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GABAergic specification in the basal forebrain is controlled by the LIM-hd factor *Lhx7*

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Abstract

We present evidence for a temporal control of GABAergic neurotransmitter specification in the basal forebrain orchestrated by the LIM-homeodomain factor Lhx7. In *Xenopus*, using in vivo overexpression experiments, we show that *x-Lhx7* and *x-Nkx2.1* inhibit GABAergic specification in the *Dlx*-expressing areas of the forebrain (subpallium and diencephalon). In addition, *x-Lhx7* almost totally represses GABAergic differentiation at early but not late embryonic stages in subpallial mouse primary neurons in culture, indicating that *x-Lhx7* is not able to withdraw the GABAergic phenotype once it is acquired. Moreover, anatomical data show striking correlations between *x-Lhx7* expression and the GABAergic/cholinergic phenotypes. These functional and anatomical observations suggest a sequential role for x-Lhx7 in neurotransmitter specification. Thus, x-Lhx7 would first prevent a pool of cells to become GABAergic early in development and then promote cholinergic differentiation later on in this pool. We propose two distinct modulatory roles for a single LIM-hd factor, depending on the developmental time window.

Keywords: Neuronal specification; Forebrain; GABAergic; Lhx7; Nkx2.1; Medial ganglionic eminence

Introduction

A current model of forebrain development proposes that the identity of the major subdivisions is established in the telencephalic progenitor zones by regionalized expression of transcription factors, which are also responsible for the production of neurons with specific neurotransmitter phenotypes (Wilson and Rubenstein, 2000). Hence, the Pax6/Ngn1/2-expressing pallium generates glutamatergic neurons, whereas the Dlx/Mash1-expressing subpallium produces GABAergic and cholinergic cells. Then waves of radial (for projection neurons) and tangential (for interneurons) migrations populate telencephalic subdivisions with appropriate neuronal types (Marin and Rubenstein, 2001; Parnavelas, 2000). In particular, the Nkx2.1-positive area (medial ganglionic eminence: mge, and anterior entopeduncular/preoptic areas: aep/poa) originates cortical and striatal GABA interneurons and striatal cholinergic neurons (Anderson et al., 1997, 2001; Marin et al., 2000; Wichterle et al., 2001).

The transcription factors responsible for subpallial neuronal patterning begin to be discovered. Ectopic expression of *Mash1* in the cortex induces ectopic *Dlx1* and GAD67 (glutamic acid decarboxylase) (Fode et al., 2000) and so does ectopic expression of *Dlx2/5* (Stuhmer et al., 2002), suggesting that *Dlx* genes are able to induce a GABAergic phenotype. In addition, the LIM-homeodomain (LIM-hd) factor *Lhx6*, which is expressed in the mge/aep/poa (Grigoriou et al., 1998) under control of *Nkx2.1* (Sussel et al., 1999), regulates the decision of an mge cell to migrate tangentially towards the cortex—but does not specify its GABAergic phenotype (Alifragis et al., 2004; Lavdas et al., 1999). On the other hand, the specification of cholinergic neurons is under control of another mge/aep/poa-specific LIM-hd factor, *Lhx7*/*Lhx8* (Grigoriou et al., 1998; Matsumoto et al., 1996). While anatomical evidences show a striking correlation between *Lhx7* expression and the cholinergic phenotype (Asbreuk et al., 2002; Marin et al., 2000), nearly all forebrain cholinergic cells are absent in $Lhx7^{-/-}$ mice, although their initial specification is preserved (Mori et

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al., 2004; Zhao et al., 2003). Interestingly, the role of *Lhx7* in cholinergic specification seems also conserved in fish and amphibians (Alunni et al., 2004; Moreno et al., 2004). Recently, a role for *Lhx7* in the control of GABAergic phenotype has also been suggested in vitro (Manabe et al., 2005).

LIM-hd family members have roles in various aspects of neuronal specification, such as axonal pathfinding, migration, or neurotransmitter choice (Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002). Moreover, they are often expressed in the differentiating zones of the neuroepithelium, and their expression patterns respect the boundaries of subdivisions imposed by regionalizing factors, suggesting that they are well placed to serve as intermediates between early regional specification and neuronal specification (Bachy et al., 2001, 2002; Retaux et al., 1999). Here, we directly investigated the possibility that *x-Lhx7* is an intermediate between basal forebrain regionalization and neuronal specification, i.e., between *x-Nkx2.1* and GABAergic/cholinergic specification. Using overexpression experiments in *Xenopus* embryos together with anatomical analyses and mouse neuronal primary cultures, we present evidence that *x-Lhx7* has a role in forebrain neuronal specification. We propose that the early inhibitory action of *x-Lhx7* on GABA would preserve a pool of future cholinergic neurons, which differentiate very late in development, to become GABAergic.

Materials and methods

In situ hybridization

Antisense digoxigenin RNA probes were synthesized according to the protocol described in Bachy et al., 2001. Embryos (staged according to the table of Nieuwkoop and Faber) were fixed overnight at 4°C in MEMFA and maintained in absolute methanol at −20°C until use. After progressive rehydration and pretreatments, hybridization step was carried out with 1 μg of a digoxigenin-labeled RNA probe, in a 50% formamide containing medium overnight at 55°C. Hybridization was detected using an alkaline phosphatase-coupled anti-digoxigenin antibody (Roche Diagnostics, Mannheim, Germany, dilution 1:1500). Alkaline phosphatase staining was developed with NBT/BCIP for a purple staining or Fast Red for an orange/fluorescent staining (Roche Diagnostics, Mannheim, Germany).

Immunofluorescence

Whole mount fixed embryos (up to st. 42) or dissected brains (for older embryos and larvae) were blocked in PBT (PBS/0.5% Triton/0.5% BSA) containing 5% fetal veal serum during 1 h at room temperature. The primary antibodies were added overnight at 4°C at the following dilutions: rabbit anti-GABA (Sigma), 1/1000; mouse anti-myc (monoclonal 9E10, Sigma), 1/1000; mouse anti-GFP (Roche), 1/500. After several washes in PBT, the secondary antibodies (Molecular Probes) were added 2 h at room temperature and washed again. In case of double immuno-ISH labeling, ISH were performed first (see above for details), embryos were postfixed 1 h in MEMFA, and processed for immunolabeling according to the same protocol.

For in toto views, brains were dissected out and mounted in PPD glycerol. For sectioning, fluorescently labeled embryos or brains were embedded in PBS containing 4% agar, incubated in PBS containing 4% paraformaldehyde overnight at 4°C, and sectioned at 30–40 μm on a vibratome (Leica). The sections were mounted in PPD glycerol. Confocal images were obtained on a Leica SP2 microscope, and classical fluorescence microscopy was performed on a Nikon E800 microscope equipped with a DXM1200 camera. Images were (occasionally) corrected for brightness/contrast and mounted into figures using Adobe Photoshop.

Expression constructs

Full-length *x-Lhx7* cDNA was obtained by 5′- and 3′-Race-PCR (Smart-Race cDNA amplification kit, Clontech). The deduced coding sequence was amplified with the following primers: forward GGCAGGGCCATGTATTG-GAAGAG, reverse CCTTTTAAGCGTTGACTTATTGGTAGGTG and subcloned into pGEM-T easy (Promega). An EcoRI-SpeI 1.2 kb full-length *x-Lhx7* fragment was then inserted in frame into the pCS2-MT (myc-tag) expression vector (from David Turner). The *x-Nkx2.1* expression vector was obtained by subcloning an 1.6 kb EcoRI-ApaI fragment from the full-length *x-Nkx2.1* cDNA (gift of Paul Krieg) into the pCS2 vector (David Turner). For co-injection experiments, the GFP reporter construct was pEGFP-N3 (Clontech).

DNA injections into 4/8 cell stage Xenopus embryos

DNA expression constructs (Quiagen DNA prep) were injected into *Xenopus* embryos at 4 or 8 cell stage, targeting a D1 blastomere fated to the anterior brain, according to Moody (1987). A volume of about 1 nl containing a maximum of 500 pg DNA was injected. For co-injection experiments, the GFP reporter was present at a 1:2 ratio. Injected embryos were maintained in MMR0.1× containing gentamycin for further development, anaesthetized in MS222 (0.4 mg/ml) and fixed in MEMFA at the appropriate stage for further processing.

Primary culture of mouse subpallial embryonic neurons

Ganglionic eminences from E12 or E16 mouse embryos were dissected and manually homogenized in HBBS (Gibco) on ice. After centrifugation, cells were resuspended in neurobasal/B27/glutamax (Gibco) containing β-mercaptoethanol and antibiotics (Sigma) and plated on polyornithine/laminin-coated coverslips at a density of 10^6/well in 24-well plates. Twelve hours later, cells were transfected with x-Lhx7-myctag or with GFP using Lipofectamin2000 (Invitrogen) according to the manufacturer's protocol. Primary neurons were then maintained in culture until the equivalent of "E21/P2" (i.e., 9 days in vitro (div) for neurons from E12 and 5 div for neurons from E16), fixed, and processed for immunohistochemistry. After blocking in PBT (PBS/0.1%Triton/0.5%BSA), primary antibody (rabbit anti-GABA or guinea pig anti-VAChT from Chemicon, 1/500; mouse anti-TuJ1 from Sigma, 1/500) was added 48 h at 4°C, followed by secondary antibody. Double labeling (anti-myc for x-Lhx7-mt transfected cells or anti-GFP for control-transfected cells) was then performed. Cells were counterstained with DAPI (Molecular Probes), mounted, and assessed for neurotransmitter phenotype under fluorescence microscopy.

Results

The definition of subpallial divisions of the developing *Xenopus* telencephalon has been previously reported, leading to the proposal of a lge-like and a mge-like territory on the basis of gene expression patterns (Bachy et al., 2002). Here, we re-examined in detail the anatomical relationships of *x-Dll3*, *x-Nkx2.1*, and *x-Lhx7* expression domains at early stages for the purpose of functional considerations relative to neurotransmitter specification (Figs. 1A–D). In toto staining for these markers at stage 35 showed a prominent expression in subpallial and diencephalic regions (Figs. 1A–C). In the subpallium, both x-Nkx2.1 and *x-Lhx7* labeled the same region which corresponds to the mge/aep/poa and is comprised in the *x-Dll3*-positive area. In the diencephalon, the band of *x-Lhx7* expression was similar although thinner than *x-Dll3*, both being included into the