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HeyL promotes neuronal differentiation of neural progenitor cells

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Abstract

Members of the Hes and Hey families of basic helix-loop-helix transcription factors are regarded as Notch target genes that generally inhibit neuronal differentiation of neural progenitor cells. We found that HeyL, contrary to the classic function of Hes and Hey factors, promotes neuronal differentiation of neural progenitor cells both in culture and in the embryonic brain *in vivo*. Further, null mutation of HeyL decreased the rate of neuronal differentiation of cultured neural progenitor cells. HeyL binds to and activates the promoter of the proneural gene neurogenin2, which is inhibited by other Hes and Hey family members, and HeyL is a weak inhibitor of the Hes1 promoter. HeyL is able to bind other Hes and Hey family members, but it cannot bind the Groucho/Tle1 transcriptional co-repressor which mediates the inhibitory effects of the Hes family of factors. Further, while HeyL expression is only weakly augmented by Notch signaling, we found that BMP signaling increases HeyL expression by neural progenitor cells. These observations suggest that HeyL promotes neuronal differentiation of neural progenitor cells by activating proneural genes and by inhibiting the actions of other Hes and Hey family members.

Keywords

Neurogenesis; neural stem cell; Hes; Hey; Notch

INTRODUCTION

Basic helix-loop-helix (bHLH) transcription factors are critical for maintenance, fate specification, and differentiation in neural stem cells. Proneural bHLH factors (*i.e.*, Neurogenin1 [Ngn1], Ngn2, and Mash1) promote differentiation, while Hes family bHLH transcription factors generally inhibit differentiation. Hes1 and Hes5 are Notch target genes expressed in the telencephalic ventricular zone (VZ) (Akazawa et al. 1992; Allen and Lobe 1999; Ohtsuka et al. 1999; Sasai et al. 1992); Hes1 and Hes5 homodimers function in a transcriptional repressor complex that includes the co-repressor Tle (mammalian ortholog of drosophila *Groucho*) (Dawson et al. 1995; Fisher et al. 1996; Grbavec and Stifani 1996;

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Paroush et al. 1994). This complex binds N-box sequences, repressing target promoters (*i.e.*, the proneural bHLH promoter, Mash1, and that of Hes1 itself) (Chen et al. 1997; Ishibashi et al. 1995; Van Doren et al. 1994). Groucho recruits Rpd3 (the ortholog of mammalian HDAC) also likely mediating Hes repression (Chen et al. 1999). Other proposed inhibitory mechanisms include formation of nonfunctional heterodimers (*e.g.*, with E12, required for proneural bHLH functioning) and of repressive heterodimers with proneural bHLH factors (Alifragis et al. 1997; Giagtzoglou et al. 2003; Hirata et al. 2000; Sasai et al. 1992).

In Hes1 and Hes5 single or double knock-out mice, these genes prevent neuronal lineage commitment by neural progenitors (Nakamura et al. 2000; Ohtsuka et al. 1999; Tomita et al. 1996). Normally, Hes expression decreases during differentiation and ectopic overexpression prevents neurogenesis (Castella et al. 1999; Ishibashi et al. 1994; Ohtsuka et al. 1999; Tomita et al. 1996). Atypically, Hes6 promotes neuronal differentiation, partly by inhibiting Hes1 repression (*e.g.*, regulating proneural neurogenin genes through positive feedback) (Bae et al. 2000; Gratton et al. 2003; Koyano-Nakagawa et al. 2000). Thus, most Hes proteins sustain a proliferative state, and inhibit differentiation.

The closely related Hey family of bHLH transcription factors (also termed Hesr, Herp, and Hrt) is also implicated in maintaining neural stem cells. Known mammalian Hey factors (Hey1, Hey2, and HeyL) share some Hes features: an N-terminal bHLH domain, and a conserved “orange” domain, C-terminal to the bHLH region. In Hes proteins however, a proline invariant in the Hey basic domain is replaced by glycine; and at the C terminus, Hes proteins contain a Groucho-interacting WRPW motif while Hey proteins contain a YXXW motif (Leimeister et al. 1999; Nakagawa et al. 1999; Steidl et al. 2000). Like Hes factors, Hey1 and Hey2 are induced by Notch signaling, and inhibit their own promoters (Nakagawa et al. 2000). Hey1 and Hey2 are expressed in the developing central nervous system (CNS), form Hes-Hey heterodimers, bind E- and N-boxes, and repress pro-neural gene transcription (Iso et al. 2002; Iso et al. 2001a; Iso et al. 2001b; Sakamoto et al. 2003; Steidl et al. 2000). Hey1 null mice are phenotypically normal (Fischer et al. 2004), like nulls for HeyL (Fischer et al. 2007), Hes6 (Koyano-Nakagawa et al. 2000) and several other Hes factors (Hirata et al. 2001; Ohtsuka et al. 1999), suggesting functional redundancy. Nevertheless, how most of these factors function in neural development was delineated through *in vivo* overexpression or *in vitro*, and little is known about HeyL function in the CNS.

HeyL is expressed in multiple neural structures (Gray et al. 2004; Leimeister et al. 1999; Leimeister et al. 2000), suggesting a role in neural development. Accordingly, in dorsal root ganglia, HeyL regulates the number of TrkC neurons (Mukhopadhyay et al. 2009). Furthermore, unlike other Hey genes, Notch signaling only weakly activates HeyL transcription, and HeyL is the weakest inhibitor of the Hey2 promoter (Nakagawa et al. 2000). Therefore HeyL likely functions differently than other Hey or Hes proteins. Accordingly, here we show HeyL promotes neuronal differentiation by activating proneural genes and inhibiting other Hes and Hey proteins.

MATERIALS AND METHODS

Preparation of expression plasmids

Plasmid expression constructs of Hes1, Hes5, Hey1 and HeyL that were used in Ngn2p luciferase reporter assays and co-immunoprecipitations were prepared by insertion of PCR fragments containing the coding region of each gene into pcDNA3.1-myc/His (Invitrogen) in frame with the myc-His tag or into pIRES-hrGFP-1a (Stratagene) in frame with the FLAG tag. Untagged constructs were also prepared for use in luciferase reporter assays and promoter binding assays. Retroviral expression vector pCLE-IRES2-eGFP was a kind gift from Dr. Jeffrey Nye based on the pCLE backbone (Gaiano et al. 1999). The coding regions

of Hey1 and HeyL were subcloned into these vectors. Additional information on oligonucleotide primers and cloning strategies used to generate each construct is available upon request.

Preparation of retrovirus and infection—Virus was produced by double transfection of GP293 cells with pCLE-IRES2-eGFP and pVSV-G constructs. Viral supernatant was collected for 3 days and 100-fold concentrated by ultracentrifugation at 25,000g for 1 hour 30 minutes. For viral infections, concentrated virus was added to the medium after neural progenitors were dissociated and re-plated in fresh medium (see below) containing 10 ng/ml FGF2 (basic FGF, BD) for 24 hours. At 48 hours after infection, neurospheres were dissociated and 10^4 cells were plated on PDL/laminin coated coverslips in fresh medium containing 0.5 ng/ml FGF2. Cells were allowed to differentiate for 5 days before immunohistochemical analysis.

Neural progenitor cell culture—Timed pregnant CD1 mice were sacrificed and the embryos were removed and processed for FGF-generated neurospheres as previously described (Gross et al. 1996). Briefly, the ganglionic eminence regions of embryonic day 13.5 or 17.5 mice were carefully dissected and dissociated by incubation with 0.05% trypsin/EDTA for 5 minutes followed by mechanical dissociation. After a wash, cells were spun down and re-suspended in DMEM/F12 (Invitrogen) supplemented with N2 (Invitrogen), B27 (Invitrogen), 2 μg/ml heparin, and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen). FGF2 was then added to the medium at a final concentration of 10 ng/ml. Cells were grown in 10 cm Petri dishes for 4 days before passage. Cells were used for retroviral infection, as described above, or were treated with no BMP4 or with 10 or 50 ng/ml of BMP4 (R&D) for the indicated times.

HeyL null neural progenitor cell culture—Frozen mouse embryos of C57BL6 background containing a targeted null mutation of the HeyL locus (Developed by Drs. Hiroki Kokubo and Randy L. Johnson) were obtained from Riken Institute (Japan) along with genotyping instructions and primer sequences provided by Dr. Yumiko Saga. The embryos were thawed and transferred to pseudo-pregnant female mice at the Northwestern University Transgenic Core Facility. The resulting heterozygous male and female mice were mated to generate homozygous null (HeyL −/−) as well as heterozygous (HeyL +/−) and wild-type (HeyL +/+) embryos. Neural progenitor cells were isolated from E13.5 HeyL −/−, HeyL +/−, and HeyL +/+ littermates and cultured as described in the previous section. After 2 passages, dissociated cells were plated on PDL/Laminin coated coverslips (10^4 cells/cm^2) and allowed to differentiate in fresh medium containing 0.5 ng/ml FGF2 and 10 ng/ml BMP4 for 7 days before immunohistochemical analysis.

HEK-293 and NIH-3T3 cell culture—HEK-293 and NIH-3T3 cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen) in humidified 5% CO_2. Cells were split on the day prior to transfection to achieve 50–60% or >90% confluencey at the time of transfection with FuGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen), respectively.

P19 cell culture and transfection—P19 embryonal carcinoma cells (ATCC) were maintained in MEM alpha medium (Invitrogen) supplemented with 7.5% newborn calf serum (Invitrogen), 2.5% fetal bovine serum (Invitrogen), and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen), in humidified 5% CO_2. Cells grown in suspension were transfected with pIRES-hrGFP-1a vector containing the *HeyL* gene or an empty vector. At 48 hours after transfection, cells were trypsinized and replated at 10^5 cells/

ml on poly-D-lysine coated coverslips. Three days later, the cells were fixed and stained with the neuronal marker β-tubulin III.

Promoter binding assay—Equal amounts of lysates of HEK-293 cells overexpressing HeyL or control plasmid were added to columns containing biotinylated Ngn2 promoter DNA fixed onto streptavidin coated magnetic microbeads following manufacturer's instructions (μMACS FactorFinder Kit, Miltenyi Biotec). After collecting the flow-through and 4 low-stringency and 4 high-stringency washes, the bound protein was washed off using warm SDS buffer and subjected to SDS-PAGE using rabbit IgG anti-HeyL (1:500, Chemicon). The various Ngn2 promoter fragments were obtained from mouse genomic DNA by performing PCR reactions using the following biotinylated primers: TGCTAAAGCCCCTCCCAAGA and TTAGGAGTTCACAGAGACGTCCCCTA (Fragment A, 574 bp), TGTTTCCCAAACAGTCATAGACAC and CTGCAGCCCAAGCTACC (Fragment B, 577 bp), CCTGGAGCGCCAACAG and GTGTCTGGCACACGACTCT (Fragment C, 311 bp), and GCAGCCACTGAACCACAAG and CGGCTCCAGATGTGTCG (Fragment D, 308 bp). The full-length 1.8 kb promoter (Ngn2p) was amplified using the first and last biotinylated primer in the above list. The DNA fragments were used in equivalent molar amounts.

Luciferase reporter plasmids and assays—A 1.8 kb genomic fragment containing the putative promoter region of the mouse *Ngn2* gene (Ngn2p) was cloned into the promoter-less luciferase reporter vector pGL3-basic (Promega). Cloning primers contained the following homology sequences: TGCTAAAGCCCCTCCCA (Forward), CGGCTCCAGATGTGTCG (Reverse). Hes1p-luciferase reporter plasmid was constructed by cloning the previously reported promoter region of the mouse *Hes1* gene into the pGL3-basic vector (Jarriault et al. 1995). For Ngn2p reporter assays, HEK-293 cells were plated in 12-well plates 24 hours before transfection. Routinely, a total of 0.5 μg/well of plasmid DNA was transfected using FuGENE 6 (Roche) according to the manufacturer's instruction. Each transfection included 0.1 μg reporter plasmid, 0.4 μg of expression plasmid(s) or empty vector, and 5 ng TK-Renilla luciferase (pRL-TK, Promega) as an internal control. For Hes1p reporter assays, NIH-3T3 cells were plated in 24-well plates 24 hours before transfection. In each well, 400 ng HeyL/control, 150 ng Hes1/control, 150 ng NICD/control, 75 ng Hes1p-pGL3, and 25 ng CMV-Renilla luciferase (pRL-CMV, a gift from Dr. Warren Tourtellotte) were transfected using Lipofectamine 2000. Cell lysates containing Ngn2p or Hes1p luciferase reporters were collected 36 or 48 hours after transfection, respectively, and luciferase activity was measured in a luminometer (Berthold). Each condition was tested as three independent measurements each composed of 2 (Ngn2p assay) or 6 (Hes1p assay) readings.

Co-immunoprecipitations and immunoblot analyses—Transfected cells were harvested and dissolved in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, and 0.5% sodium deoxycholate). To perform immunoprecipitation, manufacturer's instructions were generally followed (Roche). Briefly, lysates were pre-cleared by incubation with Protein G-agarose for 2 hours before incubation with primary antibody (anti-His, Invitrogen) for 12 hours with rotation at 4°C. After washing, bound protein was eluted and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and then probed with secondary antibodies anti-FLAG (1:1000, Sigma) or anti Tle1 (1:500, SantaCruz).

Ultrasound guided microinjection *in utero*—We generally followed the protocol outlined previously (Gaiano et al. 1999; Turnbull 1999; Wichterle et al. 2001). Briefly, timed Swiss-Webster mice were anesthetized and a sterile incision was made along the

ventral abdomen. A section of the uterus containing 1–2 embryos was pulled through a slit in a rubber membrane into a PBS-filled Petri dish where the embryos were visualized using a high frequency ultrasound probe. The 30° beveled tip (60 μm O.D.) of a pulled glass capillary was inserted into the telencephalic vesicle of each embryo *in utero* using ultrasound guidance. 1–2 μl of replication-deficient viral stock (~10^5 infectious particles) was injected through the glass capillary into the telencephalic vesicle of each embryo. The capillary was then removed, the uterus tucked back inside the mother, the abdominal wall sutured, and the skin clipped closed allowing for recovery of mother and natural growth of embryos.

Immunohistochemistry—For fluorescent immunostaining, embryonic brains were harvested, pre-fixed with 4% paraformaldehyde, cryoprotected in 15% sucrose/PBS, snap-frozen in dry ice/isopentane slurry, and sectioned using a cryostat. Cells on coverslips or brain sections were fixed with 4% paraformaldehyde, washed in phosphate-buffered saline (PBS), blocked for 1hr in goat or donkey serum, and then incubated overnight in primary antibodies, alone or in combination, at 4°C. The primary antibodies used were mouse IgG_{2b}-anti- β-tubulin III (1:1000, Sigma), mouse IgG_1-anti-GFAP (1:1000, Invitrogen), mouse O4 antibody (1:40, Chemicon), and rabbit IgG-anti-GFP (1:750, Invitrogen). Following washing in PBS 3×5 minutes at room temperature, goat secondary antibodies Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647 (1:1000, Invitrogen) were applied alone or in combination. After final washing, coverslips were mounted in anti-fade reagent (Vector Laboratories) and photographed using a Zeiss Axiovert fluorescence microscope. Fluorescent images were processed with Adobe Photoshop, and cells were counted based on the nuclei (stained with Hoechst) of GFP^+ cells.

Statistical analysis—For comparisons of two independent variables (e.g. HeyL vs Control), unpaired Student's t-test (two-tailed) was used to determine statistical significance. For comparisons of more than two independent variables (e.g. Control vs Hey1 vs HeyL, or HeyL +/+ vs HeyL +/− vs HeyL −/−), one way ANOVA with Tukey's *post hoc* test was used.

Northern blot analysis—Total RNA (15 μg) from E12.5 neural progenitor cells treated for 6 hours with low (10 ng/ml) or high (50 ng/ml) concentrations of BMP4 (R&D) or with buffer were extracted by acidic guanidinium phenol. RNA was separated on a 1.2% agarose formaldehyde gel and transferred to a nylon membrane (NEN). The full length HeyL and Actin cDNAs were used as probes.

RESULTS

HeyL promotes neuronal differentiation of cultured neural progenitor cells

To examine the role of HeyL on neural lineage commitment, we first studied the effects of overexpression of HeyL in cultured P19 mouse embryonal carcinoma cells. Pluripotent P19 cells have the capacity to generate all neural cell types (neurons, astrocytes, and oligodendrocytes) and other cells upon proper induction. Few cells transfected with a control plasmid differentiated into neurons as indicated by β-tubulin III staining or morphology. By contrast, transfection with the HeyL expression plasmid resulted in substantial neuronal differentiation after 5 days (Fig. 1A). To verify and quantify this effect in neural progenitor cells, we infected cultured murine E13.5 and E17.5 progenitor cells with either a control virus (derived from the pCLE retroviral vector (Gaiano et al. 1999;Gaiano et al. 2000) containing an IRES2-eGFP sequence, or with a pCLE-Hey1 or pCLE-HeyL virus. The cells were then plated and allowed to differentiate and were examined after 5 days for expression of the neuronal marker β-tubulin III, the astroglial marker GFAP, and the oligodendroglial

marker that binds O4 antibody. There were no apparent differences in cell survival as indicated by live/dead staining, and total cell counts did not differ significantly among the groups. Overexpression of HeyL significantly increased neuronal differentiation of E13.5 neural progenitor cells (N=12, Fig. 1B) while astroglial differentiation had a non-significant decrease. In E17.5 HeyL-overexpressing neural progenitor cells, the increase in neuronal and decrease in astroglial differentiation were both significant (N=12, Fig. 1C). Conversely, overexpression of Hey1 promoted astrocyte differentiation and inhibited neuronal differentiation by both E13.5 and E17.5 progenitor cells, although inhibition at E13.5 was not significant. In this assay, neither Hey1 nor HeyL influenced oligodendroglial lineage commitment of E17.5 progenitors, and the number of oligodendrocytes generated from E13.5 progenitors was negligible.

HeyL promotes neuronal differentiation *in vivo*

Our cell culture observations indicated that HeyL promotes neuronal differentiation *in vitro*. To determine whether HeyL exerts similar effects *in vivo*, we overexpressed HeyL in the developing mouse telencephalon by microinjecting pCLE-HeyL or control retrovirus (each containing an IRES2-eGFP sequence) into the telencephalic vesicle of E10.5 embryos using ultrasound guidance. After four days of normal growth and development of the embryo *in utero*, the brains were removed and the percentage of GFP^+ cells that expressed β-tubulin III was determined in thin coronal sections (Fig. 2A). HeyL significantly increased the proportion of neurons (GFP^+, β-tubulin III^+) generated within the telencephalic wall as compared to control (Fig. 2B). In each condition, a total of 13 different microscopic fields, each from different embryos were used for cell counting, and to minimize variability, the images were collected from similar regions of the dorsolateral telencephalon where approximately half of the thickness of the telencephalic wall was β-tubulin III^+. The cells carrying the control virus tended to yield larger colonies of progeny compared to the cells overexpressing HeyL (Fig. 2A), although there was substantial variability in apparent colony size in both groups.

HeyL null neural progenitor cells have a reduced rate of neuronal differentiation in culture

Previous reports indicate that HeyL null mice, similarly to Hey1, Hes6, and several other Hes family null mutants, have a grossly normal phenotype. However, our laboratory recently demonstrated that HeyL null mice form fewer TrkC neurons in their dorsal root ganglia and Hey1 null mice have the opposite phenotype (Mukhopadhyay et al. 2009). Immunohistochemical staining of the brain at different embryonic and postnatal ages using β-tubulin III and GFAP as markers of neurons and astrocytes on a gross level and calbindin, calretinin, and parvalbumin as interneuron markers did not show any appreciable differences between HeyL null and wild-type brains (data not shown). Nevertheless, our overexpression studies using telencephalic progenitor cells strongly suggested a role for HeyL in neuronal differentiation. To more specifically define this role, we examined HeyL null neural progenitor cells in culture. E13.5 HeyL +/+, HeyL +/−, and HeyL −/− neural progenitor cells were plated on PDL/laminin coated coverslips and differentiated for 7 days before staining for the neuronal marker β-tubulin III and astroglial marker GFAP (N=8, Fig. 3A–C). Hoechst was used as the nuclear counterstain, and percentages of neurons and astrocytes were determined. Compared to wild-type neural progenitors, HeyL −/− neural progenitors produced a significantly smaller proportion of neurons and larger proportion of astrocytes (Fig. 3D). HeyL +/− progenitors produced an intermediate phenotype although the differences were not statistically significant. Numbers of oligodendrocytes formed under all conditions were negligible, and live-dead staining indicated no apparent differences in cell survival between the three groups.

HeyL can bind other Hey and Hes proteins but not Tle1

Hes and Hey transcription factors interact with each other or with other proteins through their bHLH domains, Orange domains, or tetrapeptide motifs. The bHLH and Orange domains of Hes and Hey genes are highly homologous to one another. The C-terminal WRPW motif, which is conserved among the *Hairy* and *E(spl)* subfamilies in vertebrates and flies (Davis and Turner 2001), is involved in the recruitment of the Groucho/Tle corepressor and is required for the transcriptional repression activity (Fisher et al. 1996; Grbavec and Stifani 1996). However, in HeyL the WRPW domain is replaced by YHSW which can result in functional differences.

To examine the nature of protein-protein interactions for HeyL, we overexpressed Flag-tagged HeyL along with each of His-tagged Hes1, Hes5, Hey1, Hey2, HeyL, or the control His vector (pcDNA3.1) in HEK-293 cells and performed a series of co-immunoprecipitation experiments. HeyL protein interacted with Hes1 and Hes5 as well as all three Hey proteins (Fig. 4A). We also overexpressed Flag-tagged Hes1 along with each of His-tagged HeyL, Hes1, or control His vector (pcDNA3.1) in HEK-293 cells and performed additional co-immunoprecipitation experiments. The results confirmed that Hes1 interacts with HeyL as well as Hes1, as previously reported (Fig. 4B). Furthermore, when we used HeyL as bait in a bacterial two-hybrid screen, we also pulled down Hes5 from a mouse embryo cDNA library (data not shown). These experiments suggest that HeyL can complex with both Hes and Hey proteins. We also examined possible interactions of Hey proteins with Tle1 (mammalian ortholog of Groucho) by overexpressing His-tagged Hey1, HeyL, Hes1 and control (His) vector (pcDNA3.1) in HEK-293 cells and performing co-immunoprecipitation experiments. We found that Hey1 as well as Hes1 interacted with Tle1 but that HeyL was unable to complex with Tle1 (Fig. 4C).

HeyL binds specific regions of the Ngn2 promoter

As mentioned previously, members of the Hes and Hey families can repress transcription from promoters of proneural genes and their own promoters. As a first step to investigate the regulatory role of HeyL on proneural genes, we investigated whether HeyL can bind the promoter of Ngn2. We amplified a 1.8 kb genomic DNA fragment containing the immediate 1.5 kb Ngn2 promoter sequence and 0.3 kb exon1 sequence using biotinylated primers. This DNA sequence (Ngn2p) was fixed onto streptavidin coated magnetic microbeads in a column surrounded by a magnet. After adding the lysate of cells overexpressing the HeyL protein to the column, the flow through was collected. The column was then washed 4 times using a low stringency wash buffer and 4 times using a high stringency wash buffer, which were also collected for SDS-PAGE analysis. Only a final wash using a warm SDS buffer was able to remove the bound HeyL protein from the column presumably by denaturing the protein and disrupting protein-DNA interactions (Fig. 5B). When no DNA was fixed onto the microbeads in the column (Control) most of the HeyL protein flowed through the column and the rest was washed off in the first low-stringency wash, as was the case for the unknown protein in the lysate (non-specific band) when added to Ngn2p or Control column (Fig 8B).

To determine whether HeyL protein bound specific regions of the Ngn2 promoter, we divided the 1.8 kb sequence into smaller A, B, C, and D fragments (Fig. 5A) and subjected them to the same binding assay using magnetic microbeads. The warm SDS wash from each of these fragments was analyzed using SDS-PAGE (Fig. 5C). The results indicate that fragments A and B bound the HeyL protein most strongly. Fragment C demonstrated weak binding, and fragment D showed no binding as all of the HeyL protein was washed off in earlier washes. HeyL, therefore, binds to at least three non-overlapping sites on the Ngn2 promoter.

HeyL activates the Ngn2 promoter and is a weak inhibitor of the Hes1 promoter

To determine whether the binding of HeyL to Ngn2 promoter has any role in regulating the transcription from this promoter, the full length Ngn2p sequence was cloned into the pGL3 firefly luciferase reporter vector (Promega). This promoter reporter construct was transfected into HEK-293 cells along with various Hes or Hey expression constructs or pcDNA3.1 (Control), and a dual luciferase reporter assay was performed after 36 hours. HeyL significantly activated transcription from the Ngn2 promoter, whereas Hes1, Hes5, and Hey1 each significantly repressed transcription from this promoter. Further, co-expression of HeyL with Hes1 or Hes5 prevented the Hes mediated transcriptional repression of the Ngn2 promoter (N=10, Fig. 6A).

Hey and Hes factors are known to inhibit each other's promoters. HeyL has been shown to be a weaker inhibitor, however, for example in the case of the Hey2 promoter (Nakagawa et al. 2000). We sought to determine the regulatory effect of HeyL on the Hes1 promoter by performing luciferase reporter assays. HeyL and/or Hes1 expression constructs were transfected into NIH-3T3 cells along with a Hes1 promoter (Hes1p) reporter construct. Where appropriate, an equal amount of empty vector was used as control to maintain the total amount of transfected DNA constant. Also, to more closely replicate the natural environment of neural progenitor cells where Notch signaling continuously activates the Hes1 promoter, an expression construct for the constitutively active Notch intracellular domain (NICD) was cotransfected. Dual luciferase assay after 48 hours indicated that NICD, as expected, strongly activated the Hes1 promoter (Fig. 6B). In addition, HeyL expression weakly inhibited the Hes1 promoter as compared to Hes1 itself, and further inhibition was achieved by combining Hes1 and HeyL (N=10, Fig. 6B).

HeyL expression is induced by BMP signaling

Although Hes and Hey genes are typically regarded as Notch target genes, HeyL is only very weakly activated by Notch signaling as compared to other Hey or Hes family members (Nakagawa et al. 2000). Given that Notch signaling is not a strong inducer of HeyL expression, and having found that HeyL promotes neuronal differentiation, we hypothesized that HeyL is regulated by other signals that induce neuronal differentiation in early development. Since BMP signaling fits this profile (Li et al. 1998; Mabie et al. 1999), we examined the effects of BMP4 on HeyL expression. Cultured E12.5 neural progenitor cells were treated with BMP4 or vehicle and HeyL expression was examined by Northern (Fig. 7A) and Western (Fig. 7B) blot analyses. Treatment of cultured neural progenitor cells with BMP4 resulted in significant increases in both HeyL mRNA and HeyL protein.

DISCUSSION

Neural progenitors exit the cell cycle and commit to lineages, at least in part, by the balanced regulation by positive and negative bHLH transcription factors (Ross et al. 2003). Since knockout models of a number of Hes and Hey factors lack a discernible phenotype (Fischer et al. 2004; Fischer et al. 2007; Hirata et al. 2001; Koyano-Nakagawa et al. 2000; Ohtsuka et al. 1999), some of these factors likely exert redundant functions in maintaining this balance. Consequently, to exaggerate the effects of individual factors and elucidate their functions, one must resort to *in vivo* overexpression or culture experiments, as with Hey1 and Hes6 (Bae et al. 2000; Koyano-Nakagawa et al. 2000; Sakamoto et al. 2003).

In the case of HeyL knockout mice, our laboratory only recently elucidated a rather subtle phenotype in dorsal root ganglia, which show 19% reduction in TrkC neurons but no significant changes in TrkA or TrkB neurons (Mukhopadhyay et al. 2009). In the brain of HeyL knockout mice, no gross changes were observed in the number of neurons or

astrocytes or in specific interneuron subtypes marked by calbindin, calretinin, or parvalbumin (data not shown). One could speculate, among other reasons, that HeyL knockout mice lack a brain phenotype because their brains express little HeyL (compared to other bHLH factors) or have a higher degree of redundancy of bHLH function. Nevertheless, to delineate HeyL function, we designed a number of *in vitro* and *in vivo* overexpression and mechanistic studies, similar to those designed to study Hey1 and Hes6 (Bae et al. 2000; Koyano-Nakagawa et al. 2000; Sakamoto et al. 2003).

Overexpressing HeyL in pluripotent P19 embryonal carcinoma cells caused significant neuronal differentiation, similar to the effects of other proneural and neuron-differentiation bHLH transcription factors such as Ngn1, Mash1, and NeuroD2 (Farah et al. 2000). In cultured neural progenitor cells, which have a more restricted fate potential, overexpressing HeyL significantly increased neuronal differentiation and reduced astroglial lineage commitment. This effect was evident both in early (E13.5) and later (E17.5) embryonic progenitor cells, respectively cultured in the presence of FGF2 and EGF. In contrast, overexpressing Hey1 promoted glial differentiation while reducing the rate of neuronal lineage commitment. The opposing functions of HeyL and Hey1 are also revealed in the dorsal root ganglia of HeyL and Hey1 knockout mice; the former has fewer TrkC neurons while the latter has more. Similarly, HeyL and Hey2 exert different effects in retinal explant cultures, where overexpression of Hey2 but HeyL promotes glial differentiation (Satow et al. 2001). These observations strongly support a unique role for HeyL among Hey factors: *in vitro*, HeyL promotes neuronal differentiation; *in vivo* HeyL promotes differentiation into a specific neuronal subtype in the dorsal root ganglia. To verify the proneural activity of HeyL in the embryonic brain *in vivo*, HeyL was virally delivered to neural progenitor cells in the telencephalic VZ of mouse embryos. *In vivo* overexpression of HeyL, similar to *in vitro* overexpression, increased significantly the proportion of cells differentiating into neurons.

Complimentary culture experiments using HeyL-null neural progenitor cells indicated that loss of HeyL decreased the rate of neuronal differentiation. These gain- and loss-of-function studies, performed both *in vitro* and *in vivo*, clearly demonstrate a role for HeyL in promoting neuronal differentiation. This is the converse of the function of the closely related family members Hey1 and Hey2, as well as of most of Hes family members, which inhibit neuronal differentiation. The phenotype of HeyL null neural progenitors in culture but not in the brains of HeyL knockout mice naturally demands an explanation, but we can only speculate about the possible reasons. Conceivably, artificial culture conditions exaggerate the effects of HeyL because an imbalance of positively and negatively regulating bHLH factors (*e.g.*, respectively, Hes6, Ngn1, Ngn2, and Mash1, or, Hes1, Hes5, Hey1, and Hey2). It is also possible that in culture, expression of HeyL is artificially high, exaggerating effects like those when neural progenitor cells are exposed to serum or BMP (both typical culture additives in differentiation assays). In fact, our experiments do indicate that when neural progenitor cells are treated with BMP4, HeyL expression rises. Thus, although the artificial conditions *in vitro* might magnify the effects on cultured cells, the direction of effects are in general agreement with our overall finding that HeyL promotes neuronal differentiation. Additionally, although we looked at a number of neuronal subtypes in the HeyL knockout brain, a more subtle brain phenotype might be revealed via more specific neuronal subtype markers. Alternatively, analysis of mice with double mutations in HeyL and another Hey or Hes factor (such as Hes6, a proneural factor) may reveal a phenotype in a matter analogous to other Hes family double mutants, such as Hes1 and Hes5 (Ohtsuka et al. 1999).

Most members Hes and Hey proteins are direct targets of Notch signaling, which inhibits neurogenesis. However, HeyL is only weakly activated by Notch in comparison to other family members (Nakagawa et al. 2000), suggesting it may instead be preferentially targeted by other signals that induce neuronal differentiation. In fact, we found HeyL expression

increased significantly in cultured neural progenitor cells in response to BMP4, a factor known to induce neuronal differentiation by VZ progenitor cells (Li et al. 1998). Upon induction, HeyL can weakly repress transcription from the Hes1 promoter (beyond the self-regulatory effect of Hes1 itself) and weaken downstream Notch signaling. Also, our co-immunoprecipitation assays showed that HeyL can bind inhibitory Hes and Hey proteins, but cannot recruit the co-repressor, Tle1. Although using tagged, overexpressed proteins for these co-immunoprecipitation assays is less than ideal, we lack sufficiently specific and sensitive antibodies against different Hes and Hey factors, precluding us from performing these assays on endogenous proteins. Nevertheless, our results suggest that HeyL can, in effect, act as a dominant negative factor for inhibitory Hes and Hey transcription factors, by forming complexes with these factors that cannot recruit the required co-repressor.

Mechanistically, Hes1 maintains neural progenitor cells in the cell cycle, at least in part, by inhibiting the expression and action of proneural genes such as Ngn2. Thus, one can postulate that HeyL activation of Ngn2 promoter can result from both inhibition of Hes1 expression and interference with Hes1 function as a dominant negative factor. At the same time, it is not clear why HeyL can act as an activator for one promoter (Ngn2) and an inhibitor for another (Hes1), albeit a weaker inhibitor than other family members. Perhaps some of these effects are direct while others indirect. Alternatively, these effects might depend on the promoter context and the how other cofactors in the transcriptional regulatory complex are balanced, a balance that changes with the regulatory milieu of the cell. Currently, we do not have any evidence that HeyL recruits a direct activator to the promoter complex, but more experiments should determine how HeyL activates the Ngn2 or inhibits the Hes1 promoters. Interpretation of our Ngn2 promoter-binding assay is similarly limited because HeyL is overexpressed, and the antibodies available are not specific or sensitive enough to detect endogenous protein in this assay. Nonetheless, we believe the assay has sufficient power to establish that HeyL can bind Ngn2 promoter sequences and to distinguish between fragments that do not bind HeyL versus those that bind weakly or strongly. Moreover, our studies showing binding and activation of Ngn2 promoter by HeyL are naturally limited by the artificial conditions inherent in any *in vitro* experiment. Further studies are needed to determine specific consensus binding sites for HeyL, and whether HeyL activates Ngn2 or other proneural genes *in vivo*.

It is unlikely that Hes and Hey proteins repress transcription only by recruiting Groucho orthologs (*e.g.*, Tle1) to the tetrapeptide motif of transcription factors (Chin et al. 2000; Sun et al. 2001). For example, Hes1 requires the orange domain, but not the WRPW motif, to repress its own promoter and that of p21Cip1 (Castella et al. 2000). The difference in how Hey1 and HeyL bind Tle1 could explain the divergent effects in neural progenitors; but in our reporter assays, Hey1 and HeyL showed no significant changes when the YRPW and YHSW domains were deleted or swapped (data not shown). In zebrafish, however, injecting hey2 mRNA without the tetrapeptide motif failed to mimic wild-type Hey2 mRNA in rescuing the gridlock phenotype (Zhong et al. 2000), indicating that this motif is required for normal functioning of Hey2. The WRPW motif is also present in the proneural factor, Hes6, and although it is not required for Hes6 to promote neurogenesis (Bae et al. 2000; Koyano-Nakagawa et al. 2000). This motif can enhance the neurogenic effects of Hes6 (Gratton et al. 2003); and it mediates the effects of Hes6 on myogenesis (Cossins et al. 2002). Although Hes6 may enhance neuronal differentiation by competing with Hes1 for groucho/Tle binding (Gratton et al. 2003), this mechanism is unlikely to play a role in the case of HeyL, since we could not detect HeyL binding to groucho/Tle1. More experiments should better delineate the structural reasons for the divergent function of HeyL, and any possible roles the YHSW motif in HeyL may play in its divergent function. Additionally, Hes6 also promotes neuronal differentiation by enhancing proteolytic degradation of Hes1 (Gratton et al. 2003). Since HeyL can bind Hes1, HeyL might function, in part, through a similar mechanism. Overall,

our studies indicate HeyL is a unique member of the Hey family of transcription factors, which promotes neuronal differentiation through complex mechanisms unlike those of most other Hes and Hey factors.

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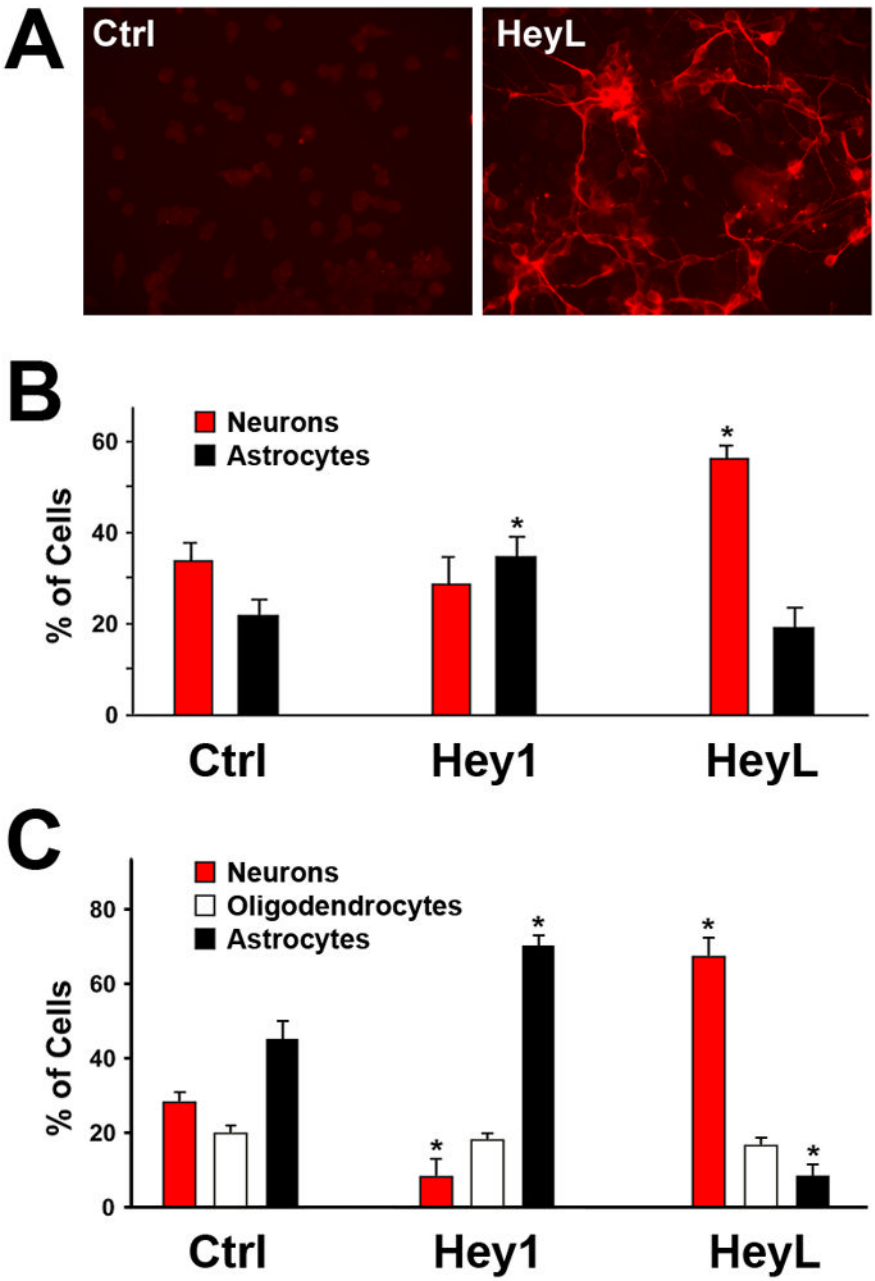

Fig. 1. HeyL promotes neuronal differentiation by cultured neural progenitor cells

(A) A significant number of P19 cells transfected with HeyL expression construct differentiated into neurons after 5 days as indicated by the neuronal marker β-tubulin III staining. There was no significant difference in the amount of cell death between the two groups. (B, C) Neural progenitor cells harvested from E13.5 (B) or E17.5 (C) mouse embryos were infected with pCLE virus containing an empty control cassette (Ctrl) or Hey1 or HeyL expression cassettes. After 5 days of differentiation, cells were subjected to immunohistochemistry. Hey1 overexpression promoted astroglial and inhibited neuronal differentiation. By contrast, HeyL overexpression promoted neuronal and inhibited astroglial differentiation. The numbers of oligodendrocytes were negligible at E13.5, and at E17.5

there was no effect on oligodendroglial differentiation (* $P<0.05$ vs control). Error bars represent s.d.

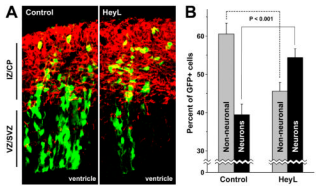

Fig. 2. HeyL promotes neuronal differentiation *in vivo*

(A) Retrovirus carrying HeyL-IRES2-eGFP or only IRES2-eGFP (Control) sequence was microinjected into the telencephalic vesicles of E10.5 mouse embryos. Embryonic brains were harvested at E14.5 and sections were immunostained for GFP (green) and β-tubulin III (red). (B) The quantification of the proportion of neuronal and non-neuronal progeny indicates that HeyL overexpression results in a significantly higher proportion of neurons. Error bars represent s.e.m. CP: cortical plate, IZ: intermediate zone, SVZ: subventricular zone, VZ: ventricular zone.

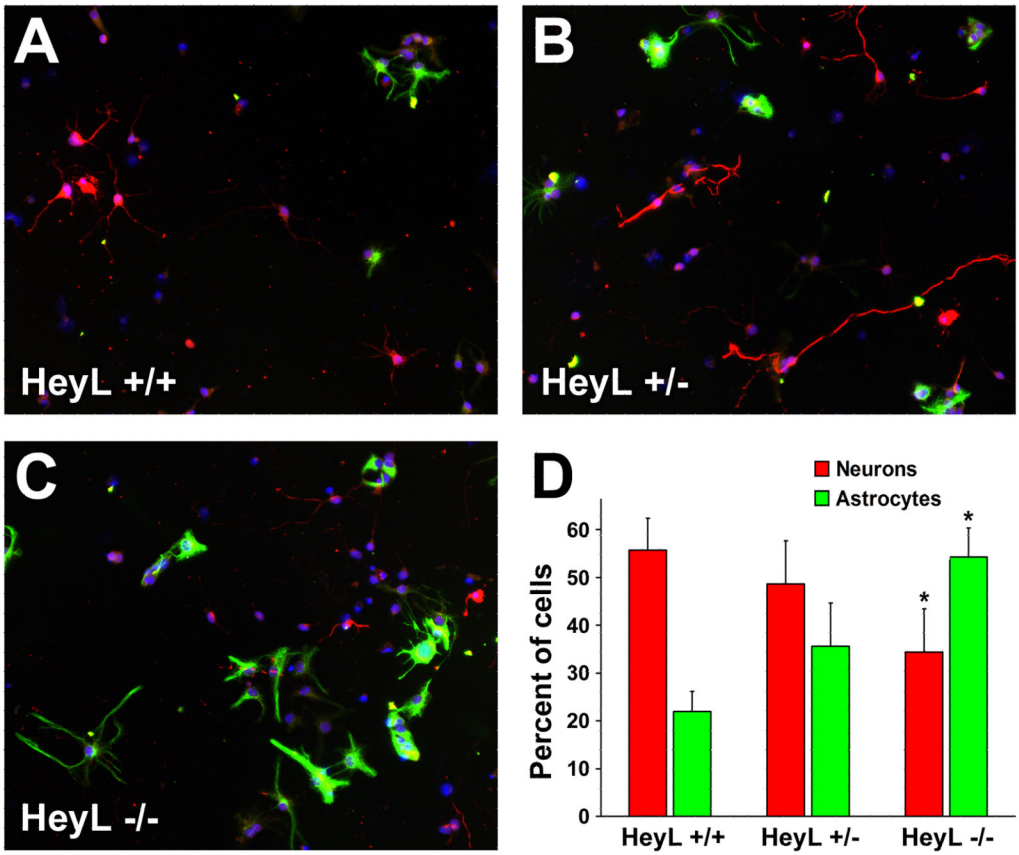

Fig. 3. Loss of HeyL in neural progenitor cells reduces the rate of neuronal differentiation in culture

(A–C) Immunohistochemistry of cells obtained from differentiation of HeyL +/+, HeyL +/−, and HeyL −/−neural progenitor cells over 7 days. Red and green fluorescence indicate β-tubulin III (neuron) and GFAP (astrocyte) staining, respectively. Blue fluorescence indicates Hoechst nuclear counterstain. (D) Quantification of the proportions of differentiated cells illustrates that, compared to wild-type neural progenitors, HeyL −/− progenitors generated a significantly smaller proportion of neurons and larger proportion of astrocytes. HeyL +/− progenitor cells generated an intermediate phenotype (* $P<0.05$ vs wild-type control). Error bars represent s.d.

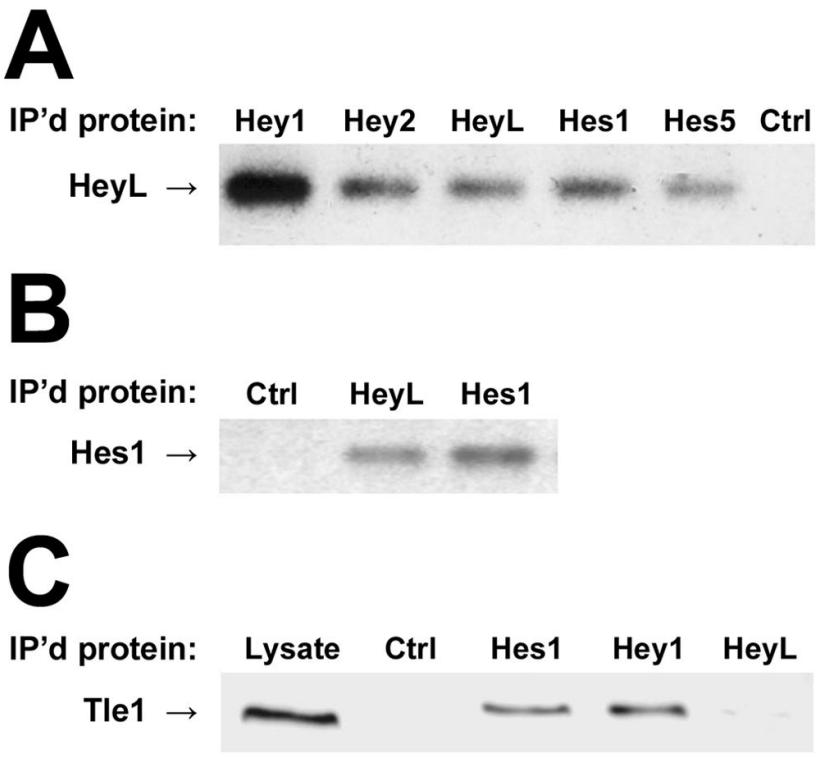

Fig. 4. HeyL associates with other Hes and Hey proteins but not Tle1

(A, B) His-tagged Hes or Hey proteins were immunoprecipitated (IP'd) with anti-His antibody. The eluted protein mixtures were separated by SDS-PAGE followed by Western blot analysis with anti-Flag antibody probing for the presence of Flag-tagged HeyL (A) or Hes1 (B). Note that HeyL can associate with Hes and Hey proteins. (C) His-tagged Hes or Hey proteins were immunoprecipitated (IP'd) with anti-His antibody. The eluted protein mixtures or the whole cell lysate (Lysate) were separated by SDS-PAGE followed by Western blot analysis with anti-Tle1 antibody. Note that Hes1 and Hey1 strongly interact with Tle1, but HeyL only very poorly interacts with Tle1. "Ctrl" lanes in (A–C) represent anti-His immunoprecipitation of contents of cells transfected with the empty His vector. Equal amounts of total protein were used for all lanes in (A–C).

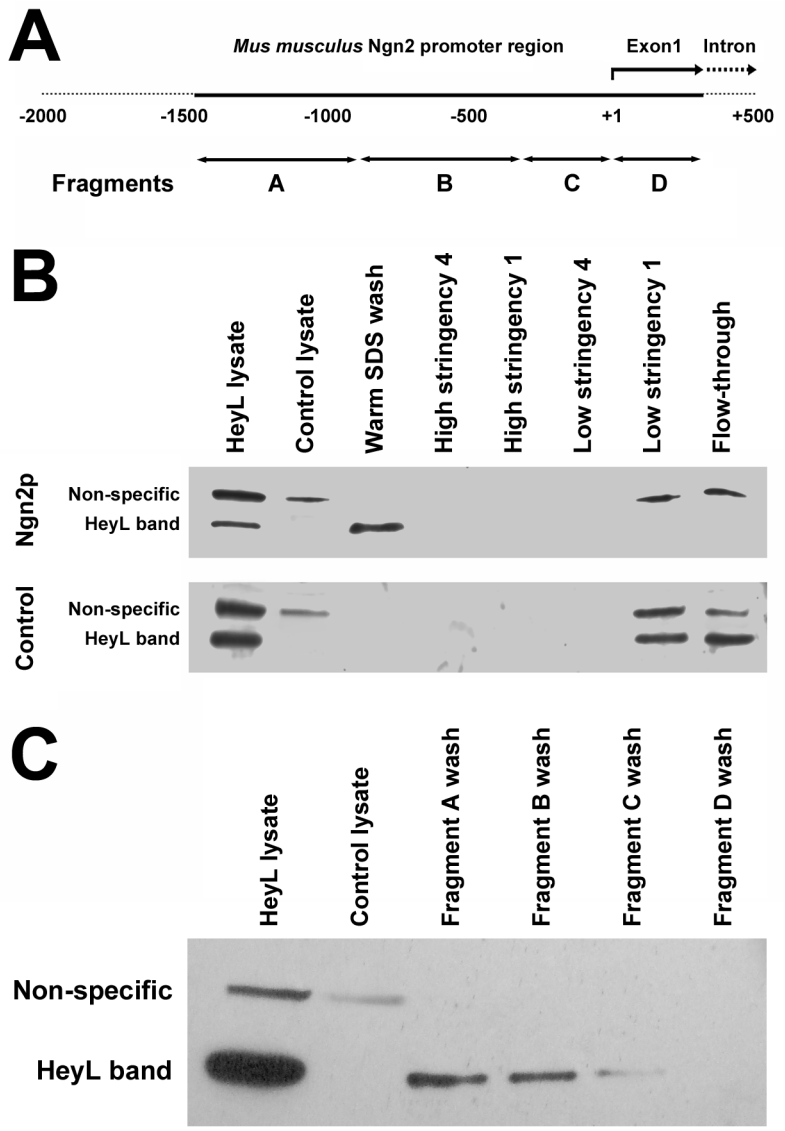

Fig. 5. HeyL binds specific regions of the Ngn2 promoter

(A) Diagrammatic depiction of murine Ngn2 genomic sequence. First base of exon1 is marked as +1. The solid bar represents the full-length Ngn2 promoter sequence (Ngn2p) used for binding assay in (B), and double-arrows represent smaller fragments A, B, C, and D used for binding assay in (C). (B) When no DNA was fixed onto the column (Control), HeyL protein flowed through or was washed off with the first low-stringency wash. When Ngn2p was fixed onto the column, HeyL was retained in the column through all the washes until the denaturing warm SDS wash. HeyL and Control lysates are lysates of cells transfected with HeyL plasmid and empty vector, respectively. (C) Fragments A, B, C, and D were subjected to the same assay as in (B), and the warm SDS wash from each fragment was subjected to SDS-PAGE. Fragments A and B showed strongest binding to HeyL. Fragments C and D showed weaker or no binding.

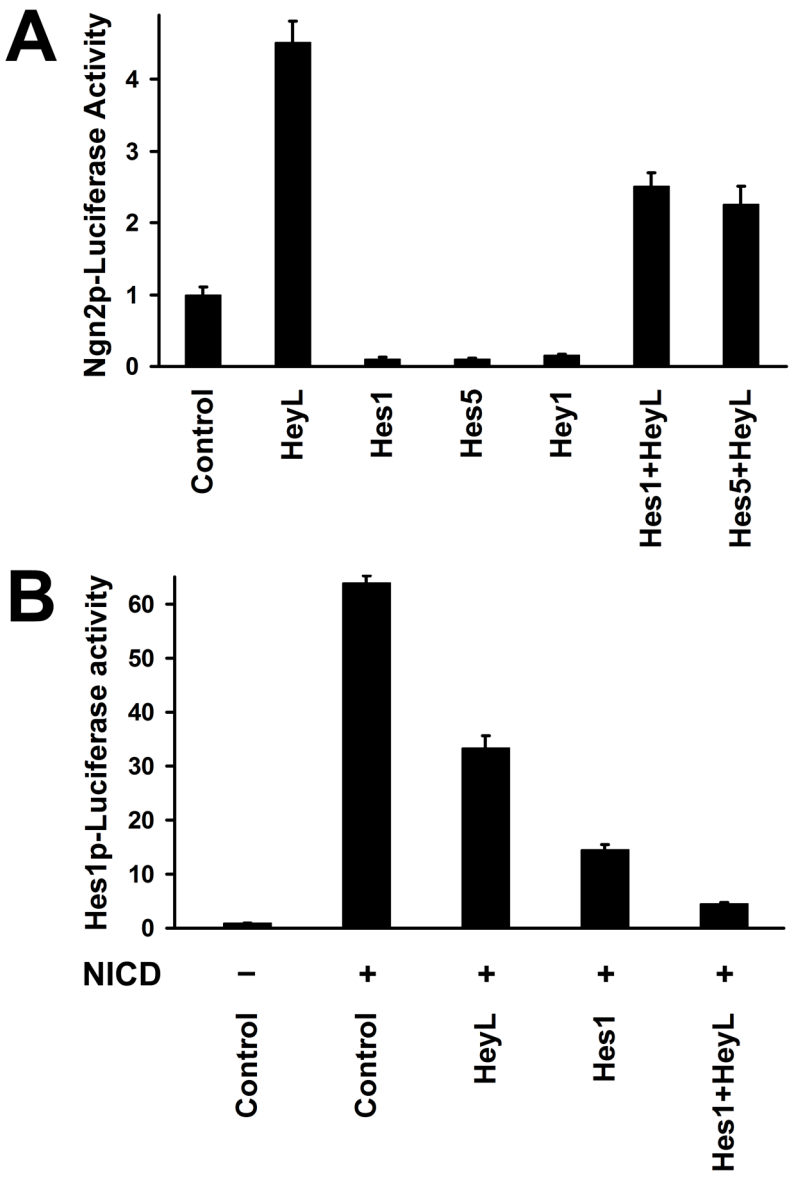

Fig. 6. HeyL activates the Ngn2 promoter and is a weak inhibitor of the Hes1 promoter

(A) HeyL significantly activates the transcription of the luciferase reporter under the control of the Ngn2 promoter, and is capable of reversing Hes1 and Hes5 mediated transcriptional repression. Values are normalized to Control. Error bars represent s.d. (B) Co-transfection of NICD is indicated by a + sign under each bar. NICD significantly activates and Hes1 significantly inhibits the transcription of the luciferase reporter under the control of the Hes1 promoter. HeyL is a weaker inhibitor compared to Hes1, and further inhibition is achieved by combining Hes1 and HeyL. Values are normalized to the no-NICD Control. Error bars represent s.d.

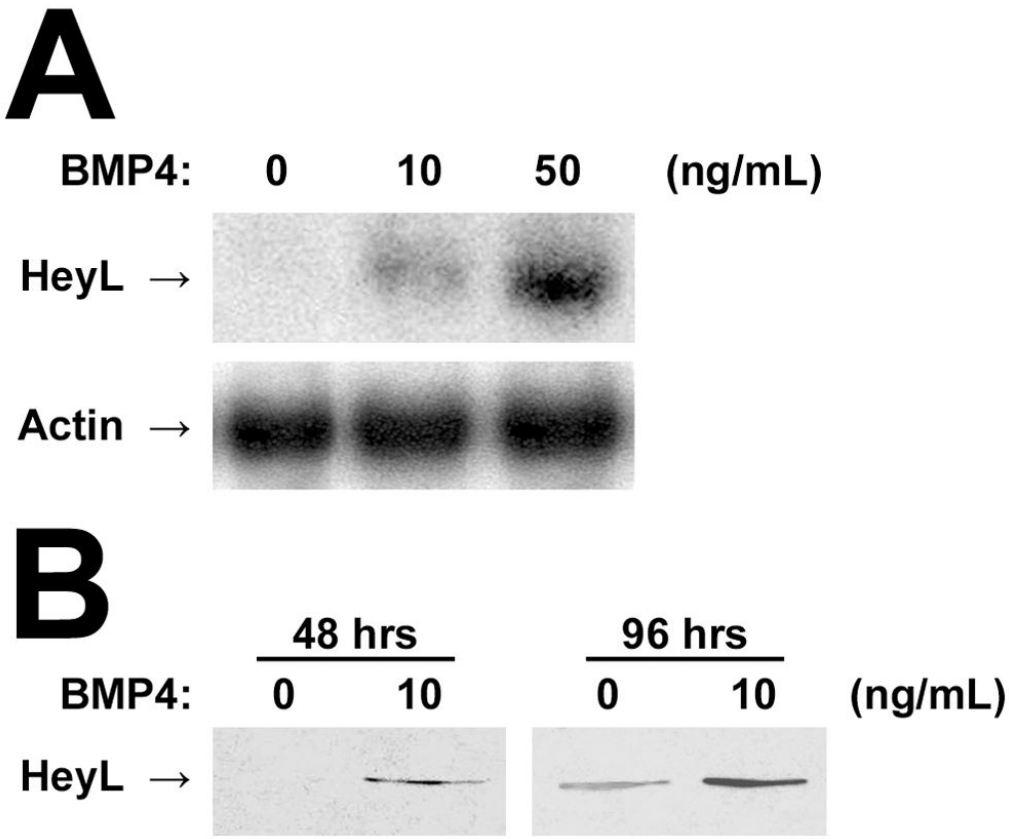

Fig. 7. HeyL expression is induced by BMP signaling

(A) Northern blot analysis of HeyL in cultured E12.5 neural progenitors following 6 hours of exposure to BMP4. Actin hybridization is provided as the loading control. (B) Western blot analysis of HeyL in cultured neural progenitors following 48 or 96 hours of exposure to BMP4. Note that the expression of both HeyL RNA and protein is increased as a result of treatment with BMP4.