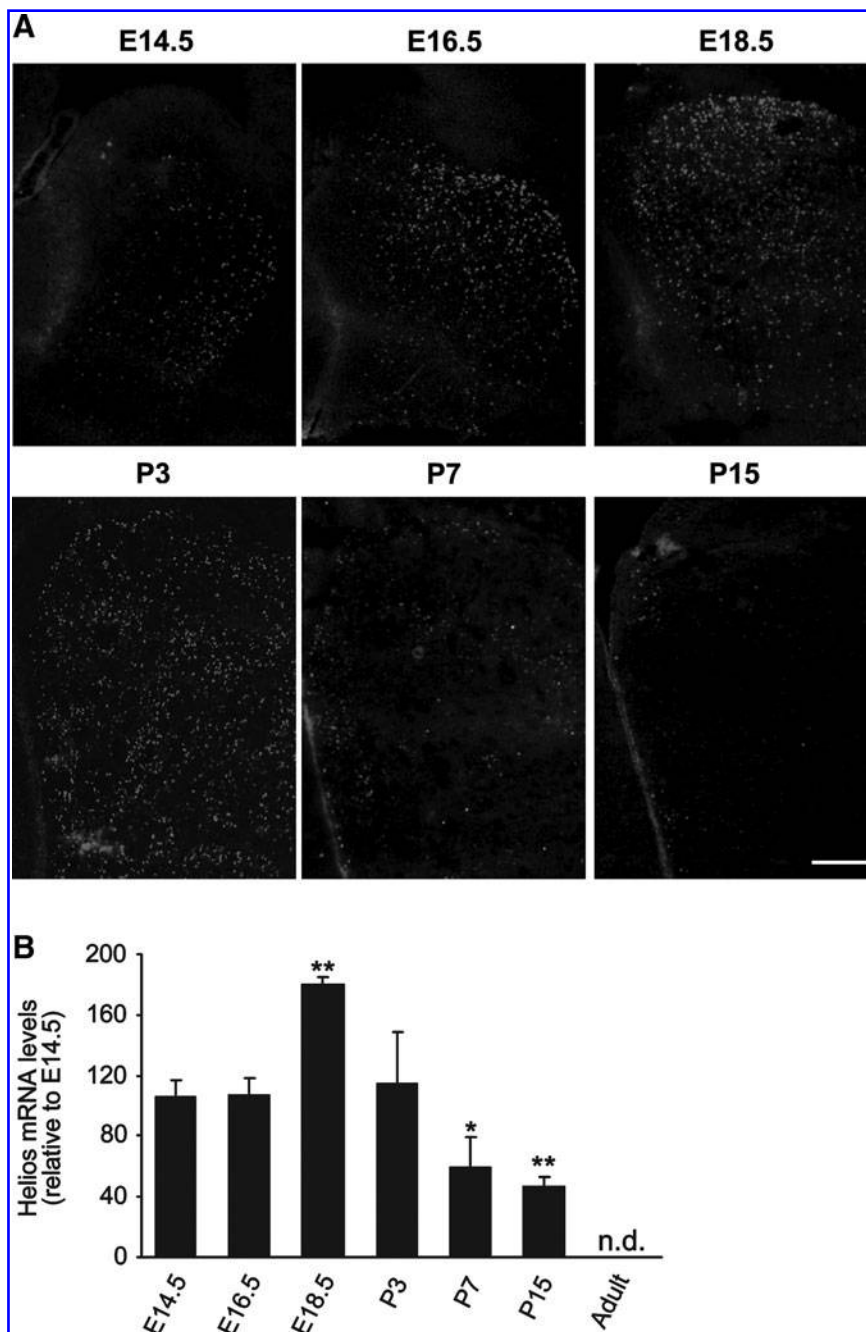


FIG. 2. *Helios* presents a dynamic spatio-temporal pattern of expression during LGE and striatal development. **(A)** *Helios* immunohistochemistry was performed in LGE coronal sections at different stages of embryonic (E14.5, E16.5, and E18.5) and postnatal (P3, P7, and P15) development. *Helios* is initially expressed at the ventro-lateral area of E14.5 mantle zone (MZ LGE) and progressively spreads to the dorso-medial area during development. The expression of *Helios* is reduced at later postnatal stages and only a few positive cells can be observed at P15. **(B)** Quantification of *Helios* mRNA levels by Q-PCR during LGE development showing a peak of expression at E18.5. Results are expressed as the mean of 3 to 5 LGE samples and error bars represents the standard error of the mean (SEM). Statistical analysis was calculated by Student's *t*-test; * $P < 0.05$; ** $P < 0.005$ relative to E14.5 expression. Scale bars: (A) 500 μm . (n.d., not detected).

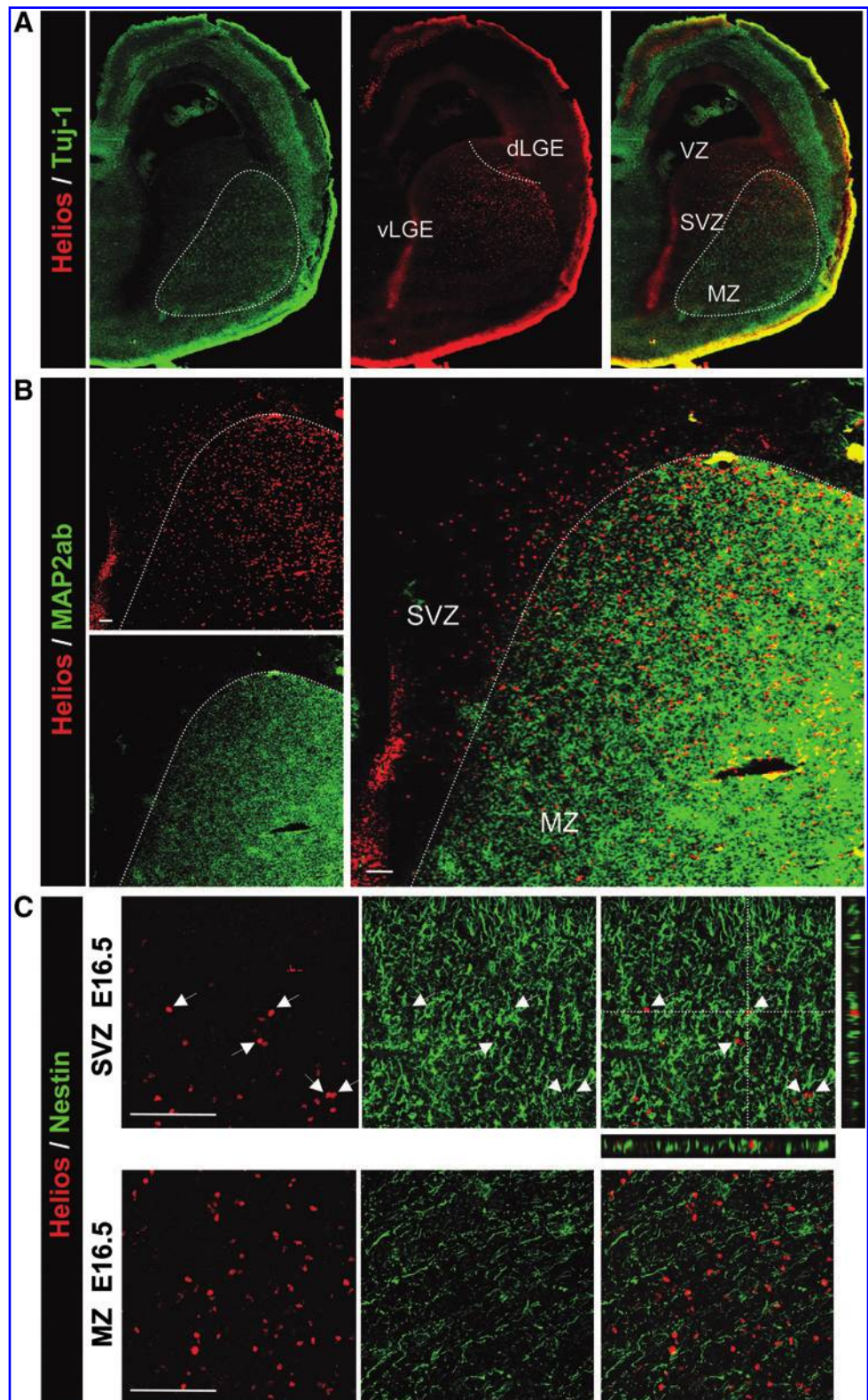
levels of expression decreased; after P15 expression was not detectable (Fig. 2A). These results were confirmed by Q-PCR (Fig. 2B). We did not detect *Helios* expression in the medial ganglionic eminence, the source of striatal interneurons (Supplementary Fig. S2).

The spatial distribution of *Helios* expression in the LGE and striatum showed 2 interesting findings. First, the pattern of *Helios* expression in the striatum changed over time, starting at E14.5 in the ventrolateral area and moving to the dorsomedial region at later stages (Fig. 2A). Secondly, we observed that this transcription factor was detected in 2 different cell populations. During embryonic development, *Helios* was expressed by a small population of cells in the SVZ (Fig. 3A and B). Within this germinal area, *Helios* colo-

calized with nestin-positive NPCs (Fig 3C). In addition, *Helios* was expressed by a larger population of cells located in the MZ at both embryonic and postnatal stages. However, in this area *Helios*-expressing cells were nestin-negative but MAP2-positive differentiating neurons.

The striatum is compartmentalized into separate matrix and striosomal (or patch) domains, which can be defined by specific markers [45]. The striosomal compartment is positive for DARPP-32 at late embryonic and early postnatal stages. During this period, *Helios* expressing cells appeared to be DARPP-32-negative (Fig. 4A). This finding is in agreement with the lack of colocalization observed at P3 between *Helios* and NeuN, showing the later a patchy distribution as well (Fig. 4B). These results suggested that *Helios* is expressed by

FIG. 3. *Helios* is expressed by nestin-positive cells in the SVZ and MAP2-positive cells in the MZ of the LGE. Double immunohistochemistry was performed in mouse brain coronal sections at E16.5 for *Helios* and β -III-tubulin (Tuj-1) (A), MAP2 (B), and nestin (C). (A, B). Note the expression of *Helios* in 2 different cell populations: a small one located in the Tuj-1- and MAP2-negative SVZ of the LGE that is spread through the ventral and dorsal part (A–B), and a larger one distributed along the MZ where it colocalizes with the neuronal marker MAP2 (B). (C) Representative high-magnification photomicrograph showing double immunohistochemistry for nestin and *Helios* both at the SVZ and MZ. Colocalization of both markers is observed in the SVZ of the LGE. Z-analysis shows the colocalization of *Helios* positive nuclei (arrows) surrounded by nestin-positive intermediate filaments. VZ, ventricular zone; SVZ, subventricular zone; MZ, mantle zone (delimited by dotted area). Scale bars: (B) 150 μ m; (C) 100 μ m.



matrix cells. This assumption was confirmed by the colocalization of *Helios* with *Bcl11b* (Fig. 4D) and *Foxp1* (Fig. 4E), 2 transcription factors mainly expressed by matrix cells [22,46]. Interestingly, we did observe double staining for *Helios* and NeuN at P7, a developmental stage at which the mature neuronal marker NeuN is spread all over the LGE (Fig. 4C).

However, the cells that express high levels of *Helios* still lack NeuN expression, while low *Helios*-expressing cells are double positive for both markers (Fig. 4C). This dynamic expression of NeuN indicated a progressive maturation of striatal neurons that is in agreement with previous papers demonstrating the early maturation of neurons located in the patches [14].

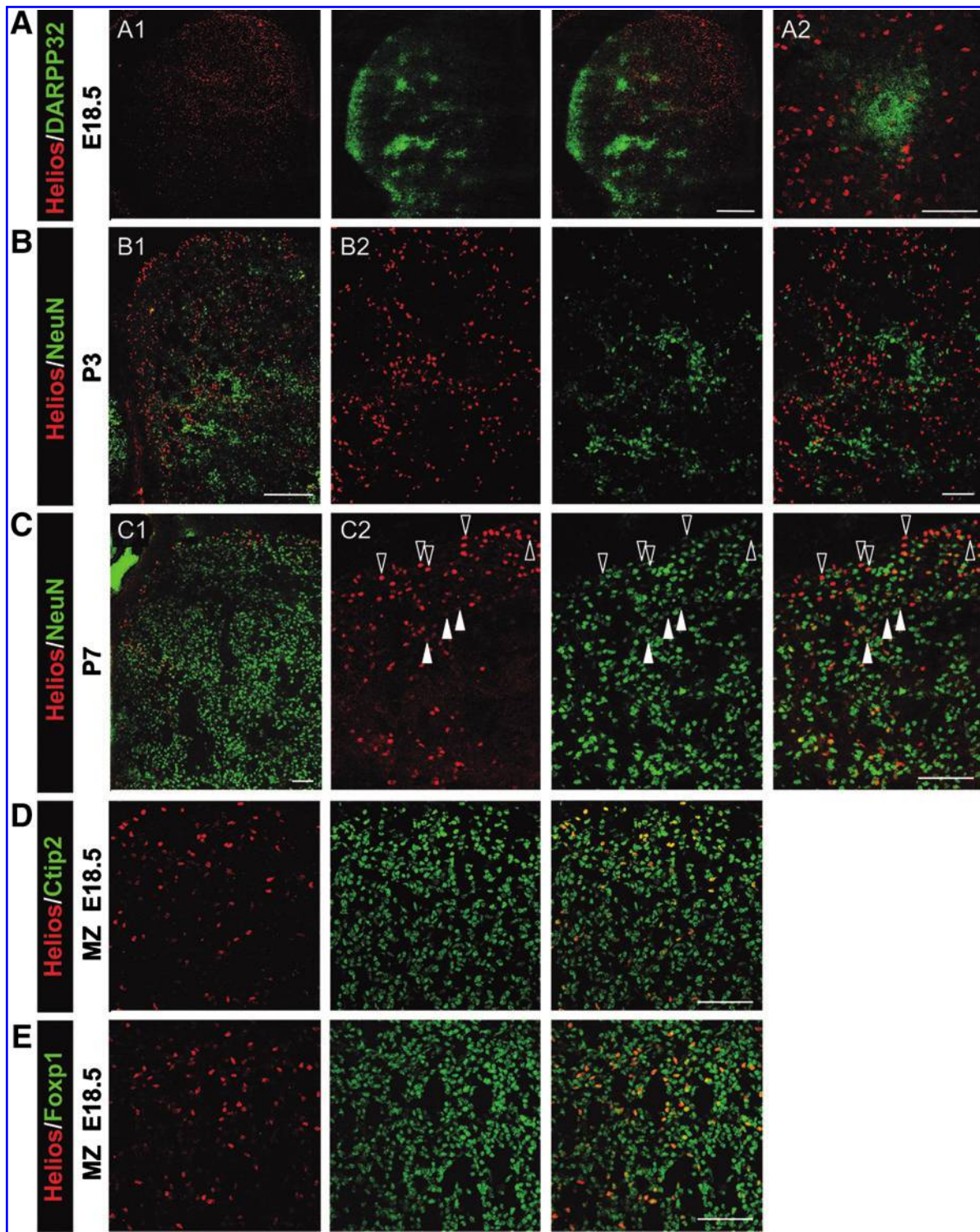


FIG. 4. *Helios* is expressed by neuronal precursors located in the striatal matrix compartment. Double immunohistochemistry was performed in mouse brain coronal sections at several embryonic and postnatal stages for *Helios* and striatal neuronal markers such as: DARPP-32 (**A**), NeuN (**B**, **C**), *Bcl11b* (*Ctip2*) (**D**), and *Foxp1* (**E**). Representative photomicrographs show that *Helios* is not expressed in the striosomal compartment, since it has a complementary distribution with DARPP-32, a striosomal marker at this stage (**A1**, **A2**; note that **A2** is a high magnification of **A1**). At P3, *Helios* does not colocalize with NeuN, a marker of mature neurons that at this stage presents a striosomal distribution (**B1**, **B2**; note that **B2** is a high magnification of **B1**). However, both markers are coincident in some cells at P7, developmental stage at which NeuN is staining most of the striatal neurons (**C1**, **C2**; note that **C2** is a high magnification of **C1**). High magnification picture shows that the cells that express high levels of *Helios* are negative or express low levels of NeuN (*open arrows*), while low *Helios*-expressing cells are positive for both markers (*closed arrows*) (**C1**). In addition, *Helios* is expressed by *Bcl11b* (*Ctip2*) (**D**) and *Foxp1*-positive neurons (**E**), two markers of striatal projection neurons. Scale bars: (**A1**, **B1**) 500 μm ; (**A2**, **B2**, **C**, **D**, **E**) 100 μm .

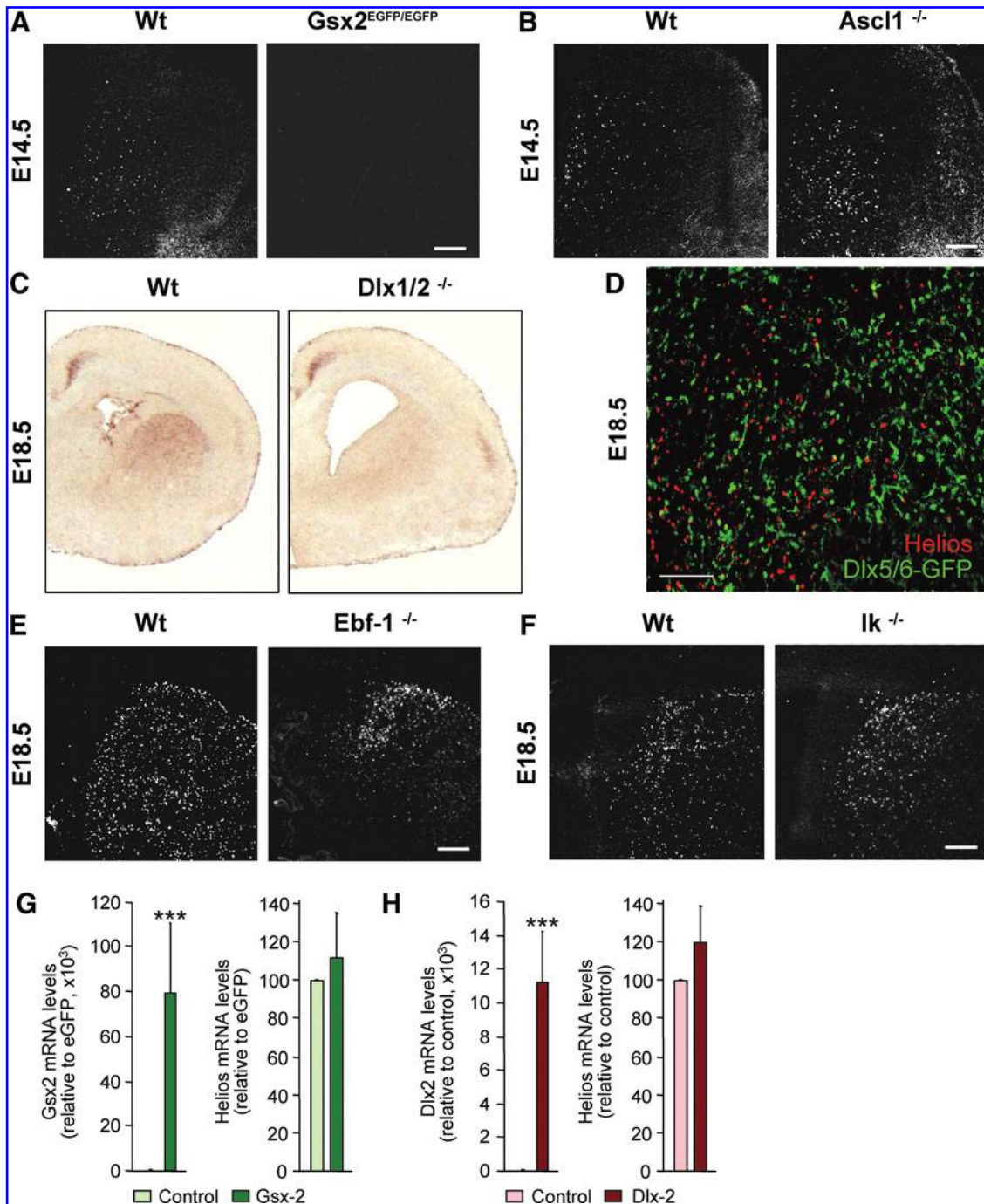


FIG. 5. *Helios* is expressed by a specific neuronal precursor downstream of *Gsx2* and *Dlx1/2* during LGE development. Immunohistochemistry and in situ hybridization analysis of *Helios* expression was performed in several knockout animals for transcription factors involved in striatal projection neurons development. *Gsx2* knock-out mice (*Gsx2*^{EGFP/EGFP}) show a complete lack of *Helios* expression (A). In contrast, normal *Helios* protein expression was detected in *Ascl1*^{-/-} mice (B). In situ hybridization analysis for *Helios* mRNA in wt and *Dlx1/2*^{-/-} mice show that the expression of this transcription factor is lost in the LGE but conserved in cortical areas at E18.5 (C). Double immunohistochemistry for *Helios* and eGFP performed in recombinant *Dlx-5/6-Cre-IRES-GFP/gtROSA* (*Dlx5/6-GFP*) mice shows that *Helios* does not colocalize with eGFP positive cells at E18.5 (D). In addition, *Helios* immunohistochemistry performed in wt and *Ebf-1*^{-/-} mice at E18.5, shows that its expression is detected in *Ebf-1*^{-/-} mice although its distribution changes due to the LGE alterations described in these mice (E). Similarly, *Helios* protein expression is also preserved in *Ikaros*^{-/-} mice (*IK*^{-/-}) at E18.5 (F). Over-expression of *Gsx2* (G) or *Dlx2* (H) in neurosphere cultures does not affect *Helios* expression levels 3 days after transduction or transfection, suggesting that this transcription factor is not directly regulated by *Gsx2* or *Dlx2*. Results are expressed as the mean of 4 to 5 neurosphere cultures and error bars represent the s.e.m. Statistical analysis was calculated by Student's *t*-test; ****p* < 0.001 relative to neurospheres transduced or transfected with the respective control vectors. Scale bars: (A, B) 500 μ m; (D) 150 μ m; (E) and (F) 500 μ m.

Helios is expressed by a specific type of neuronal precursor in the LGE

The germinal zone of the LGE is the main source of striatal projection neurons. In order to study the relationship between *Helios* and other transcription factors involved in the development of these neurons we analyzed the expression of this transcription factor in knockout mice for *Gsx2*, *Ascl1*, *Dlx-1/2*, *Ebf1*, and *Ikaros* (Fig. 5). Our results showed that *Helios*-positive cells completely disappeared in *Gsx2* knockout mice (Fig. 5A). However, *Helios* was normally expressed in *Ascl1*^{-/-} mice (Fig. 5B). On the other hand, *Dlx-1/2*^{-/-} mice lacked detectable *Helios* expression in the LGE, although its expression was not affected in the cerebral cortex (Fig. 5C). These findings indicated that *Helios* is expressed downstream of *Gsx2* and *Dlx1/2* specifically in the LGE. However, we demonstrated that *Helios* is not directly regulated by *Gsx2* or *Dlx2* since overexpression of these transcription factors in neurosphere cultures did not modify *Helios* expression (Fig. 5G and H).

Helios expression levels appeared unchanged in *Ebf1*^{-/-} (Fig. 5E) and *Ikaros*^{-/-} mice (Fig. 5F), although there was a change in the distribution of *Helios*-expressing cells in both mutants (Fig. 5E and F), probably reflecting striatal phenotypes that have been previously described [24,47].

Interestingly, the analysis of *Helios* expression in recombinant *Dlx-5/6-Cre-IRES-GFP/gtROSA* transgenic mice showed a lack of colocalization with GFP-positive cells

(Fig. 5D). Thus, *Helios*-positive cells appear to be in distinct immature striatal neurons, or alternatively, that it could be expressed upstream of *Dlx-5/6*.

Dimerization could be a prerequisite for DNA binding and function of all *Ikaros*-family members. Like *Aiolos* and *Ikaros*, *Helios* can dimerize with itself as well as with other family members [48]. To further analyze the relation between *Ikaros*-family members within the LGE, double immunohistochemistry for *Helios* and *Ikaros* was performed at E18.5. A lack of coincidence between these 2 transcription factors is shown in Fig. 6A. The same result was obtained at E14.5 and E16.5 (data not shown). In addition, the expression of *Ikaros* was not affected in *Helios* knockout mice (Fig. 6B), indicating a parallel or independent expression of these two transcription factors in different neuronal precursors.

Helios induces the expression of the neuronal marker β-III-tubulin in neurosphere cultures

All previous results suggested that *Helios* might participate in striatal development as a neurogenic factor. To test this hypothesis, we overexpressed *Helios* in neurosphere cultures, and 5 days later we tested the expression of several neural markers including vimentin, nestin, β-III-tubulin, GFAP, Olig1, and 2. Our results showed that *Helios* overexpression increased the levels of the neuronal marker β-III-tubulin without affecting the expression of precursors or glial markers (Fig. 7).

Present findings point to the hypothesis that *Helios* is a transcription factor expressed by a specific neuronal precursor in the LGE during striatal development defining a new lineage that might contribute to the generation of a matrix striatal neuron subpopulation (Fig. 8).

Discussion

We demonstrate for the first time that *Helios* is expressed in specific ectodermal and neuroectodermal-derived tissues. One of these areas is the LGE, the striatum anlage, where we

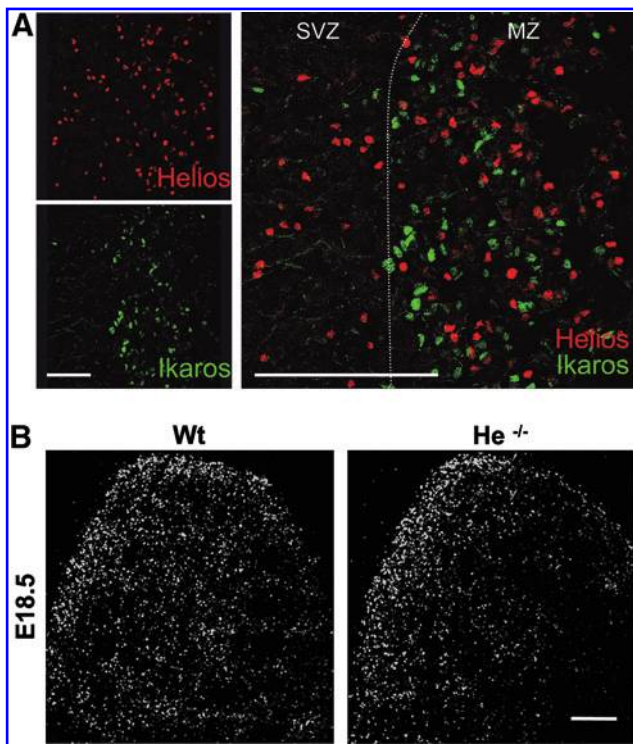


FIG. 6. *Helios* and *Ikaros* are not expressed in the same LGE cells at E18.5. Double immunohistochemistry for *Helios* and *Ikaros* performed in the LGE at E18.5 shows a lack of coincidence between these two transcription factors (A). Note that *Helios* is expressed in the SVZ and MZ whereas *Ikaros* is only expressed in the MZ (A). Immunohistochemistry for *Ikaros* in wt and *Helios*^{-/-} mice (*He*^{-/-}) demonstrate no changes in *Ikaros* expression in the absence of *Helios* (B). Scale bars: (A) 400 μm; (B) 500 μm.

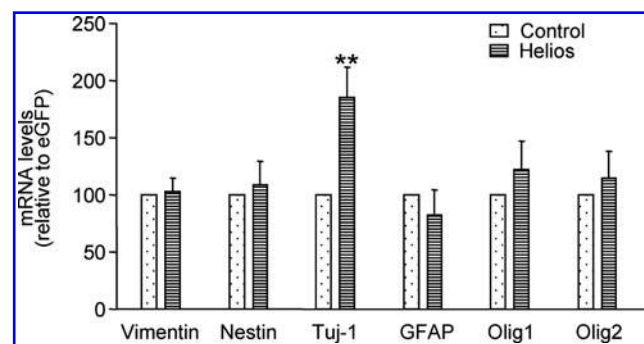


FIG. 7. *Helios* over-expression increases the levels of the neuronal marker β-III-tubulin. Over-expression of *Helios* in neurosphere cultures for 5 days induces an increase in the levels of the neuronal marker β-III-tubulin (Tuj-1). However, the expression of markers for neural precursors, vimentin and nestin, or glial cells, GFAP and Olig 1 and 2, are not modified after *Helios* over-expression in the neurosphere culture. Results are expressed as the mean of 5 neurosphere cultures and error bars represent the s.e.m. Statistical analysis was calculated by Student's *t*-test; ***p* < 0.005 relative to neurospheres transduced with the control vector.

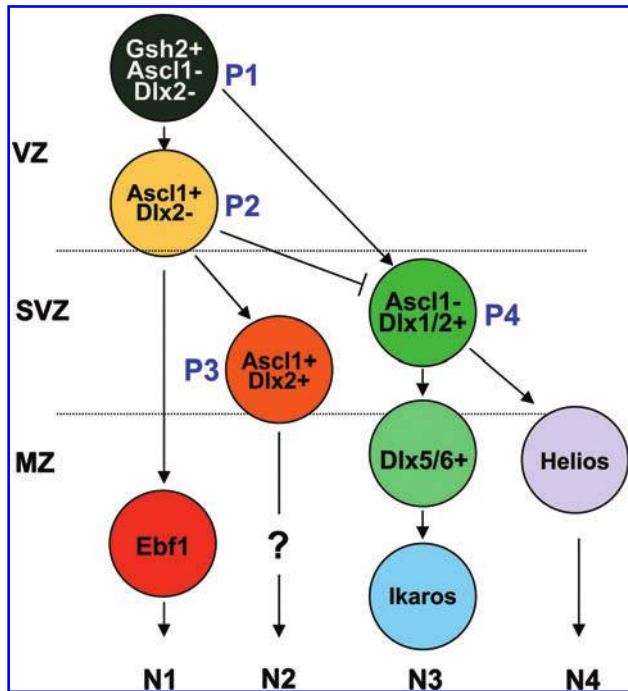


FIG. 8. Proposed model for the definition of different lineages contributing to the development of striatal projection neurons. P, NPC; N, immature neuron; VZ, ventricular zone; SVZ, subventricular zone; MZ, mantle zone. Color image available online at www.liebertpub.com/scd

observed that this transcription factor is expressed by both NPCs as well as by a subset of striatal immature neurons.

The relationship between *Helios* and other transcription factors involved in striatal development allowed us to define a new lineage that could determine the specific development of a subpopulation of matrix striatal projection neurons.

Helios is expressed by a specific precursor of striatal projection neurons during LGE development

Helios was originally described as a cell-type specific hematopoietic factor that is involved in the development of lymphocytes [37]. However, here we show that this transcription factor is expressed in several regions of the CNS and peripheral nervous system (PNS).

In the present work we focused on the characterization of *Helios* expression in the LGE, the progenitor region for the striatum. It is initially expressed in the ventro-lateral area of the LGE MZ at E14.5. This initial detection spatially coincides with the early pattern of expression of the mature markers of striatal projection neurons, such as proenkephalin [49]. In addition, as embryonic striatal development occurs, *Helios*-expressing cells appear dorsomedially following the same dynamics that has been previously described for proenkephalin [49]. These results suggest that *Helios* might be involved in the determination of a neuronal precursor toward a striatal projection neuron that would be committed to an enkephalineric phenotype. Keeping with this view, we observe that all *Helios*-expressing cells are positive for *Bcl11b*, an important transcription factor for striatal projection neurons development that is detected in NPCs at E13.5 [22].

Interestingly, *Helios* expression also colocalizes with the Forkhead transcription factor *Foxp1*. Several *Foxp*-family genes have been detected during telencephalon development [46,50,51]. Although all *Foxp* examined genes are expressed in the developing LGE, *Foxp1* shows a preferential expression for a subset of striatal projection neurons located in the matrix compartment [46] while *Foxp2* and *Foxp4* are more coincident with markers of the striosomal compartment. Therefore, these findings indicate that *Helios* and *Foxp1* are both expressed by a common neuronal precursor that will be differentiated into a matrix striatal projection neuron. Keeping with this view, *Helios* does not colocalize with DARPP32, a well known striosomal marker [52,53]. The colocalization of *Foxp1* and *Helios* is not restricted to the LGE; they are also coexpressed in the cortical plate and the CA1/2 subfield of the hippocampus (present results and references 46 and 51). Therefore, *Helios* and *Foxp1* may share functions in regulating the development of the striatum, cortex, and hippocampus.

Helios is expressed in the dLGE and the vLGE

Similarly to *Foxp1*, *Helios* is also detected in a subset of cells localized in the SVZ where it coincides with the NPC marker nestin. In this area, *Helios* is expressed in both the dorsal (dLGE) and the ventral LGE (vLGE).

The dLGE and vLGE are sources for both striatal projection neurons and olfactory bulb interneurons [12,54,55,56,57, 58]. While *Helios* was expressed in differentiating striatal neurons, we did not detect *Helios*-expressing cells migrating to the olfactory bulb, or in interneurons of the main olfactory bulb, during development or in adulthood. On the other hand, we did detect *Helios* expression in cells of the accessory olfactory bulb, which is responsible for the detection of pheromones [59,60,61]. It has recently been reported that *Bcl11b* is also expressed in the accessory olfactory bulb [62], where this transcription factor plays an important role during its development [63]. Interestingly, the expression of *Helios* is also coincident with the expression of *Bcl11b* in the respiratory epithelium of the nasal cavity (present results and reference 62).

Helios defines a new lineage of neuronal progenitors during LGE development

The expression of *Helios* in the LGE suggests that it participates as a neurogenic factor for the development of a subset of striatal projection neurons. In fact, we demonstrate that *Helios* overexpression induces an increase in the levels of neuronal markers without altering the expression of glial markers. Distinct transcription factors acting in cascades underlie the sequential steps of the determination and differentiation of the striatal projection neurons. Specification of the striatum depends on the function of the *Gsx1&2* homeobox genes, which are expressed in the VZ and SVZ of the LGE [64,65,54,66,55]. Mice lacking *Gsx2* have defects in dLGE specification [64]. Adding to the previously known defects, here we showed that *Helios* expression is not detectable in *Gsx2* knockout mice.

There is clear evidence that *Gsx2* drives the expression of *Dlx* genes and *Ascl1* in the LGE [64]. The *Dlx* homeobox gene family is essential for the development of striatal projection neurons [67,17]. *Dlx1* & 2, whose expression are first detected in the VZ and the SVZ, are the earliest *Dlx* genes expressed

during LGE development [17]. Thereafter, SVZ NPCs expressing *Dlx1* & 2 start expressing *Dlx5* & 6 [17,68]; thereafter, immature striatal neurons maintain *Dlx* expression [69]. Our results demonstrate that *Helios* is expressed downstream of *Dlx1* & 2 function but upstream or independent of *Dlx5* & 6 in the LGE.

Striatal development is not fully blocked in the *Dlx1&2^{-/-}* mutants [67], demonstrating that parallel and/or redundant pathways continue to promote the generation and migration of some striatal projection neurons. The analysis of gene expression profiles in the double *Dlx1/2^{-/-}* mice identified *Dlx*-dependent and *Dlx*-independent pathways. The *Dlx*-independent pathway depends in part on the function of the *Ascl1* transcription factor [40]. Our present results show that *Helios* is correctly expressed in *Ascl1^{-/-}* mice, indicating that this *Ikaros*-family member is expressed by *Dlx*-dependent but *Ascl1*-independent progenitors.

Ascl1 and *Dlx2* expression define different lineages of subcortical progenitors [20]. Combinatorial expression of these transcription factors, in conjunction with the expression of proliferation and differentiation markers, provided evidences for at least 3 lineages of NPCs in the germinal area (P1, *Ascl1⁻/Dlx2⁻*; P2, *Ascl1⁺/Dlx2⁻*; and P3, *Ascl1⁺/Dlx2⁺*) [20]. In the model proposed in Fig. 8, we introduced a new NPC which will be independent of *Ascl1* (P4, *Ascl1⁻/Dlx2⁺*). Our present and previous data also suggest that this new lineage will give rise to 2 different cell progenies that will define subsets of striatal projecting neurons (N3–4). One of these progenies will express *Dlx5/6* and thereafter become *Ikaros*+ neuronal precursors (N3) [24], whereas the other neuronal precursors will become *Helios*+(N4). This is in agreement with our present results demonstrating that *Helios* and *Ikaros* are not coexpressed in striatal neurons.

Here we also show that *Helios* is independent of *Ebf1*, another LGE specific transcription factor that regulates the development of SP neurons. Our previous data demonstrated that *Ebf1* is independent of *Ikaros* [24] and its expression is preserved in the *Dlx1/2^{-/-}* mice [24,40]. All these data indicate that *Ebf1* might be expressed by the P2 progenitor (*Ascl1⁺/Dlx2⁻*) to mediate the differentiation of SP striatal neurons by a cell-autonomous function exerted in NPCs as described elsewhere [20]. In fact, *Ebf1* has been closely related to the development of these striatal projection neurons [23,21]. In contrast, *Ascl1* might be indirectly involved in the development of striatal neurons by a non cell-autonomous mechanism that regulates the rate at which adjacent NPCs mature [20]. We propose that these adjacent NPCs could be the P4 that do not express *Ascl1*.

Many efforts are directed to the identification of specific transcription factors activated temporally and spatially to regulate cell fate determination during LGE development [4,5,70]. Here we describe a new player in striatal development named *Helios*. This zinc finger transcription factor defines a new lineage of neural precursors that will become a subset of matrix striatal projection neurons. Our results also provide evidences that suggest that *Helios* might be involved in the development of other specific brain areas.

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Author Disclosure Statement

No competing financial interests exist.

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