LIM homeodomain transcription factor-dependent specification of bipotential MGE progenitors into cholinergic and GABAergic striatal interneurons

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Coordination of voluntary motor activity depends on the generation of the appropriate neuronal subtypes in the basal ganglia and their integration into functional neuronal circuits. The largest nucleus of the basal ganglia, the striatum, contains two classes of neurons: the principal population of medium-sized dense spiny neurons (MSNs; 97-98% of all striatal neurons in rodents), which project to the globus pallidus and the substantia nigra, and the locally projecting striatal interneurons (SINs; 2-3% in rodents). SINs are further subdivided into two non-overlapping groups: those producing acetylcholine (cholinergic) and those producing γ-amino butyric acid (GABAergic). Despite the pivotal role of SINs in integrating the output of striatal circuits and the function of neuronal networks in the ventral forebrain, the lineage relationship of SIN subtypes and the molecular mechanisms that control their differentiation are currently unclear. Using genetic fate mapping, we demonstrate here that the majority of cholinergic and GABAergic SINs are derived from common precursors generated in the medial ganglionic eminence during embryogenesis. These precursors express the LIM homeodomain protein Lhx6 and have characteristics of proto-GABAergic neurons. By combining gene expression analysis with loss-of-function and misexpression experiments, we provide evidence that the differentiation of the common precursor into mature SIN subtypes is regulated by the combinatorial activity of the LIM homeodomain proteins Lhx6, Lhx7 (Lhx8) and Isl1. These studies suggest that a LIM homeodomain transcriptional code confers cell-fate specification and neurotransmitter identity in neuronal subpopulations of the ventral forebrain.

KEY WORDS: Basal ganglia, Cholinergic interneurons, Isl1, LIM homeodomain transcription factors, Lhx7, Striatum, Mouse

INTRODUCTION

A crucial step in the assembly of functional neuronal circuits in animals is the generation, at the appropriate position and time, of a plethora of neuronal subtypes with unique cellular and physiological properties (Gosgnach et al., 2006; Goulding and Pfaff, 2005; Jessell, 2000). The generation of diverse neuronal phenotypes is controlled by multiple extracellular and intracellular mechanisms operating in neuroepithelial progenitors and/or postmitotic neurons (Jessell, 2000). For example, in the ventral spinal cord, exposure of proliferating progenitors to the graded activity of sonic hedgehog (Shh) induces the expression of specific classes of transcription factors, such as members of the homeodomain (HD) and basic helix-loop-helix (bHLH) families, with cross-regulatory interactions that set up discrete progenitor domains (Dessaud et al., 2008). Several aspects of neuronal diversity, such as soma morphology and position, axonal trajectory and neurotransmitter phenotype often become apparent after cell cycle exit and are controlled, at least in part, by the activity of members of the HD family of transcription factors (Cheng et al., 2004; Shirasaki and Pfaff, 2002). Although diffusible signals and transcriptional regulators similar to those described in the spinal cord have been implicated in patterning and neuronal specification in the telencephalon (Wilson and Rubenstein,

2000), the molecular mechanisms operating in postmitotic forebrain neurons to activate distinct programmes of differentiation are less well understood.

The striatum is the largest nucleus of the basal ganglia, which is implicated in motor coordination and cognitive functions (Graybiel, 2000; Kreitzer, 2009). It receives most of the cortical input to the basal ganglia and, via its principal cell type, the γ-aminobutyric acid producing (GABAergic) medium-sized spiny projection neurons (MSNs), targets the substantia nigra and the globus pallidus (Bolam et al., 2000; Tepper et al., 2007). Deficits in the activity of MSNs and inadequate integration of their output underlie the motor deficits observed in conditions with dyskinesia, such as Parkinson's disease and Huntington's disease (Perez-Navarro et al., 2006; Pisani et al., 2005). In addition to MSNs, discrete subtypes of striatal interneurons (SINs) can be identified on the basis of morphological characteristics, molecular and neurotransmitter phenotype and electrophysiological properties (Kreitzer, 2009). Cholinergic SINs in rodents constitute approximately 0.3% of all striatal neurons, release acetylcholine (Ach) and can be identified by the expression of choline acetyl transferase (ChAT), an enzyme that catalyses the rate-limiting step in Ach biosynthesis. ChAT+ SINs have uniquely large cell bodies (magnocellular neurons) with a dense local axon collateral plexus and modulate the response of MSNs to cortical input (Kawaguchi et al., 1995; Tepper and Bolam, 2004). Non-cholinergic SINs constitute ~1-2% of all striatal neurons, use GABA as neurotransmitter and generate a strong inhibitory postsynaptic potential to modulate the firing pattern of MSNs. All GABAergic SINs express Gad67 (Gad1 – Mouse Genome Informatics), but are subdivided into three largely non-overlapping subtypes expressing parvalbumin (PV), calretinin (CR) or neuronal nitric oxide synthase (nNOS) (Kawaguchi et al., 1995; Tepper and Bolam, 2004). In contrast to the principal striatal neurons (MSNs), which originate

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from the ventricular zone of the lateral ganglionic eminence (LGE), SINs are born during embryogenesis in the medial ganglionic eminence (MGE) and migrate tangentially to reach the striatal anlage (Marin et al., 2000; Nobrega-Pereira et al., 2008; Olsson et al., 1995). Despite their crucial role in striatal function, the lineage relationship of the two main SIN subtypes and the molecular mechanisms that specify their distinct identities, including their neurotransmitter phenotype, are currently unclear.

The closely related LIM HD proteins Lhx6 and Lhx7 (also called L3/Lhx8) (Grigoriou et al., 1998; Matsumoto et al., 1996) have been implicated in the development of MGE derivatives, including cortical GABAergic interneurons and ventral forebrain cholinergic neurons (FCNs) (Alifragis et al., 2004; Bachy and Retaux, 2006; Du et al., 2008; Fragkouli et al., 2005; Lavdas et al., 1999; Liodis et al., 2007; Marin et al., 2000; Mori et al., 2004; Zhao et al., 2008; Zhao et al., 2003). During embryogenesis, *Lhx6* is induced in Nkx2.1-expressing MGE progenitors as they exit the cell cycle and colonise the subventricular zone (SVZ) (Flames et al., 2007; Liodis et al., 2007). Such Lhx6-expressing immature neurons also produce GABA (Liodis et al., 2007; Marin et al., 2000; Wonders and Anderson, 2006) and have been identified as proto-GABAergic neurons (Flames and Marin, 2005). Expression of *Lhx6* is maintained in postmitotic neurons in the mantle zone (MZ) of the MGE and in the majority of tangentially migrating GABAergic interneurons and is required for the specification of the PV- and somatostatin (SST)-expressing subpopulations of cortical GABAergic interneurons (Du et al., 2008; Liodis et al., 2007; Zhao et al., 2008). *Lhx7* is also induced in postmitotic cells in the SVZ and the MZ of the MGE, but unlike *Lhx6* is not expressed in tangentially migrating cortical interneurons. Instead, Lhx7 transcripts are restricted to ventral forebrain neurons, including the entire population of FCNs, which is composed of the cholinergic interneurons of the striatum and projection neurons of the basal forebrain (Asbreuk et al., 2002; Fragkouli et al., 2005; Zhao et al., 2003). Consistent with its expression pattern, mice homozygous for *Lhx7* mutations show a dramatic reduction in the number of ChAT⁺ neurons in the ventral forebrain, including the striatum, indicating that Lhx7 is required for the differentiation of the majority of FCNs (Fragkouli et al., 2005; Mori et al., 2004; Zhao et al., 2003). By analysing the expression of a nuclear β-galactosidase (β-gal) reporter driven by the null *Lhx7^{nlacZ}* allele, we have further demonstrated that upon deletion of *Lhx7* the number of β-gal-expressing cells in the forebrain of mutant animals does not change significantly (Fragkouli et al., 2005). Despite these studies, the broader role of Lhx7 in neuronal subtype specification and its potential interaction with other transcription factors in the ventral forebrain remain unknown.

Here we combine expression studies, phenotypic analysis of mutant mice, genetic fate mapping and ectopic gene expression in embryos to provide evidence that, upon deletion of *Lhx7*, cholinergic SINs are mis-specified into mature GABAergic SINs. We also demonstrate that both subtypes of SINs are derived from common *Lhx6*-expressing proto-GABAergic precursors generated in the MGE. Our experiments suggest that the choice of subtype identity by the common precursor is controlled by the combinatorial activity of the LIM HD proteins Lhx6, Lhx7 and Isl1. Expression of *Lhx6* is associated with GABAergic differentiation, whereas downregulation of *Lhx6* and combined expression of *Lhx7* and *Isl1* leads to cholinergic differentiation. These experiments advance our understanding of neuronal subtype specification in the mammalian forebrain and suggest molecular strategies for manipulating the identity of telencephalic progenitors.

MATERIALS AND METHODS

Animals

The following transgenic mouse lines have been described elsewhere: *Lhx7^{nlacZ}* (Fragkouli et al., 2005), Gad^{GFP} (Tanaka et al., 2003), *Lhx6^{nlacZ}* (Liodis et al., 2007), Nkx2.1-Cre (Kessaris et al., 2006) and Rosa26-StopYFP (Srinivas et al., 1999). To generate transgenic mice expressing Cre under *Lhx6* transcriptional control we used homologous recombination in bacteria to modify a genomic BAC containing the Lhx6 locus (RP24-384G1) (Lee et al., 2001). The iCre coding sequence was fused to the ATG of Lhx6 in exon 1 using a PCR-based approach. An SV40 polyA was inserted downstream of iCre. The construct was designed to delete the coding sequence of exon 1, as well as the first 80 bp from the first intron. Transgenic mice were generated by pronuclear injection of the modified BAC as previously described (Kessaris et al., 2006). A partial description of this line has already been reported (Fogarty et al., 2007). All animal studies were carried out under a UK Home Office Project Licence in a Home Office designated facility and under local Ethics Committee regulations.

Immunostaining

Immunostaining was performed on vibratome sections (50 μm) from adult mouse brain or on cryosections (10 μm) from embryonic mouse brain or chick embryos. Adult mouse brain was dissected from animals perfused with 4% paraformaldehyde (PFA) in PBS and immersed in the same fixative for an additional 2 hours (for immunostaining with the anti-Isl1 antibody) or overnight (for all the other primary antibodies). Embryonic tissues were fixed for 2 hours in 4% PFA in PBS, cryoprotected in 30% sucrose in PBS and kept frozen until used. Adult mouse brain sections were permeabilised with 1% Triton X-100/PBS for 1 hour at room temperature (RT), blocked with 10% fetal calf serum (FCS), 1% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS for 2 hours (RT) and incubated overnight at RT (or 36 hours at 4°C) with specific primary antibodies diluted in a solution containing 1% FCS, 0.1% BSA, 0.1% Triton X-100 in PBS. Sections were then washed with PBS, incubated for 2 hours at RT with the appropriate secondary antibodies conjugated with fluorophores and mounted with vectashield mounting medium containing DAPI (Vector). Cryosections from embryonic tissue were blocked with 1% BSA in PBS for 1 hour at RT, incubated overnight at 4°C with specific antibodies in the same solution and processed as previously described.

The following primary antibodies were used: goat anti-ChAT (1:500, Chemicon), mouse anti-β-gal (1:500, Promega), rabbit anti-β-gal (1:500, Cappel), rabbit anti-GFP (1:500, Molecular Probes), rabbit anti-DARPP-32 (1:500, Chemicom), rabbit anti-nNOS (1:100, BD Biosciences), mouse anti-PV (1:1000, Chemicon), rabbit anti-CR (1:1000, Chemicon), rabbit anti-Lhx6 (1:250) (Lavdas et al., 1999), rat-anti-GFP (1:500, nacalai tesque), mouse anti-Isl1/2 (1:50, 4D5, DSHD) mouse anti-MNR2 (1:50, 5C10, DSHB), rabbit anti-Chx10 (1:5000) (Ericson et al., 1997) and rabbit anti-Isl1 (Ericson et al., 1992).

β-gal histochemistry

Embryonic mouse brain tissue was processed for β-gal histochemistry essentially as described previously (Fragkouli et al., 2005).

DNA cloning

The electroporated constructs were generated by subcloning the entire mouse Lhx6 and Lhx7 cDNAs (Grigoriou et al., 1998) into the pCAGGs expression vector, which also encodes bicistronically nuclear targeted GFP (IRES NLS-GFP pCAGGs). The Rat Isl1 cDNA (also subcloned into the PCAGGS vector) has been described previously (Thaler et al., 2002).

Embryonic mouse brain electroporation

Ex vivo electroporations of embryonic brain and organotypic slice cultures were carried out essentially as described previously (Heng et al., 2008). Briefly, plasmids encoding GFP, Lhx7-IRES-GFP, Lhx6-IRES-GFP or Isl1-IRES-GFP (1.5 μg/μl each) were injected into one of the ventricles of E14.5 mouse embryo heads. Subsequently, brains were electroporated, embedded into 3% low melting agarose in L-15 and incubated on ice for 60 minutes. Vibratome slices 300 μm thick were then cultured at 37°C, 5% CO_2 for 4 days. At the end of the culture period slices were cryoprotected and re-

sectioned at 10 μm using a cryostat. In situ hybridisation for ChAT (Fragkouli et al., 2005) followed by immunohistochemistry for GFP was performed following standard procedures.

In ovo electroporation

DNA constructs were injected into the neural tube lumen of Hamburger and Hamilton (HH) stage 12-14 chick embryos and electroporation was performed as previously described (Briscoe et al., 2000). Chick embryos were analysed at HH stage 25-28.

Quantification

To quantify the percentage of striatal β-gal positive cells colocalising with markers of different striatal neuronal subtypes, we used coronal brain sections (50 μm each, spaced 250 μm apart) from four $Lhx7^{+/nlacZ}$ and four $Lhx7^{nlacZ/nlacZ}$ animals, employing an unbiased systematic random approach i.e. the percentage of double-positive cells over the total number of β-gal-positive cells was counted in a rostrocaudal series of equally spaced sections (every 250 μm, one section counted from every six sections cut) starting from a section in which the brain structure first appeared in each animal – according to the mouse brain atlas (Paxinos, 2001) – and spanning the full extent of the structure. In each section the entire striatum was analysed. Quantification was performed using confocal microscopy. For every section, a series of optical sections 2 μm apart were captured using a Bio-Rad/Radiance 2100 confocal laser-scanning microscope. Optical sections were then assembled into 50 μm z-stacks and analysed using the LaserSharp 2000 programme (Bio-Rad).

To quantify the total number of β-gal positive cells in the striatum we used coronal brain sections (14 μm) from four $Lhx7^{+/nlacZ}$ and four $Lhx7^{nlacZ/nlacZ}$ animals. To avoid any counting bias, the rostrocaudal extent of the areas to be examined was first defined according to the mouse brain atlas (Paxinos, 2001) and β-gal positive cells were counted in a rostrocaudal series of equally spaced sections (one section counted every ten sections cut) starting from a section in which the brain structure first appeared in each animal – according to the mouse brain atlas – and spanning the full extent of the structure. In each section the entire striatum was analysed. Brain area images were captured using a digital CCD colour video camera (TK-C1381, JVC, Japan) connected to an optical microscope (Eclipse E400, Nikon, Japan) and images were analysed using the image analysis program Image Pro Plus (Media Cybernetics).

To quantify the induction of cholinergic phenotype in the cortex, we calculated the fraction of GFP+ cells co-expressing ChAT from a minimum of nine cryosections (those containing the highest number of GFP+ cells) representing five electroporated brains. The percentage of ChAT+GFP+/GFP+ cells was assessed using an epifluorescent microscope (Axioplan2, Zeiss). Images shown in Fig. 6 were acquired using a LeicaSP5 confocal microscope.

Finally, the induction of different neuronal subtypes upon DNA electroporation in the dorsal neural tube was quantified in HH stage 26 chick embryos. Five cryosections (10 μm each) were used for each animal and the number of dorsally located cells/section was counted. For each construct or combination of constructs 15 embryos derived from at least three independent experiments were analysed.

Statistical analysis

In all cases (i.e. assessment of the effect of genotype on the phenotype of striatal β-gal+ cells or assessment of the effect of the various DNA constructs and their combinations on the induction of specific phenotypes in mouse cortical or chick spinal cord neural progenitors), statistical analysis was performed by a two-tailed Student's t-test (Excel, Microsoft).

RESULTS

Non-overlapping distribution of the LIM HD proteins Isl1 and Lhx6 define, respectively, the cholinergic and GABAergic sublineages of *Lhx7*-expressing SINs

To examine in detail the expression of *Lhx7* in the adult striatum, forebrain sections from $Lhx7^{nlacZ}$ heterozygous animals (Fragkouli et al., 2005) were double immunostained for β-gal and markers of striatal neuronal subtypes. No β-gal+ cells co-expressed DARPP-32, a characteristic marker of LGE-derived MSNs (Ouimet et al., 1984), indicating that in the striatum Lhx7 is restricted to MGE-derived SINs (see Fig. S1A in the supplementary material). Consistent with this, 64.5±1.4% of β-gal+ cells co-expressed ChAT and were characterised by relatively large nuclei and somata, distinctive features of magnocellular cholinergic SINs (Fig. 1A) (Tepper and Bolam, 2004). All ChAT+ cells in the striatum of $Lhx7^{+/nlacZ}$ mice were also positive for β-gal (Fig. 1A), demonstrating that *Lhx7* is expressed by virtually all cholinergic SINs. Immunostaining of forebrain sections from $Lhx7^{+/nlacZ};Gad67^{GFP}$ double transgenic reporter mice [in which the green fluorescent protein (GFP) marks all GABAergic neurons] (Tanaka et al., 2003) showed that 42.8±6.0% of β-gal+ cells in the striatum were positive for GFP (Fig. 1B). In contrast to the magnocellular cholinergic interneurons (Fig.
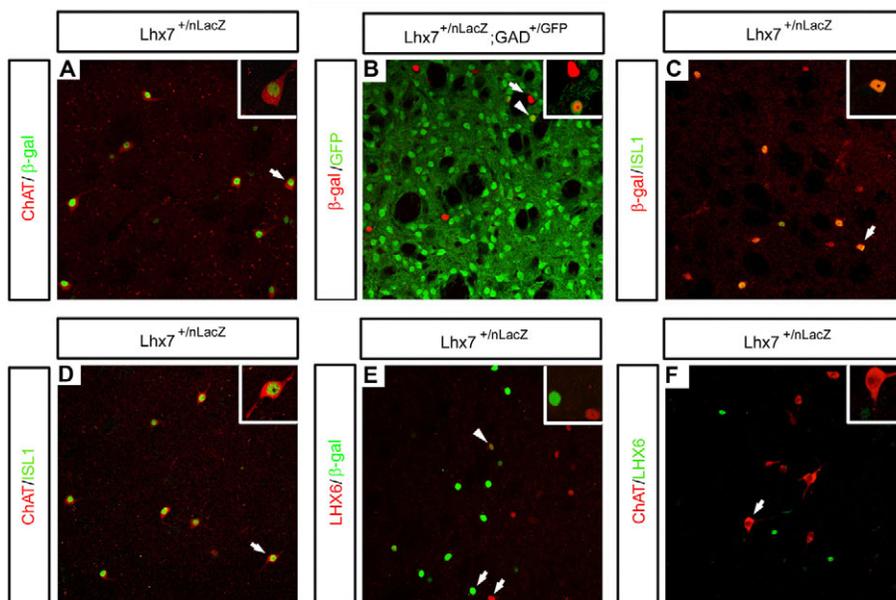

Fig. 1. Co-expression of Lhx7 and Isl1 marks cholinergic SINs, whereas Lhx6 expression is restricted to GABAergic SINs. (**A-F**) Laser confocal microscope images of adult striatal sections labelled by double immunofluorescence. Sections in A and C-F are from $Lhx7^{nlacZ}$ heterozygous animals, that in B is from an animal double heterozygous for $Lhx7^{nlacZ}$ and the knock-in reporter allele $Gad67^{GFP}$. The antibodies used for staining are listed on the left of each panel. Insets show higher magnifications of cells marked by arrows. Arrowhead in E indicates a double-positive (β-gal+/Lhx6+) neuron.

1A), β-gal[+]GFP^{+} cells had smaller nuclei that were stained relatively weakly for β-gal (Fig. 1B) and were negative for ChAT. Therefore, the β-gal-expressing population of striatal neurons in $Lhx7^{+/nlacZ}$ mice is made up of two largely non-overlapping subpopulations, namely the entire complement of cholinergic SINs and at least a subset of GABAergic SINs.

$Isl1$ is expressed in cholinergic SINs in rodents (Wang and Liu, 2001) and is required for the differentiation of subsets of FCNs in mice (Elshatory and Gan, 2008). Consistent with these reports, in $Lhx7^{+/nlacZ}$ animals Isl1 was detected in 61.8±2.0% of $LacZ^+$ striatal cells (Fig. 1C), a percentage similar to that of β-gal^{+} neurons co-expressing ChAT (Fig. 1A). Isl1 always colocalised with ChAT (Fig. 1D), indicating that in the adult striatum Isl1 specifically marks cholinergic SINs.

The LIM HD protein Lhx6 is expressed in MGE-derived cortical GABAergic interneurons and is required for their tangential migration and subtype specification (Alifragis et al., 2004; Cobos et al., 2006; Du et al., 2008; Liodis et al., 2007; Zhao et al., 2008). Immunostaining of forebrain sections from $Gad67^{GFP}$ reporter mice for Lhx6 and GFP showed that $Lhx6$ expression was restricted to approximately 2-3% of GABA^{+} striatal neurons (not shown), whereas immunolabelling striatal sections from mice heterozygous for the $Lhx6^{nlacZ}$ reporter allele (Liodis et al., 2007) for β-gal and the MSN marker DARPP-32 established that the two markers were mutually exclusive (see Fig. S1B in the supplementary material). Together, these data support the argument that in the adult striatum $Lhx6$ is expressed specifically by MGE-derived GABAergic SINs. Consistent with this, immunostaining of striatal sections from $Lhx7^{+/nlacZ}$ mice showed that $Lhx6$ is expressed in 40.6±2.0% of β-gal^{+} cells (Fig. 1E), a percentage similar to the fraction of β-gal^{+} cells co-expressing GABAergic markers (see above and Fig. 1B). Finally, no colocalisation was detected between Lhx6 and either ChAT or Isl1 (Fig. 1F and data not shown). Taken together, these studies demonstrate that the β-gal-expressing neuronal subpopulation in the striatum of $Lhx7^{+/nlacZ}$ heterozygotes encompasses all cholinergic SINs (approximately 60-65% of β-gal^{+} cells), which co-express $Isl1$ but not $Lhx6$ (β-gal[+]/ChAT[+]/Isl1^{+}/Lhx6^{−}). The remaining β-gal^{+} cells represent GABAergic SINs that are defined by the expression of Gad67 and $Lhx6$ but do not express $Isl1$ (β-gal[+]/Gad67^{+}/Isl1^{−}/Lhx6^{+}).

Upon deletion of *Lhx7*, cholinergic SIN precursors differentiate into mature GABAergic SIN subtypes

Despite the dramatic reduction of FCNs in the striatum of $Lhx7^{nlacZ}$ homozygotes, these mice maintain a normal complement of β-gal^{+} cells relative to heterozygous controls (Fig. 2A and see Fig. S2 in the supplementary material) (Fragkouli et al., 2005). This raises the possibility that upon deletion of $Lhx7$, precursors of cholinergic SINs switch fate and adopt alternative differentiation programmes. More specifically, the common origin of GABAergic and cholinergic SINs from the MGE (Marin et al., 2000; Nobrega-Pereira et al., 2008; Sussel et al., 1999) suggested that Lhx7-deficient cholinergic SIN precursors acquire GABAergic characteristics. To address this possibility, we first examined the relative proportion of cholinergic and GABAergic interneurons within the β-gal^{+} striatal cells in $Lhx7^{nlacZ}$ homozygous animals. In contrast to heterozygotes (in which cholinergic neurons represent ~65% of β-gal^{+} cells; see above and Fig. 1A), the percentage of β-gal^{+} cells co-expressing ChAT or $Isl1$ in $Lhx7^{nlacZ/lacZ}$ animals was significantly lower (25.7±3.7% and 19.4±3.3%, respectively; see Fig. 2F and compare Fig. 2A and 2B with Fig. 1A and 1C, respectively). Moreover, the majority of β-gal^{+} cells in the striatum of $Lhx7$ mutants had relatively small and weakly stained nuclei (compare Fig. 1A with Fig. 2A; see Fig. S2 in the supplementary material; data not shown). Despite the reduced representation of cholinergic SINs, residual ChAT^{+} cells in the striatum of mutant mice expressed $Isl1$ and maintained the morphological characteristics of cholinergic neurons (large, strongly stained nuclei and large somata; Fig. 2C). Together these findings suggest that in the absence of Lhx7 only a relatively small fraction of β-gal[+] SIN progenitors are capable of differentiating into cholinergic interneurons.

Interestingly, the reduced proportion of cholinergic neurons within the striatal population of β-gal^{+} cells in $Lhx7^{nlacZ}$ mutants was associated with an increase in the subset of β-gal^{+} GABAergic neurons. Thus the fraction of β-gal^{+} cells co-expressing either GFP in the striatum of $Lhx7^{nlacZ/nlacZ}$;$Gad67^{GFP}$ animals, or Lhx6 in $Lhx7^{nlacZ/nlacZ}$ mice, was 85.2±4.6% and 85.9±3.5%, respectively, significantly higher when compared to the proportion of β-gal^{+} cells co-expressing these markers in $Lhx7^{+/nlacZ}$ heterozygotes (Fig. 2F;
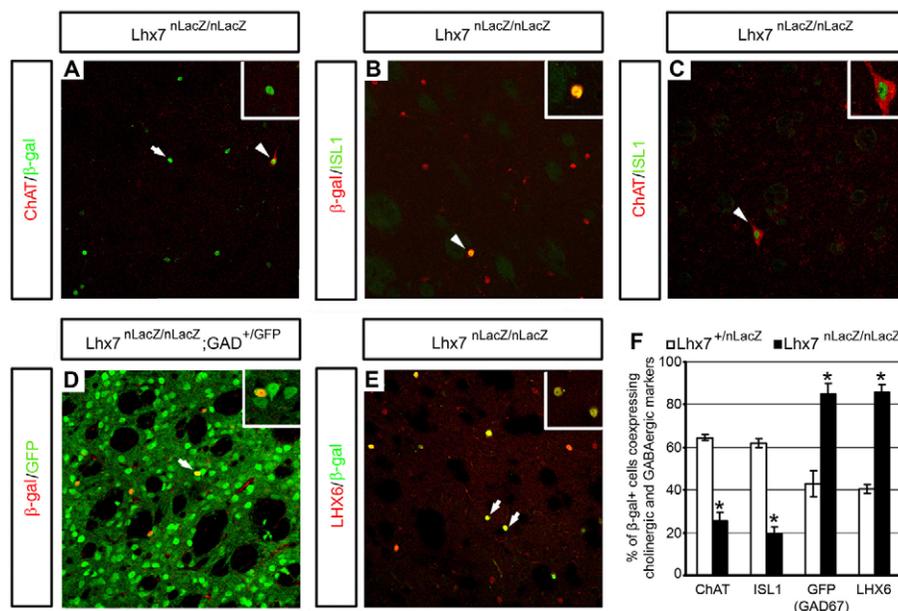

Fig. 2. Upon deletion of *Lhx7*, cholinergic SIN progenitors generate GABAergic SINs. (**A-E**) Confocal images of adult striatal sections labeled by double immunofluorescence. Sections shown in A-C and E are from animals homozygous for the $Lhx7^{nlacZ}$ null mutation, that in D from an animal homozygous for $Lhx7^{nlacZ}$ and heterozygous for the reporter allele $GAD67^{GFP}$. The antibodies used for staining are listed on the left of each panel. Insets show higher magnifications of cells marked by arrowheads (A-C) and arrows (D,E). (**F**) The percentage of adult striatum β-gal^{+} cells expressing cholinergic (ChAT, Isl1) or GABAergic (GFP=GAD67, Lhx6) markers in $Lhx7^{nlacZ}$ heterozygous and homozygous animals. *, $P<0.05$, Student's t-test.

compare Fig. 2D and 2E with Fig. 1B and 1E, respectively). Despite the increased fraction of β-gal$^+$Lhx6$^+$ cells in the striatum of Lhx7-deficient mice, all residual cholinergic neurons remained negative for Lhx6 (data not shown). Taken together, these findings suggest that in the absence of Lhx7, precursors of cholinergic SINs differentiate into GABAergic interneurons.

Upon exiting the cell cycle, MGE-derived precursors of forebrain GABAergic interneurons are specified as proto-GABAergic neurons, which produce GABA and express Gad67 and Lhx6 but generally lack molecular markers characteristic of mature inhibitory interneurons (Flames and Marin, 2005) (see Fig. S5 in the supplementary material). Mature GABA$^+$ SINs are divided into largely non-overlapping groups expressing nNOS, PV and CR (Kawaguchi et al., 1995; Tepper and Bolam, 2004). Consistent with the GABAergic identity of a subset of *Lhx7*-expressing cells, we observed that in the striatum of *Lhx7$^{+/nlacZ}$* mice 11.0±3.8%, 8.1±2.0% and 1.35±0.5% of β-gal$^+$ cells co-expressed respectively nNOS, PV or CR (Fig. 3A,C,E). By contrast, in *Lhx7^{nlacZ}* mutants

Fig. 3. Upon deletion of *Lhx7*, prospective cholinergic SINs differentiate into mature GABAergic SINs. (**A-F**) Striatal sections from adult *Lhx7$^{+/nlacZ}$* (A,C,E) and *Lhx7$^{nlacZ/nlacZ}$* (B,D,F) animals immunostained with antibodies against β-gal and either nNOS (A,B), PV (C,D) or CR (E,F). Generally, β-gal$^+$CR$^+$ cells were characterised by relatively low levels of CR, whereas CR^{+high} cells were negative for both β-gal and Lhx6 (not shown). Insets show higher magnifications of cells marked by arrows and arrowheads. Note that in *Lhx7^{nlacZ}* homozygotes, an increased proportion of β-gal$^+$ cells express mature GABAergic subtype markers.

the proportion of nNOS$^+$, PV$^+$ and CR$^+$ neurons among the β-gal$^+$ population was increased relative to heterozygous littermates (to 32.6±2.3%, 20.9±4.4% and 2.1±0.7%, respectively; *t*-test, *P*<0.05; Fig. 3B,D,F). This is consistent with the increased representation of GABAergic neurons within the β-gal$^+$ subpopulation in the striatum of *Lhx7^{nlacZ}* mutants and suggests that upon deletion of *Lhx7*, the majority of cholinergic SIN precursors differentiate into mature GABAergic interneuron subtypes.

Co-expression of *Lhx7* and *Isl1* distinguishes committed precursors of cholinergic SINs from their *Lhx6*-expressing GABAergic counterparts

The increased representation of GABAergic interneurons within the β-gal$^+$ subpopulation of SINs in *Lhx7^{nlacZ}* homozygotes could reflect a failure of specification of cholinergic SIN precursors and the default generation of GABAergic neurons or a later switch from a cholinergic to GABAergic identity. To distinguish between these possibilities, we first determined the developmental stage at which cholinergic SIN precursors were mis-specified in *Lhx7^{nlacZ}* mutants. As definitive markers of cholinergic differentiation in the forebrain (such as ChAT), are induced only at perinatal stages (Villalobos et al., 2001), we explored the possibility that the combinatorial expression of the reporter allele *Lhx7^{nlacZ}* and the LIM HD proteins Isl1 and Lhx6, which define the SIN sublineages in adult animals (see above), could also distinguish the committed precursors of cholinergic and GABAergic SINs during embryogenesis. In the striatum of E16.5 *Lhx7$^{+/nlacZ}$* embryos, in addition to MSNs (Stenman et al., 2003), *Isl1* is also expressed by a significant subpopulation of β-gal$^+$ neurons (Fig. 4A), suggesting that, similar to the adult striatum, co-expression of these markers identifies precursors of cholinergic SINs. This idea was further supported by the observation that in similar stage *Lhx7$^{nlacZ/nlacZ}$* embryos the number of β-gal$^+$Isl1$^+$ cells was drastically reduced (Fig. 4B), a finding that anticipates the reduced proportion of β-gal$^+$Isl1$^+$ cells in the striatum of mutant adult animals (Fig. 2B). β-gal$^+$Isl1$^+$ cells were also present in the SVZ of the MGE of E11.5 *Lhx7^{nlacZ}* heterozygous embryos (Fig. 4C). Despite the widespread expression of *Lhx6* in the MGE at this stage (Fig. 4D and see Fig. S5 in the supplementary material), all Isl1$^+$ cells were negative for Lhx6 (Fig. 4G). These data mirror our findings in the adult striatum and suggest that co-expression of *Lhx7* and *Isl1* and absence of Lhx6 (β-gal$^+$Isl1$^+$Lhx6$^-$) mark the earliest committed precursors of cholinergic SINs.

To further explore the relationship between Lhx6 and the cholinergic lineage of the striatum, we analysed the expression of *Lhx6* in lineally marked Isl1$^+$ cells. For this, mice expressing Cre recombinase under the control of the *Isl1* locus (*Isl1-Cre*) were crossed with the Cre-dependent *Rosa26-StopYFP* reporter mice (Srinivas et al., 2001), and striatal sections from the resulting double transgenic animals were immunostained for Lhx6 and YFP. Although the vast majority of Isl1-expressing cells in the striatum were positive for YFP (data not shown), none of the YFP$^+$ cells co-expressed *Lhx6* (Fig. 4E), further suggesting that in the ventral forebrain induction of *Isl1* is associated with irreversible commitment of SIN precursors to the cholinergic lineage. Despite the apparent switch from cholinergic to GABAergic SINs in *Lhx7^{nlacZ}* mutants, double-positive (YFP$^+$Lhx6$^+$) cells were also absent from the striatum of *Isl1-Cre; Rosa26-StopYFP;Lhx7$^{nlacZ/nlacZ}$* mice (Fig. 4F), indicating that the segregation of Isl1 and Lhx6 is maintained in Lhx7-deficient adult animals. Interestingly though, careful examination of sections from the ventral forebrain of E11.5 *Lhx7^{nlacZ}* mutant embryos immunostained for Lhx6 and Isl1 revealed a small number of Lhx6$^+$
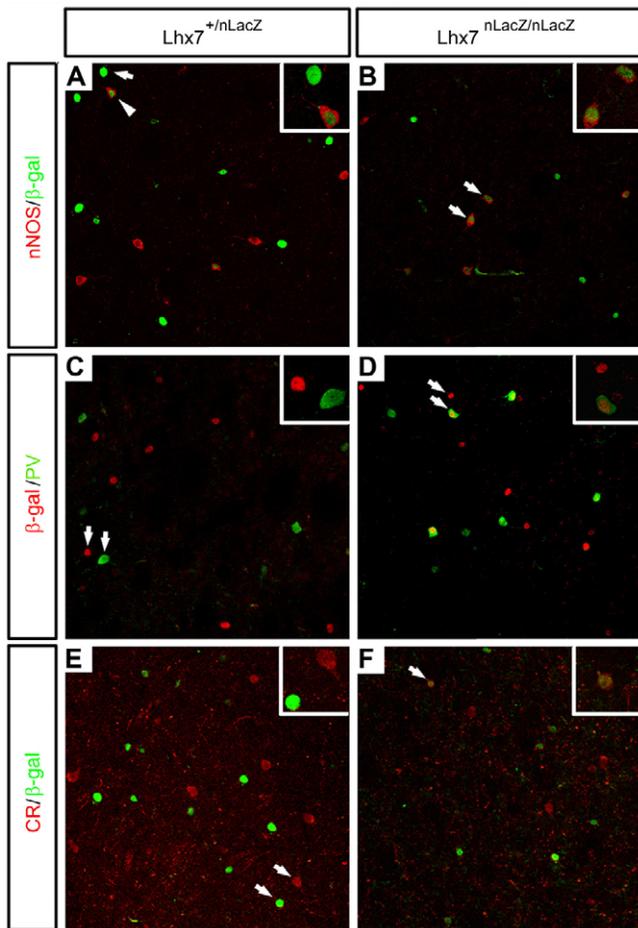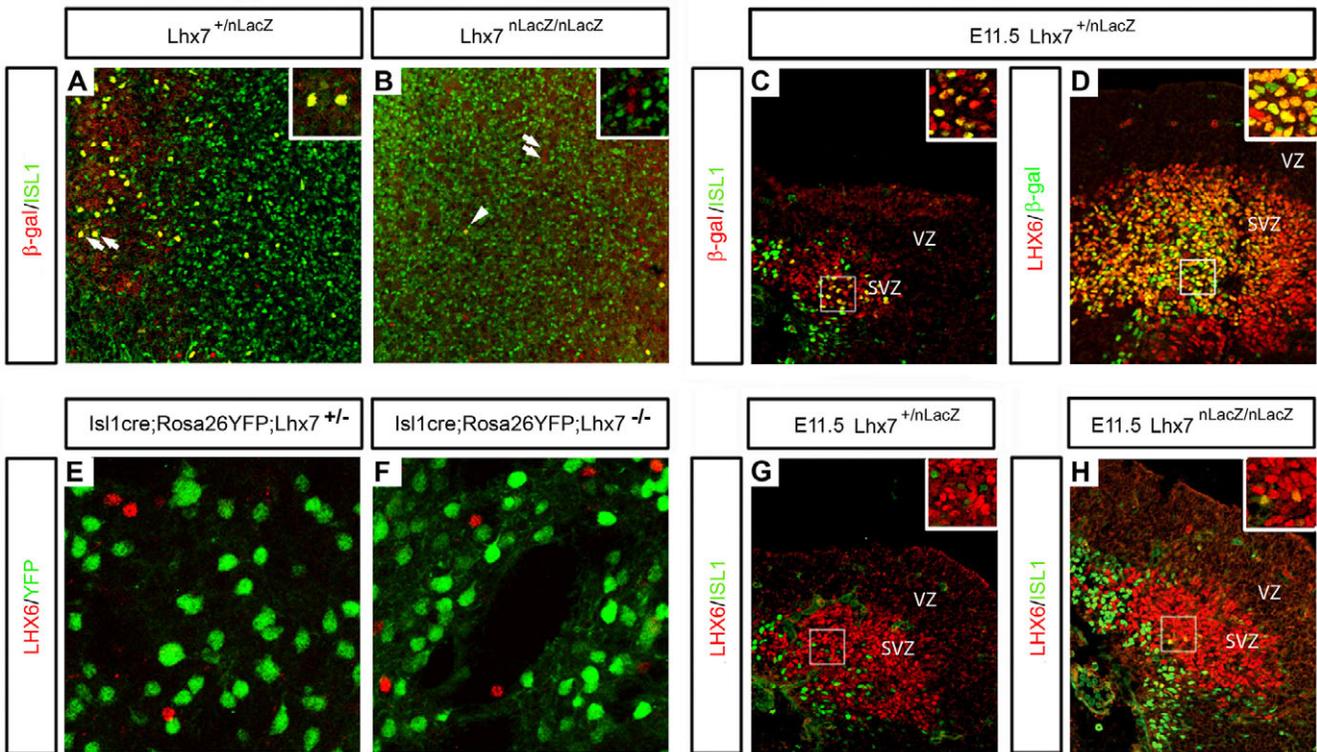
Fig. 4. Co-expression of *Lhx7* and *Isl1* marks committed progenitors of cholinergic SINs in the LGE and MGE. (**A-H**) Laser confocal microscopy images of immunofluorescence-labelled sections from: LGE of E16.5 embryos (A,B); MGE of E11.5 embryos (C,D,G,H); and adult striatum (E,F). The genotype of the animal from which the section was taken is listed above each panel; the antibodies used for staining are listed on the left. Insets show higher magnifications of cells marked by arrows (A,B) or squares (C,D,G,H).

cells in the SVZ of the MGE that co-expressed low levels of Isl1 (Fig. 4H). This clearly contrasts with our observations on similar stage wild-type embryos (Fig. 4G) and suggests that in *Lhx7^{nlacZ}* mutants *Isl1* is expressed transiently in a subset of Lhx6+ cells. Nevertheless, this expression, perhaps because of its low level or short duration, is not sufficient to activate YFP from the *Rosa26StopYFP* allele (Fig. 4F). Taken together, our studies suggest that co-expression of *Lhx7* and *Isl1* defines the cholinergic sublineage of SINs from early embryonic stages whereas persistent expression of *Lhx6* characterises GABA+ SINs.

GABAergic and the majority of cholinergic SINs originate from a common Lhx6-expressing postmitotic precursor of the MGE

The mis-specification of Lhx7-deficient cholinergic precursors into mature GABAergic SINs, along with the transient expression of *Isl1* in a subset of *Lhx6*-expressing precursors in the SVZ of the MGE of *Lhx7^{nlacZ}* mutants, suggests that both cholinergic and GABAergic SINs are derived from a common Lhx6-expressing precursor and that during normal embryogenesis prospective cholinergic neurons downregulate *Lhx6*. To directly test the common origin of GABAergic and cholinergic SINs from *Lhx6*-expressing precursors, we took advantage of a bacterial artificial chromosome (BAC) transgenic mouse strain that expresses Cre recombinase under the transcriptional control of *Lhx6* (Fogarty et al., 2007). Such *Lhx6-Cre* transgenics were crossed with the *Rosa26-StopYFP* reporter and offspring were examined for co-expression of YFP and either Lhx6 or ChAT. As expected, virtually all Lhx6+ cells in the striatum of *Lhx6-Cre;Rosa26-StopYFP* mice expressed YFP (see Fig. S3 in the supplementary material). More importantly, the majority (81.8±5.1%) of ChAT+ cells

(all of which in adult animals are negative for Lhx6; Fig. 1F) co-expressed YFP (Fig. 5A, arrow), indicating that most cholinergic SINs originate from *Lhx6*-expressing precursors. Nonetheless, approximately 18% of ChAT+ striatal neurons were negative for YFP (Fig. 5A, arrowhead), raising the possibility of an alternative origin of cholinergic SINs from Lhx6− cells. Similar analysis of *Nkx2.1-Cre;Rosa26-StopYFP* mice, in which Cre is expressed in all proliferating progenitors in the ventricular zone of the MGE (Kessaris et al., 2006), established that virtually all (96.9±1.0%) cholinergic SINs were positive for YFP (Fig. 5B, arrow), consistent with their
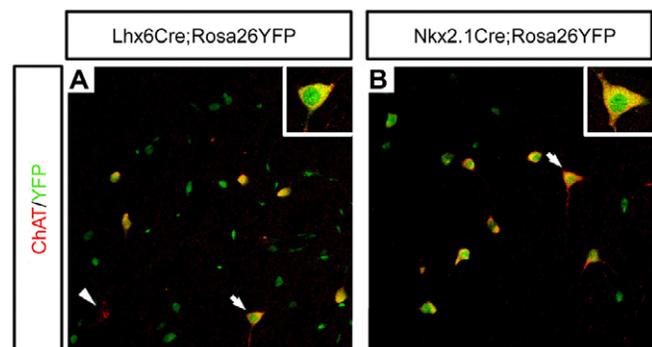

Fig. 5. Cholinergic SINs are derived from Lhx6-expressing MGE progenitors. (**A,B**) Confocal images of striatal sections from the indicated reporter mouse strains immunostained for ChAT and YFP. Despite the non-overlapping expression of ChAT and Lhx6 in the striatum of adult mice (see Fig. 1F), the majority of cholinergic SINs originate from *Lhx6*-expressing progenitors.

origin from the ventral forebrain. Taken together, these results indicate that the majority (~80%) of cholinergic SINs originate from Nkx2.1[+] progenitors of the MGE via Lhx6^{+} intermediate precursors, which also generate GABAergic SINs.

Ectopic expression of Lhx7 in dorsal telencephalic progenitors induces formation of ChAT^{+} neurons

Next we examined the possibility that Lhx7 is sufficient to induce cholinergic differentiation upon ectopic expression in central nervous system (CNS) progenitors. This was initially examined in embryonic mouse cortex, a region of the forebrain that normally does not express *Lhx7* (Asbreuk et al., 2002; Fragkouli et al., 2005; Grigoriou et al., 1998). Bicistronic DNA constructs encoding Lhx7 and GFP were introduced by ex vivo electroporation into the ventricular zone of E14.5 embryonic brain and slices derived from treated brains were cultured for several days (Fig. 6A). At the end of the culture period slices were analysed for expression of GFP and cholinergic markers such as ChAT. As shown in Fig. 6B,E,H, expression of GFP alone failed to induce cholinergic markers in cortical cells. By contrast, electroporation of Lhx7-GFP constructs resulted in many GFP^{+} cells co-expressing ChAT, a characteristic marker of cholinergic neurons (Fig. 6C,F,H). The effect of Lhx7 was specific, as similar bicistronic constructs expressing Lhx6/GFP or Isl1/GFP failed to induce cholinergic differentiation (Fig. 6D,G,H). The absence of cholinergic neurons in these cortices is unlikely to result from failure of Lhx6 or Isl1 production, as many of the GFP^{+} cells could be immunolabelled with antibodies for these LIM HD proteins (data not shown). The exclusion of Lhx6 from cholinergic SINs (Fig. 1F) raises the possibility that this LIM HD protein is capable of inhibiting cholinergic differentiation. To test this, DNA constructs encoding

Lhx6 or Lhx7 were co-electroporated into mouse cortex and slices of such brains were analysed as described above. Co-expression of Lhx6 and Lhx7 in cortical neuron progenitors resulted in reduced efficiency of ChAT induction (Fig. 6H), suggesting that under our experimental conditions Lhx6 can inhibit the cholinergic-inducing activity of Lhx7 in the dorsal telencephalon.

Our expression studies show that the cholinergic lineage of the striatum is defined from early embryonic stages by the co-expression of the LIM HD proteins Lhx7 and Isl1 (Fig. 4). This raises the possibility that Isl1 co-operates with Lhx7 to promote cholinergic differentiation in the ventral forebrain. However, co-electroporation of Isl1- and Lhx7-encoding DNA constructs did not result in an increased number of cholinergic neurons in cortical slices relative to their Lhx7-only counterparts (Fig. 6H). Together, our studies suggest that in the dorsal telencephalon Lhx7 is sufficient to induce cholinergic characteristics in dorsal telencephalic neurons and that its activity is not enhanced by Isl1.

Co-expression of Lhx7 and Isl1 in the dorsal spinal cord of chicken embryos induces formation of cholinergic motor neurons

The failure of Isl1 to enhance the cholinergic-inducing activity of Lhx7 in the cortex contrasts with the robust expression of this factor in cholinergic SINs and its requirement in FCN differentiation (Elshatory and Gan, 2008; Wang and Liu, 2001) and raises the possibility that the cooperative activity of these LIM HD proteins in the CNS is cell type specific. To provide evidence for this, we used in ovo electroporation of chick embryos to ectopically express combinations of Lhx7, Isl1 and Lhx6 in progenitors of the dorsal neural tube, a region that normally does not express these
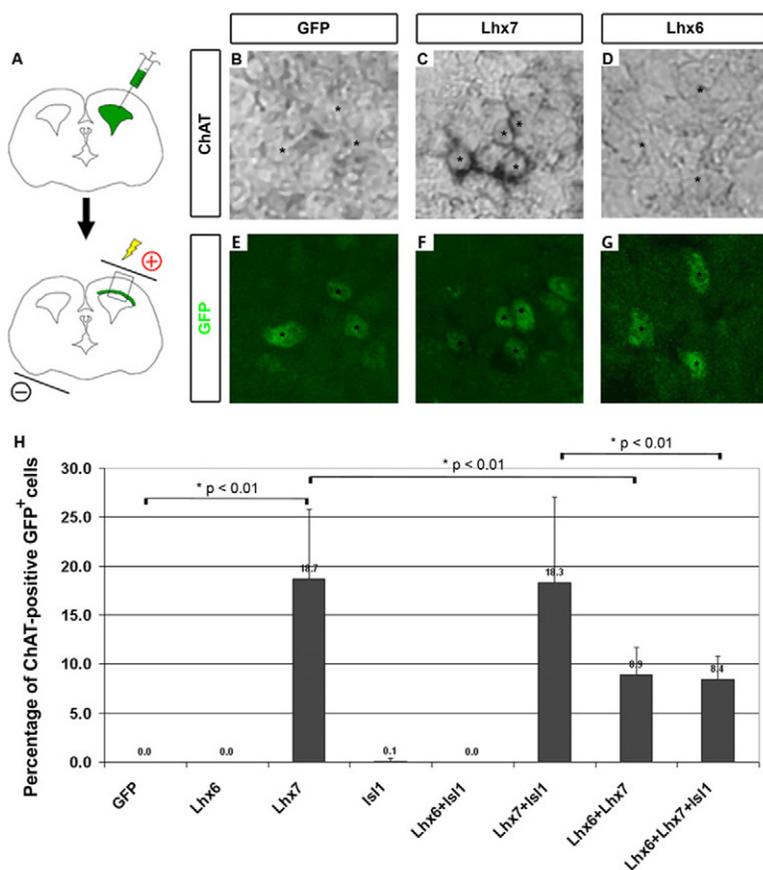

Fig. 6. Lhx7 is sufficient to induce cholinergic markers upon expression in the dorsal telencephalon. (**A**) Schematic representation of ex vivo mouse brain electroporation. (**B-G**) Sections from brains electroporated with control vector (GFP, B,E) or with vectors expressing Lhx7 (C,F) or Lhx6 (D,G). The fluorescent images shown in E-G correspond to the bright-field images shown in B-D, respectively. (**H**) Quantification of the ChAT-inducing effects of combinations of Lhx7, Lhx6 and Isl1 in the dorsal telencephalon. Asterisks mark GFP-expressing cells in E-G and the equivalent cells in B-D.

transcription factors (Grigoriou et al., 1998). Progenitors of the dorsal spinal cord, under the combinatorial activity of LIM HD proteins make cell-fate decisions analogous to those faced by MGE-derived SIN precursors (Thaler et al., 2002). More specifically, upon expression of Lhx3, dorsal neural tube progenitors generate Chx10+ V2 interneurons, whereas combined expression of Lhx3 and Isl1 induces cholinergic motor neurons (MNs; identified by expression of ChAT and MNR2) (Tanabe et al., 1998). Similar to Lhx3, electroporation of DNA constructs encoding Lhx6 induced formation of Chx10+ neurons (Fig. 7Aa,Ab), indicating that this factor can induce formation of spinal cord-appropriate neuronal subtypes. Surprisingly, electroporation of similar constructs

Fig. 7. Co-expression of Lhx7 and Isl1 promotes cholinergic differentiation of chick dorsal neural tube progenitors.
(**A-D**) Transverse sections of HH stage 26 chick embryo neural tube electroporated (+side) at HH stage 12-14 with DNA constructs expressing Lhx6 (Aa,b and Ca,b) or Lhx7 (Ba,b and Da,b) and immunostained for GFP (to identify electroporated cells; Aa,Ba,Ca,Da) and Chx10 (to identify V2 interneurons; Ab,Bb) or Mnr2 (to identify cholinergic MNs; Cb,Db). Aa, Ba, Ca and Da correspond to the sections shown in Ab, Bb, Cb and Db, respectively. Expression of Lhx6 or Lhx7 in the dorsal neural tube induces V2 interneurons (Lhx6: n=15, 26.4±7.7 dorsal Chx10+ cells/section; Lhx7: n=15, 23.8±7.6 dorsal Chx10+ cells/section). Note that neither Lhx6 nor Lhx7 alone is sufficient to induce cholinergic neuron differentiation (Ca,b and Da,b). (**E-H**) Sections of HH stage 26 chick embryo spinal cord co-electroporated at HH stage 12-14 with a combination of Lhx6, Lhx7 and Isl1 and double immunostained for GFP (Ea,Fa,Ga,Ha) and Mnr2 (Eb,Fb,Gb) or Chx10 (Hb). Co-expression of Isl1 and Lhx7 (Ea,b) induces dorsal spinal cord MNs (Lhx7/Isl1: n=15, 9.6±2.9 dorsal MNR2+ cells/section). By contrast, co-expression of Lhx6 and Isl1 (Fa,b) did not induce MNs (Lhx6/Isl1: n=15). Note that co-expression of Lhx6 with Lhx7 and Isl1 prevents MN differentiation and instead promotes V2-interneuron differentiation (Lhx6/Lhx7/Isl1: n=15, 11.8±3.9 dorsal Chx10+ cells/section). Ea, Fa, Ga and Ha correspond to the sections shown in Eb, Fb, Gb and Hb, respectively.

expressing Lhx7 also induced Chx10+ V2 interneurons with similar efficiency (Fig. 7Ba,Bb). By contrast, expression of either factor in the dorsal spinal cord failed to induce cholinergic markers, such as ChAT or MNR2 (Fig. 7Ca,Cb,Da,Db and data not shown). Therefore, neither Lhx6 nor Lhx7 are sufficient to promote cholinergic differentiation when ectopically expressed in dorsal spinal cord but instead promote V2 interneuron differentiation.

To explore the possibility that in the dorsal spinal cord Lhx7 co-operates with Isl1 to promote cholinergic differentiation, Lhx7- and Isl1-encoding DNA constructs were co-electroporated into the dorsal neural tube of chick embryos. Contrary to Lhx7-only electroporations, co-expression of Lhx7 and Isl1 failed to induce Chx10+ cells (see Fig. S4Aa,Ab in the supplementary material) and instead led to the formation of ectopic MNR2+ and ChAT+ neurons (Fig. 7Ea,Eb and data not shown). Therefore, combination of Lhx7 and Isl1 promotes cholinergic differentiation of dorsal neural tube progenitors. Interestingly, expression of Isl1 alone (see Fig. S4B1,B2 in the supplementary material) or co-expression of Lhx6 and Isl1 did not induce MNR2+ or ChAT+ neurons (Fig. 7Fa,Fb). Therefore, in the dorsal spinal cord Isl1 interacts specifically with Lhx7 to promote cholinergic differentiation of neural progenitors.

To examine whether Lhx6 can inhibit the cholinergic-inducing effect of Lhx7/Isl1 combinations, we co-expressed Lhx6, Lhx7 and Isl1 in the dorsal spinal cord. Consistent with our findings in cortical electroporations, Lhx6 inhibited the Lhx7/Isl1mediated-induction of ChAT+ and MNR2+ neurons (Fig. 7Ga,Gb). Instead, co-expression of all three factors resulted in induction of V2-interneurons (Fig. 7Ha,Hb). Together, these data support the argument that in the dorsal neural tube Lhx6 is a potent inhibitor of Lhx7/Isl1-induced cholinergic differentiation.
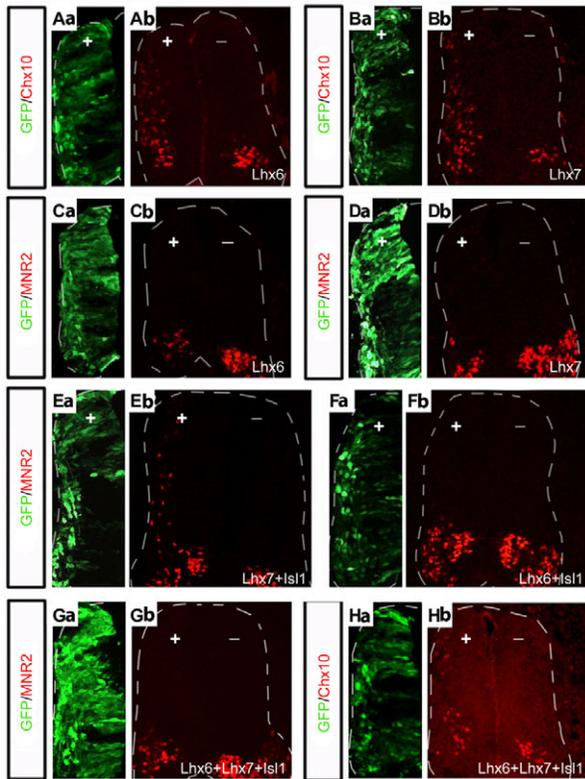
DISCUSSION
Combinatorial activity of LIM HD proteins determines the subtype identity of SINs
The generation of GABAergic and cholinergic SIN subtypes is crucial for the formation of functional neuronal circuits in the basal ganglia (Bolam et al., 2000; Kreitzer, 2009; Tepper and Bolam, 2004; Wang et al., 2006). Our current experiments demonstrate that precursors of the two SIN sublineages can be distinguished by the combinatorial expression of the LIM HD transcription factors Lhx6, Lhx7 and Isl1 shortly after they are born in the MGE and before their final settlement in the striatal anlage. Moreover, activity of these factors drives the differentiation of a common precursor along the mature GABAergic or cholinergic SIN phenotype. Such precursors are derived from *Nkx2.1*-expressing progenitors and express *Lhx6* indicating that they correspond to proto-GABAergic neurons generated in the SVZ of the MGE (Flames and Marin, 2005). These immature neurons express early GABAergic interneuron markers (such as Gad67 and Lhx6) but lack molecular markers (such as SST, PV, CR and nNOS) and functional characteristics of mature GABAergic interneurons (Butt et al., 2005; Flames and Marin, 2005; Kawaguchi et al., 1995; Liodis et al., 2007; Tepper and Bolam, 2004; Wonders and Anderson, 2006) (see Fig. S5 in the supplementary material). Previous reports have demonstrated that terminal differentiation of proto-GABAergic neurons into mature inhibitory interneurons in the cortex and the striatum is critically dependent on the activity of Lhx6 (Liodis et al., 2007; Zhao et al., 2008) (and our unpublished observations). Our present studies suggest that such proto-GABAergic cells retain their developmental plasticity and can be diverted towards cholinergic differentiation, a process that requires Lhx7 activity and is associated with downregulation of *Lhx6*. In the absence of Lhx7 (as in *Lhx7nlacZ/nlacZ* animals), *Lhx6* expression is maintained and

GABAergic SINs are generated as a default developmental option (Fig. 8). Finally, our findings suggest that cholinergic differentiation in the ventral forebrain is driven by the combined activity of Lhx7 and Isl1. This is based on the synergistic effect of these factors in motoneuron differentiation in the dorsal spinal cord, the robust expression of Isl1 in cholinergic SINs [this report and (Wang and Liu, 2001)] and the requirement of Isl1 for FCN differentiation (Elshatory and Gan, 2008). Taken together, these data support the argument that the combinatorial activity of *Lhx6*, *Lhx7* and *Isl1* within bipotential precursors of SINs controls their differentiation along cholinergic or GABAergic identity.

Cross-regulatory interactions between Lhx6, Lhx7 and Isl1 LIM HD proteins

Despite the segregated expression and subtype-specific activity of Lhx6, Lhx7 and Isl1 in SINs, similar signalling mechanisms are likely to induce the early expression of these transcription factors in the ventral forebrain. Thus, mice deficient for Nkx2.1 fail to induce *Lhx6* and *Lhx7* in the MGE and homozygous mutant animals are characterised by severe reduction in SINs (Sussel et al., 1999). A recent report has shown that *Lhx6* is one of the direct transcriptional targets of Nkx2.1 (Du et al., 2008), but it is currently unclear whether *Lhx7* and *Isl1* are also under the direct transcriptional control of Nkx2.1. Nevertheless, these studies clearly suggest that transcriptional regulators expressed in the ventricular zone of the ventral forebrain and/or its postmitotic progeny control the expression of interneuron subtype specification genes, such as *Lhx6*, *Lhx7* and *Isl1*.

Although their initial activation could be controlled by common regulatory mechanisms, the final expression profile and activity of *Lhx6*, *Lhx7* and *Isl1* are SIN-subtype specific. Lhx6 is detected in GABAergic SINs but is excluded from their cholinergic counterparts and their committed precursors, whereas co-expression of *Lhx7* and *Isl1* was detected only in cholinergic SINs. The significance of the subtype-specific expression of *Lhx6* and *Lhx7/Isl1* is highlighted by our present studies demonstrating that Lhx6 can inhibit the Lhx7/Isl1-dependent cholinergic differentiation in the cortex and the dorsal spinal cord, suggesting that the sustained expression of this factor could have a cholinergic-inhibiting effect in proto-GABAergic neurons of the ventral forebrain. Additional rationale for the differential expression of *Lhx6* and *Lhx7/Isl1* is also provided by our misexpression experiments demonstrating that expression of Lhx7 (alone in the case of the cortex, or in combination with Isl1, in the case of dorsal spinal cord) is sufficient to drive cholinergic differentiation of CNS progenitors. Moreover, Lhx6 is necessary and sufficient for the induction of genes that define the neurochemical and electrophysiological properties of GABAergic interneuron subtypes [(Du et al., 2008; Liodis et al., 2007; Zhao et al., 2008) and our unpublished data]. Based on these observations, we expect that co-expression of Lhx6, Lhx7 and Isl1 would result in the generation of interneurons of mixed (GABAergic and cholinergic) phenotype. As such interneurons are not normally observed (see Fig. S1C in the supplementary material), we propose that the segregated expression of *Lhx6* and *Lhx7/Isl1* ensures the subtype-specific expression of the non-overlapping characteristics of cholinergic and GABAergic SINs, including their distinct morphologies, neurochemical codes and electrophysiological profiles.

But what are the mechanisms by which Lhx6-expressing precursors in the MGE generate two divergent subtypes with distinct gene expression profiles? The mutual exclusion of Lhx6 and Isl1 from mature SIN subtypes together with the origin of Isl1-expressing cholinergic SINs from Lhx6$^+$ progenitors, suggest that a crucial step in the diversification of SINs is the induction in a subset of proto-
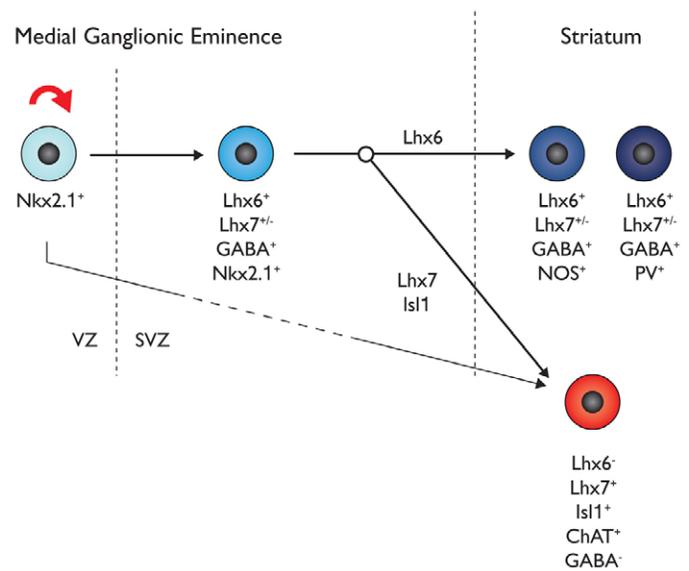

Fig. 8. A proposed model for the generation of cholinergic and GABAergic SINs from progenitors of the MGE. Nkx2.1-expressing progenitors in the VZ of the MGE exit the cell cycle and enter the SVZ, where they induce expression of Lhx6 and GABA. Many of the Lhx6$^+$ cells co-express Lhx7. Under the continued activity of Lhx6, many of these proto-GABAergic precursors differentiate into mature GABAergic SIN subtypes, such as those expressing NOS and Pv. By contrast, induction of Isl1 in postmitotic proto-GABAergic precursors and the combined activity of Lhx7 and Isl1 result in downregulation of Lhx6 and commitment along the cholinergic SIN sublineage. According to our lineage studies, less than 20% of cholinergic SINs are derived from precursors that never induce *Lhx6*.

GABAergic precursors of the LIM HD Isl1. We propose that the combined activity of Isl1 and Lhx7 downregulates *Lhx6* and specifies cholinergic SIN precursors, thus contributing crucially to the resolution of cellular phenotypes and the establishment of their non-overlapping gene expression profile. The mechanisms controlling expression of *Isl1* in cholinergic SIN precursors are currently unknown. The detection of a small transient population of Lhx6$^+$Isl1$^+$ cells in the MGE of Lhx7-deficient embryos suggests that Lhx7 is not necessary for the initial induction of *Isl1*. However, our failure to identify Lhx6$^+$YFP$^+$ cells in the striatum of *Lhx7^{nlacZ}* mutants transgenic for the Isl1-Cre;Rosa26StopYFP reporter system indicates that the co-expression of *Isl1* with *Lhx6* in Lhx7-deficient animals is short-lived and that Lhx7 may be required for maintaining the expression of *Isl1*. Irrespective of the mechanisms controlling their induction, our current findings are consistent with the hypothesis that cross-repressive interactions between Lhx6 and Lhx7/Isl1 underlie many of the non-overlapping features of SIN subtypes.

Lhx7 promotes cholinergic differentiation is a cell type-dependent context

Lhx7 appears to induce cholinergic characteristics in a cell-context-dependent manner: it is sufficient to activate the cholinergic marker ChAT in cortical progenitors, whereas in chick embryo spinal cord cholinergic differentiation (induction of motor neurons) requires the combined expression of Lhx7 and Isl1. Based on our current data and previous studies that demonstrate expression and requirement of both Isl1 and Lhx7 for FCN differentiation (Elshatory and Gan, 2008; Fragkouli et al., 2005; Mori et al., 2004; Wang and Liu, 2001; Zhao et al., 2003), we suggest that the spinal cord misexpression system mirrors more faithfully the situation in the ventral forebrain.

The reasons for the cell type-dependent requirement of Isl1 for cholinergic differentiation are currently unclear. It is possible that other LIM HD factors present within the dorsal telencephalon compensate for the absence of Isl1. For example, Lhx2 is expressed at high levels in dividing and postmitotic cortical progenitors and could synergise with Lhx7 to induce cholinergic markers (Bulchand et al., 2003). Alternatively, non LIM HD factors, such as members of the bHLH family of transcription factors, which are differentially expressed in dorsal [predominantly expressing Ngn2 (Neurog2 – Mouse Genome Informatics)] and ventral [expressing mostly Mash1 (Ascl1 – Mouse Genome Informatics)] telencephalon (Guillemot, 2007), could contribute to the differential requirement of Isl1 for cholinergic differentiation in different CNS progenitors. In that respect, it is interesting that cholinergic (motor neuron) differentiation in the spinal cord is selectively enhanced by specific members of the bHLH family of neurogenic proteins (Lee and Pfaff, 2003). Finally, the differential requirement of Isl1 in dorsal and ventral telencephalon could be related to the distinct signalling environments of the dorsal and ventral regions of the forebrain.

Based on the analysis of forebrain cholinergic differentiation in amphibian embryos, it has been suggested that the main activity of Lhx7 is to inhibit GABAergic differentiation of ventral telencephalic progenitors, thus directing them towards a default cholinergic pathway (Bachy and Retaux, 2006). Our present studies, indicating that Lhx7, either alone or in conjunction with Isl1, induces cholinergic characteristics, together with the robust expression of an *Lhx7* reporter allele (*Lhx7^{nlacZ}*) in a large subset of GABAergic SINs (and in other regions of the ventral forebrain, such as the hypothalamus, where expression of this reporter is restricted to GABA+ neurons; A. Martin and V.P., unpublished observations), are not consistent with the hypothesis that the primary role of Lhx7 is to repress GABAergic differentiation. Instead, these data suggest that, in addition to its role in cholinergic differentiation of SIN precursors, Lhx7 could also control aspects of GABAergic SIN differentiation. Such a role has been demonstrated for the closely related *Lhx6* gene (Du et al., 2008; Liodis et al., 2007; Zhao et al., 2008), but it is presently unclear whether Lhx7 functions, possibly in conjunction with Lhx6, to regulate the mature phenotype of distinct subtypes of GABAergic SINs.

Cholinergic differentiation along the anteroposterior axis of the neural tube

The implication of Lhx3 and Isl1 in motor neuron differentiation (Lee and Pfaff, 2003; Thaler et al., 2002) and Lhx7 and Isl1 in the generation of cholinergic SINs (this study), suggest that the activity of an Isl1/Lhx(n) module is involved in cholinergic differentiation along the entire length of the neural axis. Although all cholinergic neurons along the CNS share common features (such as expression of ChAT and production of Ach), cholinergic SINs and motor neurons have discrete properties, including distinct axonal trajectories and synaptic targets. Such differences could result from the unique properties of putative LIM HD protein complexes forming in the two regions of the CNS (Thaler et al., 2002). However, our studies demonstrating that co-expression of Lhx7 and Isl1 in dorsal neural tube induced spinal cord-appropriate cholinergic markers (such as MNR2), raise the possibility that additional region-specific factors contribute to the differentiation and specificity of cholinergic neuronal subpopulations along the neural axis. Consistent with this view, a recent study has shown that regulators such as Lmo4, Chx10 (Vsx2 – Mouse Genome Informatics) and Hb9 (Mnx1), modulate the transcriptional outcome of LIM HD complexes and contribute to their cross-regulation and cell type-specific effects (Lee et al., 2008).

LIM HD-encoding genes as selector genes during neuronal differentiation

Selector genes belong to a family of genes that control cell fate during animal development (Garcia-Bellido, 1975). Our present findings suggest that *Lhx6* and the combination of *Lhx7* and *Isl1* function as selector genes to promote GABAergic or cholinergic differentiation of a common progenitor of the striatal interneuron lineage (see Fig. S5 in the supplementary material). A similar role has been attributed to other members of the homeobox gene family in mammals, including the closely related *Tlx3* and *Tlx1* genes, which determine the glutamatergic over GABAergic cell fate of postmitotic precursors in the dorsal spinal cord of mice (Cheng et al., 2004). The mechanisms by which such 'terminal selector genes' (Hobert, 2008) control the emergence of distinct neuronal subtypes are currently unclear. Several studies suggest that terminal selector genes recognise and activate common regulatory elements in diverse loci, the collective activity of which defines the identity of a particular neuronal cell type (Hobert, 2008). The identity of cell-type-specific factors that potentially synergise with or antagonise the activities of *Lhx6*, *Lhx7* and *Isl1* would help us understand the molecular mechanisms that establish the diverse features of GABAergic and cholinergic SINs. Such an understanding will also facilitate the design of judicious strategies for directing the differentiation of telencephalic progenitors as a means of restoring the activity of diseased striatal neuronal circuits.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/22/3841/DC1

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