The SWI/SNF chromatin remodeling protein Brg1 is required for vertebrate neurogenesis and mediates transactivation of Ngn and NeuroD

Seongjin Seo, Genova A. Richardson and Kristen L. Kroll*

Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA
*Author for correspondence (e-mail: kkroll@molecool.wustl.edu)

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Summary

Chromatin remodeling complexes play crucial roles in transcription and are implicated in processes including cell proliferation, differentiation and embryonic patterning. Brg1 is the catalytic subunit of the SWI/SNF chromatin remodeling complex and shows neural-enriched expression. Although early lethality of *Brg1*-null mice reflects its importance in embryogenesis, this phenotype precluded further study of specific Brg1-dependent developmental processes. Here, we have identified a requirement of Brg1 for both *Xenopus* primary neurogenesis and neuronal differentiation of mammalian P19 embryonic carcinoma cells. In *Xenopus*, loss of Brg1 function did not affect neural induction or neural cell fate determination. However, the *Sox2*-positive, proliferating neural progenitor cell population was expanded, and expression of a terminally differentiated neuronal marker, *N-tubulin*, was diminished upon loss of Brg1 activity, suggesting that Brg1 is required for neuronal differentiation. The ability of the bHLH transcription factors Ngnr1 and NeuroD to drive neuronal differentiation was also abolished by loss of Brg1 function, indicating that Brg1 is essential for the proneural activities of Ngnr1 and NeuroD. Consistent with this, dominant-negative interference with Brg1 function in P19 cells suppressed neuronal differentiation promoted by NeuroD2, showing the requirement of Brg1 for neuronal differentiation is conserved in mammalian cells. Finally, we discovered that Brg1 physically associates with both Ngnr1 and NeuroD and that interference with Brg1 function blocks Neurogenin3- and NeuroD2-mediated reporter gene transactivation. Together, our results demonstrate that Brg1 (and by inference the SWI/SNF complex) is required for neuronal differentiation by mediating the transcriptional activities of proneural bHLH proteins.

Key words: Brg1, Chromatin remodeling, Differentiation, NeuroD, Neurogenesis, Neurogenin, P19, SWI/SNF, *Xenopus*

Introduction

Formation of the vertebrate nervous system requires temporal and spatial coordination of multiple processes, including neural induction, cell proliferation, regulated cell cycle withdrawal and neuron-specific gene expression accompanying differentiation. Induction of neural tissue from naïve ectoderm initially demarcates the future neural plate as distinct from the non-neural ectoderm through activation of markers such as *Sox2* and *Sox3* in proliferating neural progenitors (Bylund et al., 2003; Graham et al., 2003; Hardcastle and Papalopulu, 2000; Miyagi et al., 2004). As these neural progenitors proliferate to generate the number of cells needed to form the nervous system, subsets of these cells begin to express proneural genes such as vertebrate homologs of *Drosophila Achaete-scute* and *Atonal* (Bertrand et al., 2002; Kintner, 2002). These proneural genes subsequently induce neuronal-differentiation genes such as *NeuroD*, which regulate cell cycle withdrawal and terminal neuronal differentiation (Bertrand et al., 2002). Both the proneural and neuronal-differentiation genes are transcription factors of the basic helix-loop-helix (bHLH) class and act as heterodimers with ubiquitously expressed E2A proteins (including its alternative products E12 and E47). These proteins share the ability to bind sites with the consensus CANNTG, termed E boxes, in enhancer and promoter regions of target genes.

Non-amniotic vertebrates such as *Xenopus laevis* generate a simple pattern of primary neurons regulating early larval behavior, which represents an attractive experimental system for analyzing molecular aspects of neurogenesis. Primary neurons are observed in three longitudinal domains on either side of the dorsal midline. Cells differentiating in these domains correspond to the three classes of primary neurons – motoneurons, interneurons and sensory neurons – in a medial-to-lateral order. Neurogenesis within these domains is regulated by bHLH proneural genes. The earliest proneural gene expression is that of *Neurogenin-related-1* (*Ngnr-1*), which induces later-acting bHLH factors including NeuroD. Ngn also activates the Notch ligand Delta-1 and the Zn-finger

transcription factor MyT1 in neuronal precursor cells. Activation of *Delta-1* expression stimulates lateral inhibition, a negative feedback loop mediated by the Notch pathway, in neighboring cells, whereas MyT1 renders neuronal precursors resistant to Notch signaling (Bellefroid et al., 1996). Therefore, sequential activities of Ngn, MyT1 and NeuroD promote neuronal differentiation of some competent neural precursors, while activated Notch signaling maintains neighboring cells in an undifferentiated state (Bellefroid et al., 1996; Chitnis et al., 1995; Lee et al., 1995; Ma et al., 1996; Wettstein et al., 1997).

Factors modulating chromatin structure, such as chromatin-remodeling complexes, histone acetyltransferases (HATs) and deacetylases (HDACs) play crucial roles in transcriptional regulation and participate in diverse processes, including cell proliferation, differentiation, embryonic patterning and tumorigenesis. The SWI/SNF complex was the first chromatin-remodeling complex identified and its biochemical properties have been actively characterized (Kadonaga, 1998; Martens and Winston, 2003). The SWI/SNF complex consists of 7-13 subunits with a total molecular mass of ~2 MDa and uses energy provided by ATP hydrolysis to locally disrupt histone-DNA associations and relocate nucleosomes to alternate positions (Kingston and Narlikar, 1999; Whitehouse et al., 1999). Various sequence-specific transcription factors, HATs and HDACs are known to interact with the SWI/SNF complex to activate or repress target genes (Kadam and Emerson, 2003; Peterson and Logie, 2000). SWI/SNF complexes in mammalian cells have either one of the two catalytic subunits, Brahma (Brm) or Brahma-related gene 1 (Brg1), but not both (Martens and Winston, 2003). Brm- and Brg1-containing complexes share most other subunits and have similar in vitro biochemical activities but appear to have some target gene specificity in vivo (Kadam and Emerson, 2003).

Prior evidence has suggested that Brg1 (and by inference the SWI/SNF complex) may be involved in neural development. First, although *Brg1* is ubiquitously expressed in early stage mouse embryos, its expression becomes enriched in neural tissue during embryogenesis (Randazzo et al., 1994). For example, at stage E15, *Brg1* is abundantly expressed within the brain, spinal cord and retina. Within the spinal cord, *Brg1* is more abundant in the mantle zone, where differentiating neurons exist, compared with the ventricular zone. Second, *Brg1*-null mice die at peri-implantation stages, but 15-30% of heterozygotes display exencephaly, a neural tube abnormality (Bultman et al., 2000). In addition, heterozygotes of *Brg1* or other essential SWI/SNF complex components are predisposed to tumors of neural origin (Bultman et al., 2000; Kim et al., 2001; Klochendler-Yeivin et al., 2000). Finally, *Brg1* mutant zebrafish have defects in terminal differentiation of retinal cells (Gregg et al., 2003; Link et al., 2000). These observations suggest Brg1 is involved in neural development, but the precise role of Brg1 in neural development had not previously been defined.

Here, we describe cloning and characterization of a *Xenopus Brg1* homolog, and determination of Brg1 requirements for vertebrate neuronal differentiation. *Brg1* is ubiquitously expressed in *Xenopus* embryos until the neurula stage but its expression is gradually restricted to neural tissues at later stages. Roles of Brg1 in *Xenopus* neural development were studied by either reducing its expression by antisense morpholino oligonucleotide (MO) injection or by introducing a well-characterized dominant-negative form. Upon reduction of Brg1 activity, *Sox2*-positive neuroectoderm was properly induced, but neuronal differentiation was blocked. Neural precursors appeared to remain as proliferating progenitors. Ectopic neurogenesis driven by Ngn and NeuroD was also blocked by loss of Brg1 function. We further demonstrated that Brg1 physically interacts with the proneural bHLH proteins, Ngn and NeuroD, and mediates their transcriptional activities for neurogenesis. Our results define Brg1 as an essential regulator of neuronal differentiation during vertebrate nervous system formation.

Materials and methods

Cloning of xBrg1 cDNA and phylogenetic analysis

Gene-specific primers encompassing full-length *xBrg1* cDNA were designed based on available EST sequences: F, 5′-agctcaattgtcttctccct-gcagtgatgtcc-3′; and R, 5′-aatctcgagaggttctatggggtgggtcttg-3′. A 5031 bp *xBrg1* cDNA was obtained from a *Xenopus laevis* stage 11.5-15 embryo cDNA library by PCR using *Pfu turbo* polymerase (Stratagene). This fragment was digested with *Mfe*I and *Xho*I, and cloned into *Eco*RI and *Xho*I sites of pCS2+MT (Turner and Weintraub, 1994). The full cDNA sequence was determined and deposited into GenBank (AY726636). Accession numbers for phylogenetic analysis are in Fig. 1 or as follows: *Mus musculus* Brg1 was derived from NM_011417 and BC026672; mouse Brm was deposited as BK005591. All proteins are full length except for *Xenopus laevis* Brm, which is a C-terminal partial protein deposited as BK005590. Sequence alignment was generated using Clustal W and MegAlign software (DNASTAR).

Mutagenesis

A dominant-negative version of xBrg1 was made by site-directed mutagenesis with the QuikChange mutagenesis protocol (Stratagene) and *Pfu* turbo polymerase (Stratagene), using oligos 5′-gagatgggcctgggagcggctattcagaccattg-3′ and its complementary sequence. This mutation changed the highly conserved K770T771 residues in the ATP binding pocket to A770A771. After sequence verification, the *Eco*RI-*Not*I fragment containing the desired mutation was exchanged with the corresponding region of wild-type xBrg1 in pCS2+MT.

Embryos and RNA/morpholino oligonucleotide injections

Embryos were obtained by in vitro fertilization and raised as described previously (Kroll et al., 1998). When indicated, hydroxyurea was added to media at a final concentration of 30 mM at stage 12.5 onwards until embryos were fixed. Embryos were staged after Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For generating capped mRNAs, vectors encoding xNgnr1 (Ma et al., 1996), xNeuroD (Lee et al., 1995), xMyT1 (Bellefroid et al., 1996), xBrg1, DN-xBrg1 and β-galactosidase were transcribed in vitro (mMessage mMachine kit, Ambion). RNAs for xNgnr1 (30 pg), xNeuroD (50 pg), xMyT1 (50 pg), DN-xBrg1 (1 ng) or antisense morpholino oligonucleotides (20 ng of xBrg1MO; 5′-tcactgctaacctgtccccgaatcc-3′) (Gene Tools LLC) were co-injected with 30 pg of β-galactosidase mRNA in a volume of 10 nl into one blastomere of stage 2 embryos. For rescue experiments, wild-type *xBrg1* mRNA (900 ng) was co-injected with xBrg1MO. In parallel with xBrg1MO injections, embryos were injected with 20 ng of standard control MO 5′-cctcttacctcagttacaatttata-3′ from Gene Tools LLC; injection of up to 40 ng of this MO did not cause any apparent embryonic defects.

Xenopus whole-mount in situ hybridization, TUNEL assay, and phospho-histone H3 immunostaining

Embryos were raised until indicated stages, fixed in MEMFA for 1

hour, X-gal (5-bromo-4-chloro-3-indolyl β-galactopyranoside) stained and analyzed by whole-mount in situ hybridization (Harland, 1991). Probes for *Sox2* (Mizuseki et al., 1998) and *N-tubulin* (Oschwald et al., 1991) were generated by in vitro transcription with digoxigenin-11-UTP (Roche) and detected using alkaline phosphatase (AP)-conjugated anti-digoxigenin antibodies (Roche) with Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; Roche). Whole-mount TUNEL assay was performed after (Hensey and Gautier, 1998). Labeled cells were visualized as described for whole-mount in situ hybridization. Phosphorylated histone H3 (PH3) immunostaining was carried out essentially as previously described (Saka and Smith, 2001) using a 1:1000 dilution of primary antibody (Upstate). Secondary antibody was AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted to 1:1000. Labeled cells were visualized as described for TUNEL. After staining, some specimens were embedded in 4% low-melting agarose and vibratome sectioned (50 μm). TUNEL or PH3-positive cells were counted within regions of equal surface area on injected and uninjected bilateral halves of each embryo; fold changes were then determined for each embryo as the ratio of injected/uninjected values. Fold change values shown represent averaged results from at least five embryos.

P19 cell culture and immunohistochemistry

Maintenance of P19 cells and transfection of bHLH plasmids was performed according to Farah et al. (Farah et al., 2000). For immunohistochemistry, cells were grown on poly-L-lysine-coated coverslips and transfected with mouse *NeuroD2* (800 ng), mouse *E12* (300 ng), *eGFP* (700 ng) and *DN-hBrg1* (1200 ng) using FuGENE6 (Roche). When applicable, pCS2+MT was included in transfection to maintain constant levels of transfected DNA. Three days post-transfection cells were fixed for 6 minutes in 4% formaldehyde/PBS, permeabilized for 6 minutes in 0.2% TritonX-100/PBS, washed and then blocked in 5% BSA/PBS. TuJ1 (Covance) and anti-GFP antibody (BD Biosciences) were used at a dilution of 1:600 and 1:1500, respectively. Secondary antibodies used were Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG (Molecular Probes) diluted to 1:1500 and 1:500, respectively. Digital images were captured using a Zeiss Axioskop and Axiovision software and overlaid in Adobe Photoshop.

Luciferase assays

The E1X3-TATA reporter contains three copies of the E1 E-box from the *neuroD1/β2* promoter in a luciferase reporter vector (Huang et al., 2000). Transfections were performed in six-well plates using FuGENE6 (Roche) as described previously. Plasmid amounts were: 400 ng pCS2+Ngn3 or pCS2+NeuroD2, 1200 ng pCS2+DN-hBrg1, and 1 μg E1X3-TATA. pSV40 β-gal (Promega) (300 ng) was co-transfected to normalize transfection efficiency. When applicable, pCS2+MT was included to bring total DNA content up to 3 μg. Cells were harvested 60 hours post-transfection and analyzed using the Luciferase assay system (Promega) and the β-galactosidase enzyme assay system (Promega). Samples were assayed in triplicate and experiments were repeated multiple times with similar results. Representative experiments are shown in Fig. 8.

In vitro translation, co-immunoprecipitation (co-IP) and western analysis

Expression plasmids for *Xenopus laevis Brachyury* (Smith et al., 1991), *ESR1* (Wettstein et al., 1997), *Ngnr1*, *NeuroD* and *Brg1* were all in pCS2+ and contained a 6xMyc or 3xFLAG(FL)-epitope tag. Proteins were produced by in vitro translation using the TNT Coupled Reticulocyte Lysate System (Promega). After translation, 20 μl of Myc-xBrg1 containing lysate was added to 20 μl of each FLAG-tagged protein and incubated for 1 hour at 30°C for binding. Dilution buffer [360 μl of 20 mM HEPES (pH 7.2), 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA] supplemented with Protease inhibitor cocktail (Roche) was added and IP was performed using 20 μl of anti-Myc antibody-conjugated beads (Santa Cruz Biotechnology) at 4°C for 4 hours. Beads were collected and washed four times with dilution buffer. Bound proteins were analyzed by SDS-PAGE followed by western blotting using standard protocols.

HeLa cells were grown in DMEM supplemented with 10% FBS, transfected using PolyFect transfection reagent (Qiagen) and harvested 30-48 hours after transfection. Cells were disrupted in lysis buffer [20 mM HEPES (pH 7.2), 150 mM NaCl, 0.3% Triton X-100, 3 mM EDTA] supplemented with Protease inhibitor cocktail (Roche) on ice. After clarification by centrifugation, lysates were applied to co-IP as described above.

Results

Cloning and expression profile of *xBrg1*

To study the developmental role of Brg1 in *Xenopus* embryos, we cloned the *Xenopus laevis* homolog of *Brg1* (*xBrg1*) using a cDNA library from stage 11.5-15 embryos (see Materials and methods). The predicted amino acid sequence of xBrg1 showed 87.1% identity with hBrg1 and 74.8% with hBrm by alignment with the Clustal W algorithm. xBrg1 has an 87.1-90.6% amino acid identity with various vertebrate Brg1 orthologs versus a 73.0-75.4% identity with vertebrate Brm orthologs. Homology between xBrg1 and hBrg1 was observed throughout the protein but to a lesser extent in Domain I (78.3%), which is divergent in different Brg1/Brm homologs (Fig. 1A). Domain IV, the bromodomain, showed the highest sequence identity (98.7%), and Domain II and Domain III (ATPase domain) showed 95.5% and 94.1% identity, respectively (Fig. 1A). Sequence distances between Brg1 and Brm orthologs in different species are schematized in Fig. 1B.

To determine the expression profile of *xBrg1* during embryonic development, we performed whole-mount in situ hybridization (Fig. 1C-I) using a probe corresponding to a region of Domain I (Fig. 1A), which is relatively less conserved among various Brg1 and Brm homologs. *Xenopus Brg1* was expressed maternally and its mRNA was detected throughout the animal hemisphere (Fig. 1C). During gastrulation, *xBrg1* transcripts were still detected throughout the entire embryo except the yolk plug (Fig. 1D). However, as RNA in situ signals can be quenched in the yolk-rich vegetal hemisphere, lack of signals in the vegetal hemisphere and yolk plug does not exclude a ubiquitous distribution of maternal transcripts. No dorsoventral bias was observed until late gastrulation (stage 13; data not shown). At stage 14, for example, *xBrg1* was expressed broadly in dorsal tissues by comparison with more restricted expression of *N-tubulin* (Fig. 1I,J). From the late gastrula stage, however, *xBrg1* expression began to be enriched in the neural plate and this bias became obvious by stage 16 (Fig. 1E). At stage 20-22, *xBrg1* expression was maintained at high levels throughout the neural tube (Fig. 1F). Cranial and trunk neural crest cells also expressed *xBrg1*, whereas expression in epidermal cells decreased dramatically by these stages (Fig. 1F). At tailbud and tadpole stages, *xBrg1* mRNA was detected throughout the CNS, including the eye, brain and spinal cord, and additionally in the branchial arches and otic vesicle (Fig. 1G,H). A similar expression pattern was also observed at stage 33/34, using alternate, non-overlapping probes corresponding to Domain II or 3′ coding sequences (Fig. 1A; data not shown). The *xBrg1* expression pattern is in accordance with expression of mouse and *Drosophila* homologs, showing ubiquitous expression at early stages but

later neural-enriched expression (Elfring et al., 1998; Randazzo et al., 1994). This expression pattern suggests that Brg1 may play a general role in early development but could have a more specific function in neural development at post-gastrula stages.

Loss-of-function approaches to characterize Brg1 function during neural development

To study requirements for Brg1 in *Xenopus* embryogenesis, we used two independent and complementary approaches. First, we used a well-characterized dominant-negative form of Brg1 (DN-xBrg1). The ATPase domain of Brg1 is highly conserved in all Brg1 homologs, and point mutations in the ATP-binding pocket are known to disrupt ATPase activity (de la Serna et al., 2001a; Khavari et al., 1993). This mutant protein can still associate with the other proteins of the SWI/SNF complex, but the mutant Brg1-containing complex is enzymatically inactive. Thus, mutant Brg1 behaves in a dominant-negative manner. Here, we used this mutant to perturb function of wild-type xBrg1. At tadpole stages, injection of DN-xBrg1 consistently caused a series of related morphological defects ranging from apparent truncation of anterior structures to reduction of the eye (Fig. 2A). As a second, independent method for interfering with Brg1 function, we designed morpholino oligonucleotides (MOs) complementary to the 5′ untranslated region of *xBrg1* mRNA (xBrg1MO). After titration experiments, using doses from 1 ng to 33 ng, we found dose-dependent morphological defects in a range between 15 ng and 25 ng, and used 20 ng for experiments. Morphological defects observed in xBrg1MO-injected embryos (Fig. 2B) were similar to those observed in *DN-xBrg1*-injected embryos (Fig. 2A). These defects were not obtained following control injections of standard MO (Fig. 2C) or lineage tracer mRNA (data not shown) performed in parallel. The similarity of the defects produced by either xBrg1MO or *DN-xBrg1* injection suggests that these effects are specific to reduction of xBrg1 activity.

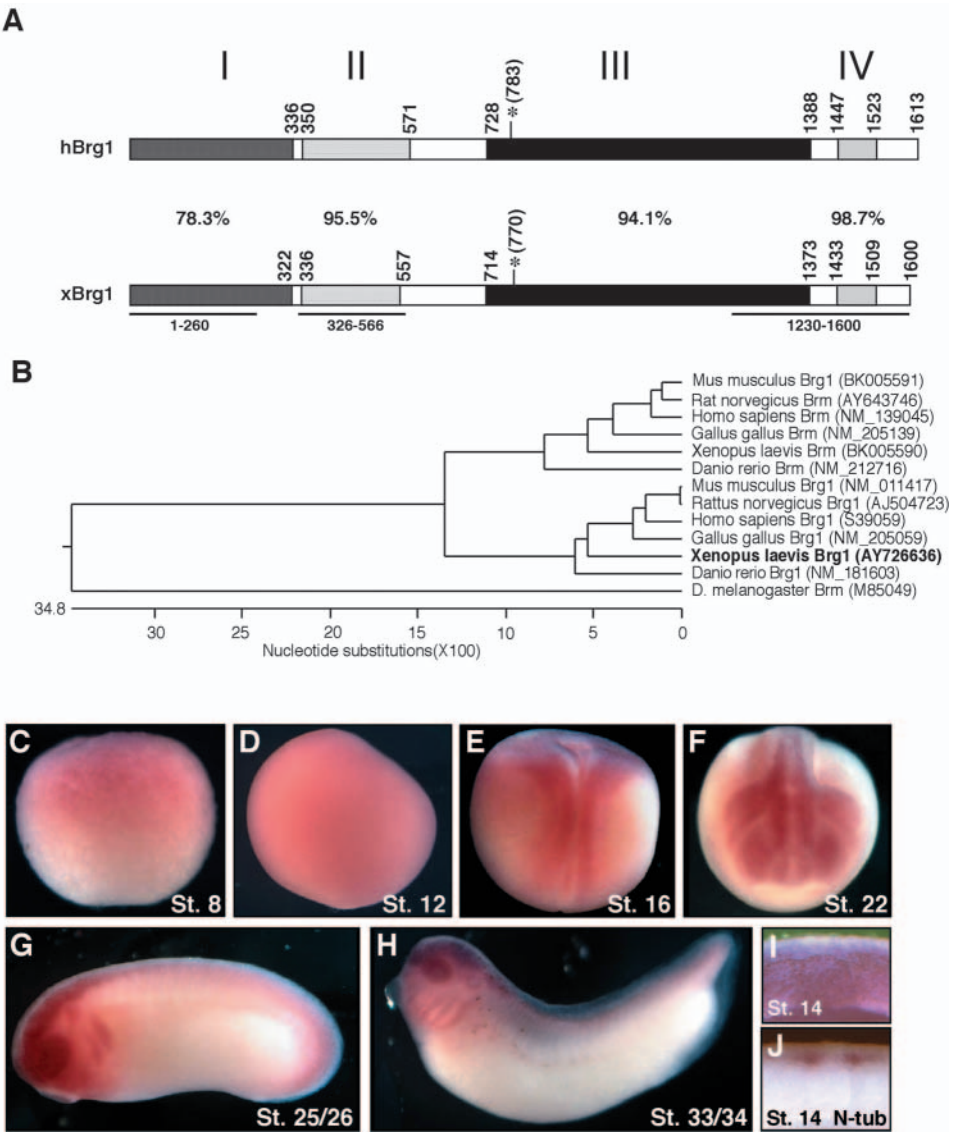

Fig. 1. Cloning and expression profile of *xBrg1*. (A) Structure of hBrg1 and xBrg1. Domains are labeled after (Khavari et al., 1993) with percent amino acid identity shown. Asterisks mark the ATP binding pocket targeted by mutagenesis to generate a dominant-negative form. Lines under xBrg1 indicate probes used for in situ hybridization. (B) Phylogenetic analysis of Brg1 and Brm orthologs. Units indicate the number of substitutions. Distance between any two sequences is the sum of horizontal branch length separating them. (C-I) Expression profile of *xBrg1*. (C) Stage 8 and (D) stage 12, side views (vegetal pole toward bottom). (E) Dorsal view, anterior towards the top (stage 16). (F) Anterior view (stage 22). (G) Stage 25/26 and (H) stage 33/34, lateral views. (I-J) Cross-sectional views of stage 14 embryos stained for *xBrg1* (I) or *N-tubulin* (J).

To examine whether the MOs we designed indeed block xBrg1 translation in embryos, we injected 25 ng of xBrg1MO into each blastomere of two-cell stage embryos and examined endogenous xBrg1 protein levels at later stages (Fig. 2D). Injection of 25 ng of standard MO was performed in parallel to assay for non-specific effects. Maternal xBrg1 protein was detected in both xBrg1MO and standard MO injected embryos from early stages. xBrg1MO-injected embryos showed a significant reduction in Brg1 protein levels; decline in xBrg1 protein levels was initially visible at stage 8 and became more pronounced by stage 12-15. By contrast, xBrg1 protein levels increased from stage 8-15 in embryos injected with 25 ng of standard MO.

To determine specific Brg1 requirements during neural development, embryos were injected with either *DN-xBrg1* or xBrg1MO and neural marker expression was examined. At stage 13, both *DN-xBrg1* and xBrg1MO-injected embryos

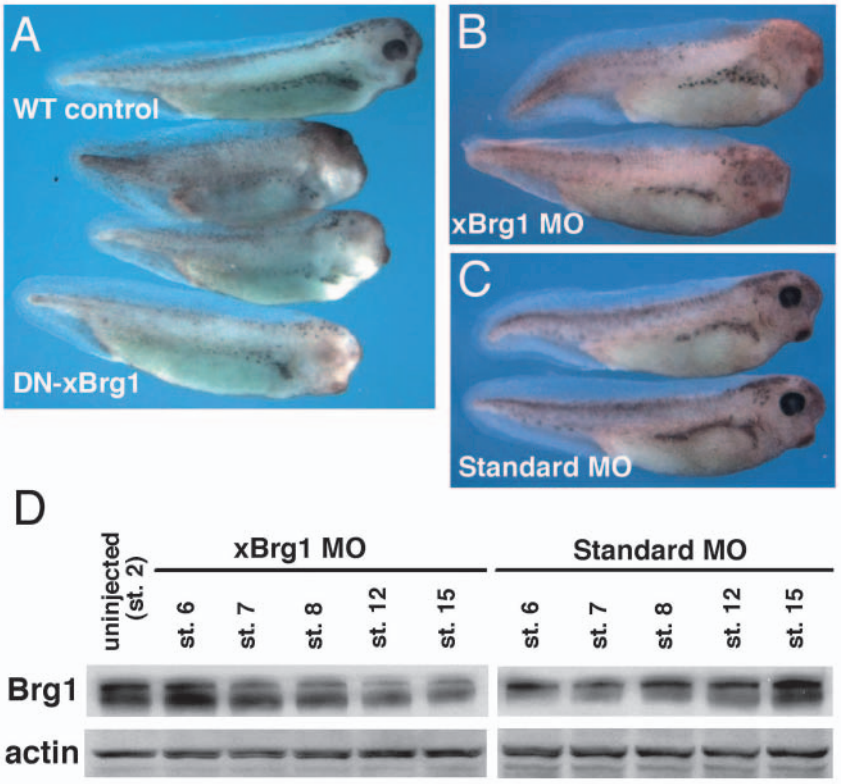

Fig. 2. *DN-xBrg1* and xBrg1MO injections have similar effects on embryonic morphology. (A-C) Morphology of tadpoles (stage 37/38) injected with *DN-xBrg1*, xBrg1MO or standard MO. Embryos were injected in both blastomeres at the two-cell stage and raised to tadpoles. (A) Bottom three embryos were injected with *DN-xBrg1*, while the top embryo was uninjected. (B) xBrg1MO (20 ng) injected tadpoles. xBrg1MO-injected tadpoles display similar defects to *DN-xBrg1*, while standard MO (C) injected ones do not show apparent defects. (D) Reduction of endogenous xBrg1 protein by xBrg1MO. Embryos were injected with 25 ng of xBrg1 or standard MO in both blastomeres at stage 2 and harvested at the indicated stages. Ten embryos were used per sample, with one embryo-equivalent of lysate loaded per lane for western blotting.

showed normal expression of *Sox2*, an early neural marker expressed in proliferating neural progenitors (Fig. 3A,D). These results imply that early aspects of neural induction and neural cell fate determination occurred normally. However, at stage 15-16, the *Sox2*-positive domain was expanded on the *DN-xBrg1* (67%, *n*=43) or xBrg1MO (80%, *n*=51) injected side of the embryo (Fig. 3B,E). By contrast, expression of type II neuron-specific tubulin (*N-tubulin*), which marks terminally differentiated neurons, was severely reduced or abolished in *DN-xBrg1*-injected embryos (Fig. 3C; Table 1). *N-tubulin* expression was also severely reduced or abolished in xBrg1MO-injected embryos (Fig. 3F; Table 1). However, injection of xBrg1MO along with wild-type *xBrg1* restored normal or near normal *N-tubulin* expression (Fig. 3K; Table 1). These data indicate that loss of *N-tubulin* expression is specific to reduction of Brg1 activity. Likewise, co-injection of wild-type *xBrg1* with xBrg1MO suppressed the *Sox2* expansion previously observed (no expansion in 78%, *n*=60) (Fig. 3J), indicating that this defect was also specific to loss of Brg1 activity. Injection of standard MO, a negative control, at doses up to 30 ng did not change either *Sox2* or *N-tubulin* expression (Fig. 3G-I). These results suggest that initial specification of the neural territory occurs normally but that neuronal differentiation is blocked by reduction of Brg1 activity.

Loss of Brg1 function increases cell proliferation

Brg1 has been implicated in cell cycle withdrawal (Klochendler-Yeivin et al., 2002; Muchardt and Yaniv, 2001). Thus, expansion of the *Sox2*-positive territory in xBrg1MO-injected embryos may be related to increased cell proliferation. To analyze this possibility, we assayed cell proliferation by immunostaining for phosphorylated histone H3 (PH3) following xBrg1 depletion. As previously reported (Saka and Smith, 2001), in uninjected embryos most PH3-positive cells were detected within the neural plate at stage 14-15, whereas only a few cells were PH3-positive in the non-neural territory (Fig. 4A,C). In xBrg1MO-injected embryos, PH3-positive cells were detected at a higher density within the neural plate of the injected side, relative to the uninjected side (28.5±6.4% increase, *n*=5 embryos; Fig. 4B,E). Intriguingly, an even greater increase in cell proliferation was observed in epidermal ectoderm (81.8±32.9% increase, *n*=5 embryos; Fig. 4D). These data demonstrate that additional cell proliferation, in both neural and non-neural tissue, accompanies reduction of Brg1 activity.

We further investigated whether the *Sox2* expansion observed previously could be abolished by forceful cell cycle arrest. For this purpose, xBrg1MO-injected embryos were treated with hydroxyurea (HU), which arrests the cell cycle in S phase. Treatment with 30 mM HU was performed from stage 12.5 and this treatment efficiently blocked cell proliferation, as measured by PH3 immunostaining (Fig. 4F). Interestingly, forceful cell cycle arrest suppressed *Sox2* expansion on the xBrg1MO-injected side of the embryo (no expansion in 92% and weak expansion in 8%; *n*=25; compare Fig. 4G with Fig. 3E). This result indicates that the *Sox2* expansion seen upon reduction of xBrg1 activity is linked to increased cell proliferation. However, *N-tubulin* expression was not rescued by forceful cell cycle arrest in xBrg1MO-injected embryos (moderate to severe *N-tubulin* reduction was observed in 85% of embryos; *n*=26; Fig. 4H).

Table 1. Reduction of Brg1 activity blocks neuronal differentiation

Injection	Complete or severe loss	Moderate loss	No change	Total (*n*)
DN-xBrg1	48%	29%	23%	52
xBrg1MO	64%	27%	9%	67
xBrg1MO + wt-xBrg1	9%	30%	60%	43

The number of *N-tubulin*-positive cells on the injected side of the embryo was scored relative to the uninjected side. In embryos scored as 'complete or severe loss', the injected side had fewer than 30% of the *N-tubulin*-positive cells seen on the control side; those scored as 'moderate loss' had 30-80% of the *N-tubulin*-positive cells seen for the control side, and embryos were scored as 'no change' if more than 80% of the *N-tubulin*-positive cells remained on the injected side.

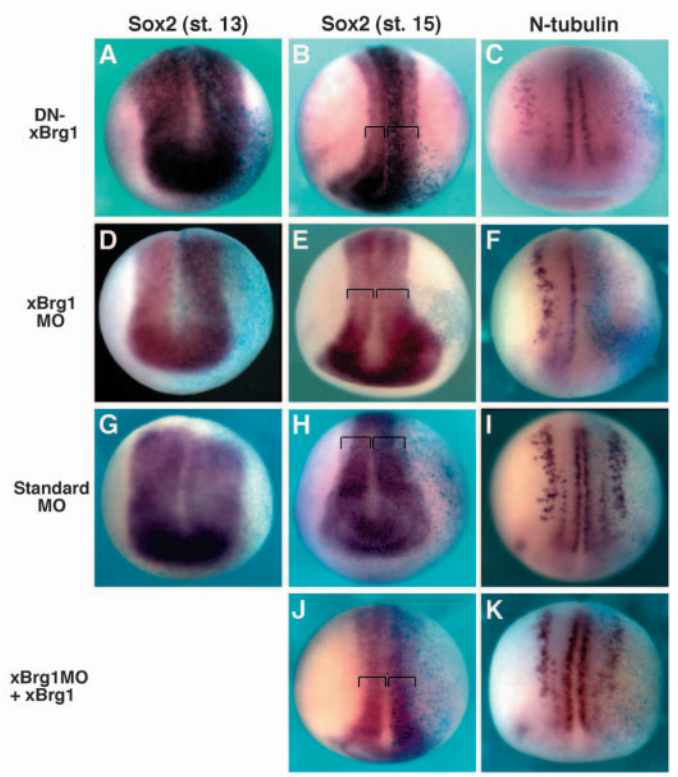

Fig. 3. Embryos depleted of xBrg1 fail to produce primary neurons. Embryos were injected with *DN-xBrg1* (A-C), xBrg1MO (D-F) or standard MO (G-I) in one blastomere at stage 2. *β-galactosidase* mRNA was co-injected and X-Gal staining (blue) was performed to reveal distribution of injected materials. Embryos were analyzed for expression of *Sox2* (A,B,D,E,G,H,J) and *N-tubulin* (C,F,I,K; stage 15). Embryos in J and K were injected with xBrg1MO and *xBrg1* mRNA. Dorsal views with injected side facing rightwards.

These data indicate that Brg1 is required for additional aspects of neurogenesis that contribute to neuron-specific gene expression as well as for cell cycle withdrawal.

We next examined whether loss of *N-tubulin*-positive cells could have resulted from apoptosis by performing TUNEL assays. Although the standard MO did not cause significant changes in apoptosis (Fig. 4I), injection of xBrg1MO resulted in a twofold increase in the number of apoptotic cells (Fig. 4J). However, the actual number of apoptotic cells that appeared following xBrg1MO injection was much fewer than the number of primary neurons normally produced. Furthermore, most apoptotic cells were observed in the anterior neural plate rather than the posterior neural plate, where the primary neurons arise. Therefore, quantitatively and qualitatively, increased apoptosis was not sufficient to explain the almost complete loss of N-tubulin positive neurons obtained in xBrg1MO-injected embryos. The lack of differentiated neurons appeared to be due to a failure of differentiation rather than selective cell death.

Brg1 is required for neurogenesis by proneural bHLH transcription factors

To further analyze requirements for and the position of Brg1 within the proneural pathway, we examined whether loss of Brg1 function affected proneural activities of Ngnr1, NeuroD and MyT1. RNAs encoding Ngnr1, NeuroD or MyT1 were

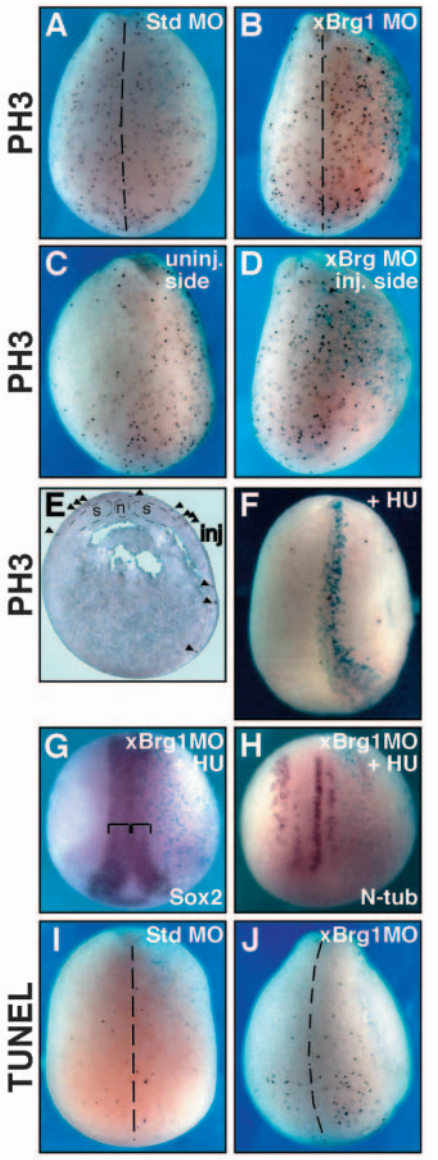

Fig. 4. Loss of Brg1 increases cell proliferation. (A-D) Whole-mount stage 14 embryos immunostained (brown) to detect phosphorylated histone H3 (PH3) after injection of standard MO (A) or xBrg1MO (B-D). (C,D) Lateral views of an xBrg1MO-injected embryo: (C) uninjected side; (D) xBrg1MO-injected side. (E) Transverse section of embryo in B, showing the injected side facing rightwards. Black arrowheads mark PH3-positive cells. n, notochord; s, somite. (F-H) Cell division was blocked by adding hydroxyurea (HU) from stage 12.5 until fixation at stage 15. (F) PH3-immunostained embryo showing cell cycle is efficiently blocked by HU treatment (xBrg1MO+β-galactosidase injection, blue; PH3 stain, brown). (G,H) xBrg1MO-injected embryos were raised in the presence of 30 mM HU from stage 12.5 and analyzed for *Sox2* (G) and *N-tubulin* (H) expression. (I,J) Whole-mount TUNEL staining (brown) after injection of standard MO or xBrg1MO. A,B,F-J are dorsal views with injected side facing rightwards.

injected alone or together with xBrg1MO. In agreement with previous studies (Lee et al., 1995; Ma et al., 1996), Ngnr-1 and NeuroD induced strong ectopic expression of *N-tubulin* (Fig. 5A,B). However, ectopic expression of *N-tubulin* induced by Ngnr1 and NeuroD was greatly reduced by co-injection of

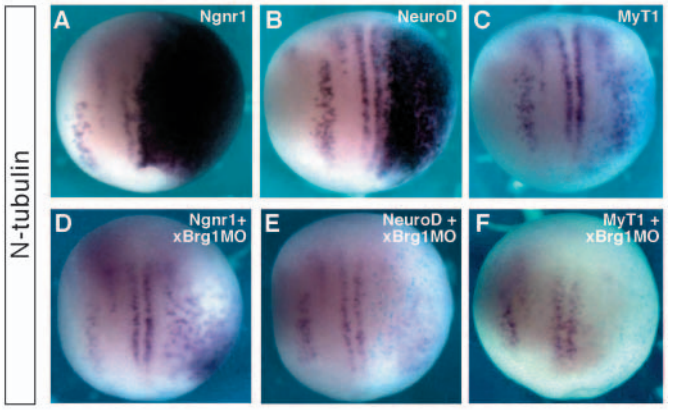

Fig. 5. Brg1 is required for the proneural activities of xNgnr1 and xNeuroD. Embryos were injected with *xNgnr1* (A), *xNeuroD* (B) or *xMyT1* (C) alone or together with xBrg1MO (D-F) and analyzed for *N-tubulin* expression (stage 15). Views are dorsal with injected side (X-Gal, blue) facing rightwards.

xBrg1MO (Fig. 5D,E). MyT1 injection caused an increase of *N-tubulin*-positive cells within the primary neuronal stripes (Bellefroid et al., 1996) (Fig. 5C), and co-injection of Brg1MO also blocked *N-tubulin* induction by MyT1 (Fig. 5F). These results suggest that the proneural activities of Ngn1, NeuroD and MyT1 are Brg1-dependent, and that Brg1 acts in concert with and/or downstream of NeuroD.

Requirement of Brg1 in mammalian neuronal differentiation

To determine whether the Brg1-requirement for *Xenopus* primary neurogenesis was also conserved in mammalian neurogenesis, we investigated effects of interfering with Brg1 function in P19 embryonic carcinoma cells. Pluripotent mouse P19 cells have been used extensively as a model system to study in vitro differentiation (Bain et al., 1995; McBurney, 1993). After retinoic acid (RA) treatment and aggregation, P19 cells differentiate into neurons and glia, whereas, under other conditions, these cells form muscle, endoderm or other cell types. Transient expression of proneural bHLH proteins such as NeuroD and MASH1 is also sufficient to induce neuronal differentiation from P19 cells in the absence of RA and aggregation (Farah et al., 2000).

To test the requirement of Brg1 for neuronal differentiation in mammalian cells, P19 cells were transiently transfected with plasmids expressing mouse *NeuroD2* and mouse *E12* together with or without *DN-hBrg1*. To identify transfected cells, a green fluorescent protein (*GFP*) expression vector was co-transfected. Three days after transfection, cells were fixed and examined for the expression of neuron-specific class III β-tubulin protein, detected by the antibody TuJ1. Consistent with previous reports (Farah et al., 2000), *NeuroD2* transfection efficiently induced formation of TuJ1-positive neurons (75.1±4.5% of transfected cells: average data from three experiments; Fig. 6A-C,J). By contrast, co-transfection of *DN-hBrg1* decreased the generation of TuJ1-positive cells by NeuroD2 to 35.0±12.1% (Fig. 6D-F,J). Overexpression of *GFP* or *E12* alone did not induce TuJ1-positive cells (Fig. 6G-I). These results indicate that normal Brg1 function is essential for neuronal differentiation driven byNeuroD2 and that the role of Brg1 in neurogenesis is likely to be evolutionarily conserved in vertebrates.

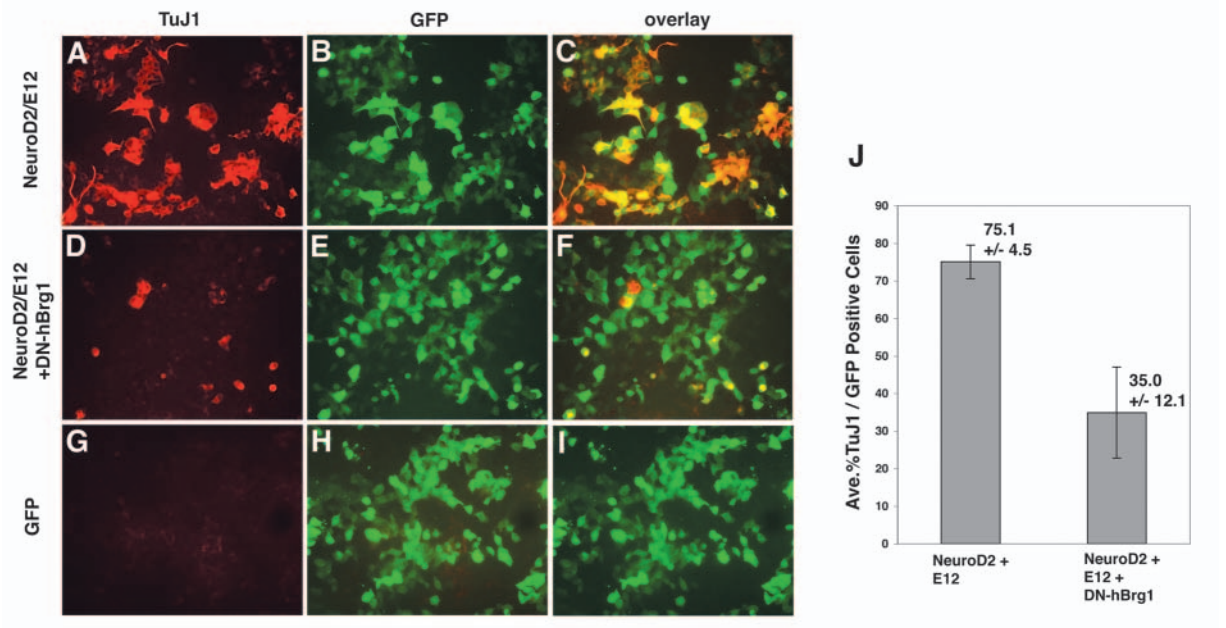

Fig. 6. The requirement of Brg1 for neurogenesis is conserved in mammalian P19 cells. P19 cells were transfected with plasmids encoding mouse NeuroD2 (A-C), mouse NeuroD2 and DN-hBrg1 (D-F), together with mE12 and GFP expression vectors. Three days after transfection, cells were analyzed by TuJ1 immunostaining (A,D,G) and *GFP* expression (B,E,H). C, F and I are overlays of images A and B, D and E, and G and H, respectively. (J) TuJ1-positive cells were scored as a percentage of GFP-positive transfected cells. A total of between 750 and 2200 GFP-positive cells were counted for each type of transfection. P19 cells transfected with *GFP* vector alone (G-I) or with *GFP* and *E12* vectors (but not with NeuroD2) yielded less than 1 TuJ-positive cell per 1.8×10^5 cells assayed in each experiment. Data shown in J are the average of three experiments.

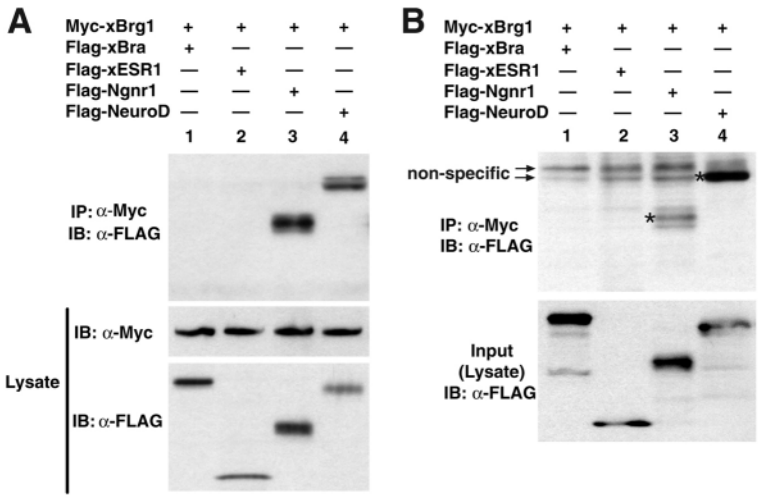

Fig. 7. Brg1 physically interacts with xNgnr1 and xNeuroD. (A) HeLa cells were transfected as indicated and applied to co-IP assay. Lysates were immunoprecipitated (IP) with anti-Myc antibodies and immunoblotted (IB) with anti-FLAG antibodies. Protein expression levels were monitored by western blotting of direct lysates. (B) Myc-xBrg1, FL-xBra, FL-xESR1, FL-xNgnr1 and FL-xNeuroD proteins were produced separately using reticulocyte lysate. After in vitro translation, an equal amount of lysate containing Myc-xBrg1 was added to FLAG-tagged proteins and subjected to co-IP with anti-Myc antibodies. Input of FLAG-tagged proteins was monitored by western blotting. Asterisks in B indicate specific bands corresponding to Ngnr1 and NeuroD.

Physical interaction of Brg1 and proneural bHLH proteins

As we had found Brg1 to be required for neurogenesis by Ngnr1 and NeuroD, and Brg1 can associate with various transcription factors (Kadam and Emerson, 2003; Peterson and Logie, 2000), it was possible that Brg1 may interact, either directly or indirectly, with Ngnr-1 and/or NeuroD and mediate their transcriptional activities in neurogenesis. To test this possibility, FLAG-tagged *xNgnr-1* and *xNeuroD* were co-transfected with Myc-tagged *xBrg1* in HeLa cells, and lysates were applied for co-immunoprecipitation (co-IP) assay. In this assay, xBrg1 was able to associate with both xNgnr1 and xNeuroD (Fig. 7A, lanes 3, 4). By contrast, xESR-1 (lane 2), another bHLH class protein that inhibits neurogenesis, and xBrachyury (lane 1), a T-box transcription factor involved in mesoderm formation, did not interact with Brg1 in this assay. We monitored the expression level of each protein by western blotting of the lysate. These results indicated that Brg1 can associate with Ngnr1 and NeuroD.

To further investigate whether Ngnr1 and NeuroD directly interact with Brg1, we conducted co-IP assays with in vitro translated proteins (Fig. 7B). In this assay, both xNgnr1-xBrg1 (lane 3) and xNeuroD-xBrg1 (lane 4) associations were detected, indicating that direct interaction between these proteins can occur. Neither xESR-1 (lane 2) nor xBrachyury (lane1) were found in complex with Brg1 in this assay.

Brg1 mediates transactivation of proneural bHLH proteins

As Ngnr1 and NeuroD were found to interact with Brg1, we next investigated whether Brg1 is required for transcriptional activation by proneural bHLH proteins. Previously, it has been shown that a multimerized E-box derived from the mouse *NeuroD1* promoter stimulated reporter expression in response to *Ngn3* and *NeuroD2* in transfected P19 cells (Farah et al., 2000). We tested whether this transcriptional activation was sensitive to interference with Brg1 activity. P19 cells were transfected with plasmids for *Ngn3*, *NeuroD2* and E-box reporter (E1X3-TATA-luc) together with or without *DN-hBrg1* and promoter activities were measured by luciferase assay. Transient expression of proneural bHLH proteins, Ngn3 or NeuroD2, robustly increased the transcription of the reporter gene (Fig. 8). However, the presence of DN-hBrg1 decreased the transcriptional activity of Ngn3 by 55.6±7.9% on average and that of mNeuroD2 by 59.9±2.4% (Fig. 8). Taken together with our data regarding the association of Brg1 with proneural bHLHs, these results suggest that Brg1 might act by mediating the transactivation of proneural bHLH proteins.

Discussion

The work presented here demonstrates that Brg1 (and by inference the SWI/SNF complex) plays an essential role in vertebrate neurogenesis. Although previous observations in mice and zebrafish (Bultman et al., 2000; Gregg et al., 2003; Link et al., 2000; Randazzo et al., 1994) were suggestive of a role for Brg1 in neural development, specific requirements for Brg1 were not defined. In the present study, interference with Brg1 activity did not affect initial neural cell specification but caused a failure of cell cycle exit and concomitant expansion of the proliferating neural precursors in *Xenopus* embryos. In addition, *Xenopus* embryos and mammalian cells blocked for Brg1 function showed a loss of differentiated neurons. Expansion of neural progenitors could be abolished by arresting the cell cycle with HU but neuronal differentiation was not restored by the same treatment, suggesting that Brg1 is required for neuron-specific gene expression as well as cell cycle withdrawal. The ability of the proneural bHLH proteins Ngnr1 and NeuroD to drive ectopic neurogenesis in *Xenopus* ectoderm was dependent on Brg1 activity. In addition to these functional requirements for Brg1 during neurogenesis, we determined that Brg1 physically interacted with both Ngnr1 and NeuroD, and that Brg1 activity was required for the transcriptional activities of the proneural bHLH proteins Ngn3 and NeuroD2 in mammalian cells. Collectively, our results indicate that Brg1 is specifically required for neuronal differentiation during neural tissue formation and that this Brg1 requirement is likely to be, at least in part, due to its interaction with and ability to mediate the transcriptional activities of proneural bHLH proteins.

Brg1 requirements for cell cycle withdrawal during neurogenesis

The process of cell differentiation is tightly linked to cell cycle withdrawal. Expansion of the *Sox2*-positive territory at stage 15 but not at stage 13 combined with loss of *N-tubulin* expression is interesting because it suggests that the neural progenitor cells which failed to differentiate in the absence of Brg1 remain as progenitor cells. However, as *Sox2* expansion

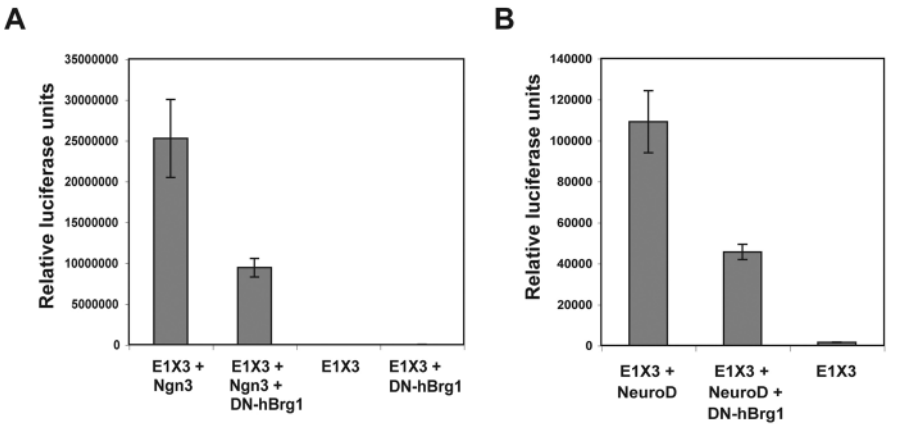

Fig. 8. Brg1 mediates transcriptional activities of Ngn3 and NeuroD2. (A) P19 cells were transfected with expression vectors for *Ngn3*, *E12* and E1X3 E-box reporter together with or without *DN-hBrg1* in triplicate and analyzed for luciferase activity. Data shown are a representative result from five independent experiments. Standard errors are indicated. (B) *NeuroD2* was used, otherwise same as A. Data shown are a representative result from two independent experiments.

was observed in not only posterior but also anterior neural plate where no *N-tubulin*-positive cells arise at this stage, expansion of neural progenitors cannot be explained solely by a failure of differentiation. Further study is required to understand this phenomenon.

Proneural genes not only determine neuronal fate but also promote cell cycle withdrawal, at least partly by inducing cyclin-dependent kinase (Cdk) inhibitors, and are therefore involved in coupling these two processes (Ohnuma et al., 2001). For example, *Ngn2* overexpression in the chick neural tube resulted both in premature neuronal differentiation of neuroepithelial cells and premature cell cycle exit (Mizuguchi et al., 2001; Novitch et al., 2001). Similarly, NeuroD associates with the promoter of $p21^{Cip1}$, a Cdk inhibitor, and can induce its expression upon RA treatment in neuroblastoma cells (Liu et al., 2004). Brg1 also appeared to associate with promoter regions of the Cip/Kip family of Cdk inhibitors and was required for their induction (Hendricks et al., 2004). The implication of both Brg1 and proneural bHLHs in cell cycle withdrawal and the physical and functional interaction of Brg1 with proneural bHLHs (our study) suggest Brg1 and bHLHs may cooperate on the same target genes to coordinate cell cycle withdrawal during neurogenesis. We have examined whether expression of $p27^{Xic1}$, which is the only currently available Cdk inhibitor in *Xenopus*, is reduced in the absence of Brg1 activity (Vernon et al., 2003). However, we did not observe a significant change in $p27^{Xic1}$ transcript levels in the neural plate (data not shown). It is unclear whether other Cdk inhibitors are involved or whether $p27^{Xic1}$ activity is post-transcriptionally regulated in *Xenopus*.

Brg1 mediates the transcriptional activities of Neurogenin and NeuroD

How does Brg1 mediate the activity of bHLH transcription factors during neurogenesis? The physical interaction we observed between proneural bHLHs and Brg1 raises the possibility that Brg1 (and the SWI/SNF complex) is recruited to Ngn and NeuroD target loci and remodels the chromatin structure to activate transcription of these target genes. Alternatively, chromatin remodeling might be a prerequisite for binding of proneural bHLHs to their target loci. In this case, the SWI/SNF complex binds target loci before bHLH factors and exposes bHLH target sites, permitting recruitment of bHLH proteins and transcriptional activation of target genes. Currently, only a few genes are known to be direct targets of Ngn and NeuroD, and chromatin remodeling at these loci during neurogenesis has not yet been analyzed. It will therefore be of interest to investigate the recruitment of the SWI/SNF complex to Ngn target genes such as *NeuroD1* (Huang et al., 2000) and to NeuroD target genes such as $p21^{Cip1}$ (Liu et al., 2004) and the recruitment order of SWI/SNF complex and proneural bHLHs to target promoters. In addition to interacting with Brg1, both Ngn and NeuroD are also known to interact with p300/CBP HATs and HAT activity has been shown to be necessary for neurogenesis (Koyano-Nakagawa et al., 1999; Mutoh et al., 1998). Thus, neurogenesis might be a process that requires extensive chromatin remodeling.

Parallels between neurogenesis and myogenesis

In many respects, molecular regulation of neurogenesis parallels that of myogenesis, with a cascade of myogenic bHLH transcription factors regulating myoblast cell fate determination, cell cycle withdrawal and upregulation of muscle-specific gene expression (McKinsey et al., 2001; Pownall et al., 2002). During myogenesis, myogenic regulatory factors (MRFs), such as MyoD, induce muscle-specific gene expression, and a subset of MRF target genes shows Brg1 dependent-expression. Brg1 mediates MyoD transactivation of some targets through recruitment of SWI/SNF chromatin remodeling activity, and Brg1 has also recently been shown to interact with MyoD and Mef2C during myogenesis (de la Serna et al., 2001a; Roy et al., 2002; Simone et al., 2004). Thus, our finding of an interaction between Brg1 and proneural bHLH proteins suggests that myogenic and proneural bHLH proteins may use similar mechanisms to activate their targets and induce differentiation. Intriguingly, recruitment of Brg1 and chromatin remodeling activity to MyoD targets has also recently been shown to be under additional regulatory controls. For example, activated p38 kinase recruits Brg1 to a subset of MyoD target genes (Simone et al., 2004). Transcriptional activation of an overlapping set of MyoD targets depends on cooperative interactions between the Pbx homeodomain protein and the C/H and helix III domains of MyoD that have been previously shown to recruit chromatin remodeling activity to MyoD target genes (Berkes et al., 2004; Gerber et al., 1997). Conversely, transactivation of other MyoD targets (notably those involved in cell cycle withdrawal) is independent of Brg1 activity (de la Serna et al., 2001b; Roy et al., 2002), and some MyoD target gene transactivation is not regulated by p38 kinase or Pbx-dependent mechanisms (Bergstrom et al., 2002; Berkes et al., 2004). Therefore, distinct subsets of MyoD targets have different requirements both for Brg1-dependent chromatin remodeling and for the additional regulatory controls that may impinge on that remodeling.

By comparison with myogenesis, we still know very little about how proneural bHLH proteins activate transcription of their targets in a chromatin context during neurogenesis. It remains to be seen whether, as for myogenesis, subsets of proneural bHLH targets show Brg1-dependent versus -independent transcriptional activation during neurogenesis. Additionally, although proneural bHLH proteins act as general regulators of neuronal cell fate, specification of distinct neuronal subtypes involves cooperative functioning of proneural bHLHs and homeodomain proteins at particular loci (Lee and Pfaff, 2003). It will therefore be of great interest to determine whether target gene subsets regulated by particular proneural bHLH and homeodomain protein combinations show differential Brg1 requirements in a manner potentially analogous to the MyoD-Pbx cooperativity, which was hypothesized to recruit chromatin remodeling activity to some loci during myogenesis (Berkes et al., 2004).

In summary, our results show that Brg1 is required for cell cycle arrest and for neuronal differentiation and can bind to and mediate transcriptional activities of Ngn and NeuroD. Although future work will enable a more complete understanding of the role of chromatin remodeling in regulating target gene activation by proneural bHLH factors, our findings here have defined Brg1 (and the SWI/SNF complex) as essential for neurogenesis.

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