



Published in final edited form as:

Dev Dyn. 2006 August ; 235(8): 2260–2265. doi:10.1002/dvdy.20858.

Barhl1 Is Required for Maintenance of A Large Population of Neurons in the Zonal Layer of the Superior Colliculus

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Abstract

The mammalian superior colliculus of the midbrain is a brainstem center that integrates sensorimotor signals involved in the control of orienting behaviors. Its structure is characterized by seven well-organized cellular and fibrous layers associated with distinct physiological properties. To date, however, little is known about the molecular bases governing the lamination, differentiation and survival of superior collicular neurons. *Barhl1* is a homeodomain transcription factor that has been demonstrated to play an essential role in maintaining inner ear hair cells, cerebellar granule cells and precerebellar neurons. We show here that *Barhl1* exhibits a select expression pattern in the superior colliculus with positive neurons largely restricted to the zonal layer, as visualized by the β -galactosidase activity expressed from the *lacZ* reporter knocked in the *Barhl1* locus. Targeted disruption of *Barhl1* results in the loss of a large population of neurons from the zonal layer of the superior colliculus, as indicated by reduced β -galactosidase staining and marker gene expression as well as by increased apoptotic cell death. Taken together, these data suggest that *Barhl1* is crucially required for the survival but not for the specification of zonal layer neurons in the superior colliculus.

Keywords

Barhl1; homeodomain; transcription factor; superior colliculus; midbrain

INTRODUCTION

The mammalian superior colliculus of the midbrain is a brainstem center that integrates sensorimotor signals involved in the control of orienting behaviors. It is a site where signals from the visual, auditory and somatosensory modalities converge and neurons involved in the generation of movements of the eye, head, trunk, pinnae and vibrissae reside (Wurtz and Albano, 1980; Sparks, 1986; Redgrave et al., 1993). During brain development, the superior colliculus differentiates into seven cellular and fibrous layers. These are, from the surface, the zonal, superficial gray, optic, intermediate gray and white, and deep gray and white layers. These layers can be functionally divided into two major entities based on anatomical, behavioral and electrophysiological properties: 1) the superficial layers consisting of zonal, superficial gray and optic layers; and 2) the deep layers composed of intermediate gray and white, and deep gray and white layers. The superficial layers are innervated by axons from the retina and visual cortex, thus receiving inputs almost exclusively related to vision. By contrast, the deep layers receive inputs mostly from nonvisual sensory modalities and are encoded with multisensory response properties.

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Despite the advancement in our understanding of the anatomy and physiology of the superior colliculus, scarcely little is known about the molecular bases underlying the lamination, differentiation and survival of superior collicular neurons. Birthdating studies have revealed that neurons in all collicular layers are generated simultaneously although subtle gradients of neurogenesis may exist (Mustari et al., 1979; Altman and Bayer, 1981; Edwards et al., 1986). In agreement with this, lineage-tracing analysis uncovers radial cell dispersion as the predominant form of cell migration from the ventricular zone and tangential migration as a minor form (Tan et al., 2002). *Reelin*, the gene responsible for the *reeler* mouse phenotype (D'Arcangelo et al., 1995; Hirotsune et al., 1995), is expressed in the developing superior colliculus and has been found to control lamination of the superior colliculus (Ikeda and Terashima, 1997; Sakakibara et al., 2003). *Reelin*-deficiency causes a complete disorganization of the superficial layers which result in tangled projections of retinal ganglion cells but has no effect on the deep layers (Sakakibara et al., 2003). The bHLH (basic helix-loop-helix) transcription factor *Tal2*, on the other hand, is essential for the formation of the superficial layers. Targeted deletion of *Tal2* leads to a diminished superior colliculus with the zonal and superficial gray layers almost completely missing and the optic layer reduced in size (Bucher et al., 2000). *Grg4*, a member of the Gro/Grg/TLE family of transcriptional repressors (Koop et al., 1996), appears also to play an important role during lamination of the superior colliculus (Sugiyama and Nakamura, 2003; Nakamura and Sugiyama, 2004).

The *Barhl1* and *Barhl2/Mbh1* genes, mammalian homologs of the *Drosophila BarH* genes, encode two closely related homeodomain transcription factors involved in sensorineural development (Kojima et al., 1991; Higashijima et al., 1992a; Higashijima et al., 1992b; Saito et al., 1998; Bulfone et al., 2000; Li et al., 2002; Mo et al., 2004). They display distinct and yet overlapping patterns of expression in the central nervous system and sensory organs during mouse embryogenesis. Of these two genes, *Barhl1* is specifically expressed by inner ear hair cells where it is required for long-term maintenance of these sensory cells (Bulfone et al., 2000; Li et al., 2002); whereas, *Barhl2* is uniquely expressed in the retina where it appears to play a role in the specification of glycinergic amacrine cells (Mo et al., 2004). In addition, both genes are commonly expressed by cerebellar granule cells and developing precerebellar neurons and at least *Barhl1* has been shown to be required for their migration and survival (Bulfone et al., 2000; Li et al., 2004b). In the developing spinal cord, *Barhl1* and *Barhl2* are similarly expressed by the dI1 interneurons, and gain-of-function studies have implicated a role for *Barhl2* in the differentiation of these dorsal commissural sensory neurons (Bulfone et al., 2000; Saba et al., 2003; Mo et al., 2004). Another common site of expression for these two genes is the developing midbrain where they are expressed in both superior and inferior colliculi at embryonic and postnatal stages (Bulfone et al., 2000; Li et al., 2004b; Mo et al., 2004). However, it is not known at present whether *Barhl* genes also play a role during midbrain development. In this work, we analyzed developmental defects in the midbrain of *Barhl1* null mice and found that a large set of neurons within the zonal layer of the superior colliculus became lost, thereby indicating an essential role for *Barhl1* in the maintenance of these neurons.

RESULTS AND DISCUSSION

Gross Abnormality in the *Barhl1*^{-/-} Superior Colliculus

Given the expression of *Barhl1* within the superior and inferior colliculi, we investigated in *Barhl1*^{-/-} mice any anomalies in the midbrain, first by X-gal staining of wholemount embryos and brains to detect the expression of a *lacZ* reporter gene knocked in the *Barhl1* locus (Li et al., 2002). At E14.5, strong β -galactosidase (β -gal) activity appeared within both superior and inferior colliculi in the mesencephalon of *Barhl1*^{+/-} mice (Fig. 1A), consistent with the mesencephalic expression pattern of *Barhl1* revealed by RNA in situ hybridization

(Bulfone et al., 2000; Li et al., 2004b). The pattern of β -gal expression in the *Barhl1*^{-/-} mesencephalon did not differ from that in the heterozygote (Fig. 1A, B), indicating that the neurons that would normally express *Barhl1* were appropriately generated in the mutant mesencephalon at early embryonic stages. At P1, P5 and P19, X-gal staining revealed that both the superior and inferior colliculi were equally heavily labeled in the *Barhl1*^{+/-} midbrain (Fig. 1C, D). By contrast, the superior colliculus in the *Barhl1*^{-/-} midbrain looked pale due to light labeling although the strong staining in the mutant inferior colliculus remained unchanged (Fig. 1C, D). These results suggested that there might be a significant neuronal loss in the mutant superior colliculus at postnatal stages.

Neuronal Loss in the Zonal Layer of the *Barhl1*^{-/-} Superior Colliculus

To confirm a possible neuronal loss in mutant superior colliculi, we compared the number of cells that express β -gal in midbrain sections of *Barhl1*^{-/-} and control *Barhl1*^{+/-} animals. In postnatal superior colliculi of heterozygous mice, the zonal layer was densely populated with β -gal-expressing cells whereas only sparse β -gal-positive cells were scattered within deeper layers (Fig. 2A,C,E). In *Barhl1*^{-/-} mice, however, few of the β -gal-positive cells were seen in the zonal layer (Fig. 2B,D,F), indicating a great loss of β -gal-expressing neurons from the zonal layer of the mutant superior colliculus. Thus, in the absence of *Barhl1*, the neurons that would normally express *Barhl1* appear to be produced in the zonal layer of the superior colliculus during embryogenesis but may not be properly maintained at later stages. In the inferior colliculi of *Barhl1*^{+/-} mice, β -gal-expressing cells were also prominently distributed to the superficial layers at postnatal stages (Fig. 2C,G). Consistent with the result of wholemount brain staining (Fig. 1C,D), there was no reduction of β -gal-positive cells in mutant inferior colliculi (Fig. 2D,H).

To further investigate neuronal loss in *Barhl1* null superior colliculi, we used the calcium binding protein calbindin D28K and GABA_C receptor ρ 2 subunit (*Gabrr2*) to mark neurons in different layers (Kim and Jeon, 1999; Park et al., 2004; Alakuijala et al., 2005). In P4 wildtype superior colliculi, we found that calbindin-immunoreactive neurons were distributed to both the superficial and deep layers but concentrated more in the zonal and upper superficial gray layers (Fig. 3A). In the mutant, however, calbindin-immunoreactive neurons were barely seen in the zonal layer even though they were normally present in other layers (Fig. 3B), indicating a near complete loss of calbindin-expressing neurons from the zonal layer. In postnatal superior colliculi, RNA in situ hybridization revealed *Gabrr2* expression in a subset of neurons scattered within the superficial gray and zonal layers in the control (Fig. 3C). In the mutant, there was a decrease of approximately 20% of *Gabrr2*-expressing neurons (Fig. 3D), consistent with a neuronal loss in the zonal layer of the superior colliculus.

We next examined by TUNEL labeling whether neuronal loss within the mutant zonal layer resulted from increased apoptotic cell death. At E15.5, cell death occurred at very low levels in the superficial layer of control heterozygous superior colliculi (Fig. 3E), whereas in the mutant, it increased by approximately 80% (heterozygote: mean \pm SD, 2.02 \pm 0.25 cells/section, n = 4; mutant: 3.61 \pm 0.89 cells/section, n = 4) (Fig. 3F). At P2 and P4, no significant difference in cell death was seen in the superior colliculi between control and mutant mice (data not shown), consistent with the observation that most zonal layer neurons already got lost as early as P1 in the mutant. These results suggest that in the mutant, zonal layer neurons degenerate primarily during late embryonic stages.

Normal Survival of Retinal Ganglion Cells in *Barhl1*^{-/-} Mice

In the mouse, the great majority of retinal ganglion cells project to the superficial layers of the superior colliculus and are thought to depend on trophic factors supplied by superior

collicular neurons for their long-term survival (Hofbauer and Drager, 1985). Given the significant neuronal loss in the zonal layer of *Barhl1*^{-/-} superior colliculi, we examined whether it could in turn cause a secondary loss of ganglion cells. Ganglion cells in the retina were visualized by immunostaining with an antibody against Brn3a, a POU domain transcription factor expressed in differentiated ganglion cells (Xiang et al., 1995). At P6 and in the adult, the number of Brn3a-immunoreactive ganglion cells did not change in the mutant retina compared to the control (Fig. 4), suggesting that ganglion cell survival is not affected by the loss of neurons within the zonal layer of the superior colliculus in *Barhl1* null mice.

Consistent with the previous finding that *Barhl1* is expressed in the developing midbrain (Bulfone et al., 2000; Li et al., 2004b), our present work reveals that targeted inactivation of *Barhl1* results in the loss of a large population of neurons from the zonal layer of the superior colliculus, as indicated by alterations in β -gal staining, marker gene expression, and cell death (Figs. 1–3). Since neurons that would normally express *Barhl1* are properly generated in the mutant superior colliculus at early embryonic stages (Fig. 1A,B), it is likely that *Barhl1* is mainly required for the maintenance of neurons in the zonal layer but not for their fate determination or early differentiation. Such a role has already been attributed to *Barhl1* during development of other neuronal/sensory cells. Our previous studies have demonstrated that inner ear hair cells, cerebellar granule cells and precerebellar neurons all depend on *Barhl1* for their proper survival but not for their fate specification or initial differentiation (Li et al., 2002; Li et al., 2004b). Therefore, *Barhl1* plays an essential role during the maintenance of neurons/sensory cells in the superior colliculus, cerebellum, precerebellar nuclei, and inner ear. Interestingly, we observed no neuronal loss in the inferior colliculus of the *Barhl1* mutant even though *Barhl1* is expressed in the superficial layers of the inferior colliculus in a pattern similar to that in the superior colliculus. Although it is formally possible that *Barhl1* expression in the inferior colliculus may represent some fortuitous expression, we favor a possibility that the outcome may manifest a redundant function between *Barhl1* and another regulatory factor. A most likely candidate is the other *Barhl* family member, *Barhl2/MBH1* (Saito et al., 1998; Bulfone et al., 2000), which has been shown to be expressed in the developing inferior colliculus and hence may play a redundant role (Mo et al., 2004).

The superior colliculus is characterized by a well-organized laminar structure with different layers associated with distinct physiological properties. It is known that in rodents, the great majority of retinal ganglion cells innervate target neurons within the superficial gray and optic layers, thereby depending on them rather than neurons within other layers for their long-term survival (Hofbauer and Drager, 1985). In agreement with this, in *Barhl1* null mice, retinal ganglion cells appear to be properly maintained in spite of the significant neuronal loss within the zonal layer of the superior colliculus (Fig. 4). Our finding that *Barhl1* has a specific role for zonal layer neurons together with the demonstrated function for *Tal2* during the formation of the superficial layers have implicated that transcriptional regulation may be an important mechanism governing the lamination of the superior colliculus (Bucher et al., 2000). Conceivably, more transcriptional regulators may be identified by molecular genetic approaches that control the formation and maintenance of the laminar structure of the superior colliculus.

EXPERIMENTAL PROCEDURES

Animals

The *Barhl1* knockout mice were generated previously and maintained in our laboratory (Li et al., 2002). The stage of mouse embryos was determined by taking the morning when the

copulation plug was shown as E0.5. All genotypes described were confirmed by PCR (Li et al., 2002).

X-Gal Staining, TUNEL labeling and Immunohistochemistry

β -Galactosidase staining was carried out essentially as described (Li et al., 2004b). Briefly, dissected mouse brains/embryos were fixed in 4% paraformaldehyde/PBS at 4°C for 3 hrs to overnight depending on the stage and tissue size and then rinsed 3 times for 10–20 min each in PBS containing 0.02% Nonidet P-40 and 0.01% sodium deoxycholate. Staining was performed for a few hours to overnight at 30°C or 37°C in PBS containing 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5 mg/ml X-gal. TUNEL labeling and immunostaining using rabbit anti-calbindin D28K (1:800, Swant) and mouse anti-Brn3a (1:50, Chemicon) antibodies were performed also as previously described (Li et al., 2004a).

RNA In Situ Hybridization

The *Gabrr2* anti-sense riboprobe was prepared using the sequence between the NcoI and Hind III sites (1160 nt) of a mouse *Gabrr2* EST clone (GenBank accession no. BI730082). In situ hybridization was carried out using this probe on brain sections according to a previous description (Li et al., 2002).

Acknowledgments

We thank members of the Xiang lab for helpful discussions. This work was supported by the National Institutes of Health (DC04594 and EY12020 to M.X.).

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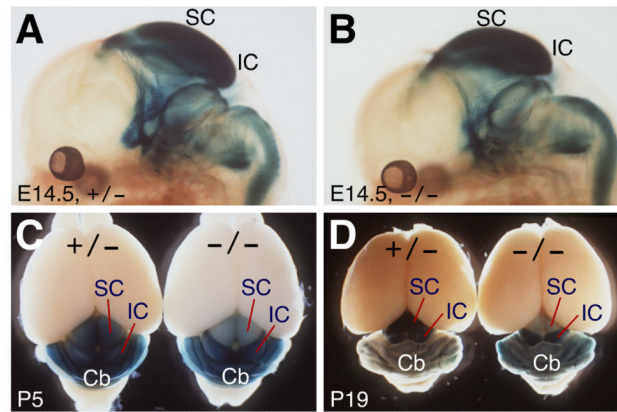


Fig. 1. Abnormal β -gal activity in the superior colliculi of *Barhl1*^{-/-} mice. **A–D:** β -gal activity was visualized by X-gal staining in E14.5 wholemount embryos (A,B) and P5 (C) and P19 (D) wholemount brains. Embryos in A and B were cleared in benzyl alcohol/benzyl benzoate following X-gal staining. Compared to *Barhl1*^{+/-} midbrains, the labeling in the superior colliculus (SC) became pale in P5 and P19 *Barhl1*^{-/-} midbrains but the staining within the inferior colliculus (IC) was not affected (C, D). At E14.5, on the other hand, the superior and inferior collicular regions in the tectum were equally labeled in both *Barhl1*^{+/-} and *Barhl1*^{-/-} embryos (A,B). Cb, cerebellum.

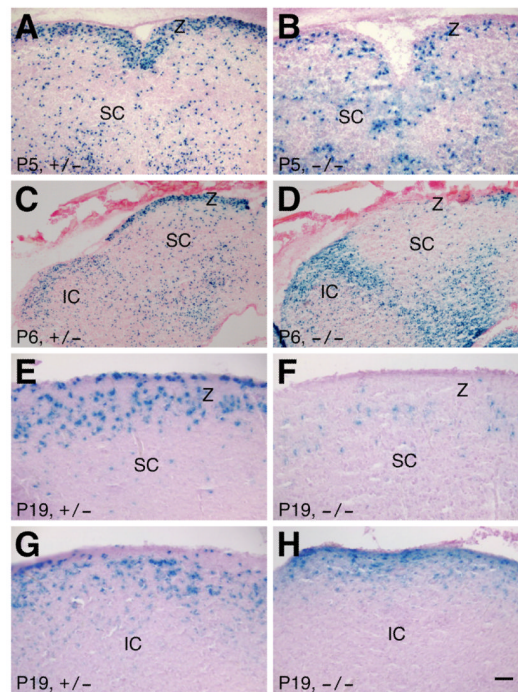


Fig. 2. Neuronal loss in the superior colliculi of *Barhl1*^{-/-} mice. **A–H:** β -gal activity was visualized by X-gal staining in P5 (A, B) and P6 (C, D) coronal and P19 (E–H) sagittal brain sections (counterstained with Fast Red). Compared to *Barhl1*^{+/-} midbrains, β -gal-expressing cells in the zonal layer (Z) largely disappeared from *Barhl1*^{-/-} superior colliculi (SC) at P5 and P6 (A–D) and were hardly seen at P19 (E,F). By contrast, there was no reduction in the number of β -gal-expressing cells in *Barhl1*^{-/-} inferior colliculi (IC) at P6 and P19 (C,D,G,H). Scale bar = 50 μ m (EH), 100 μ m (A,B), and 250 μ m (C,D).

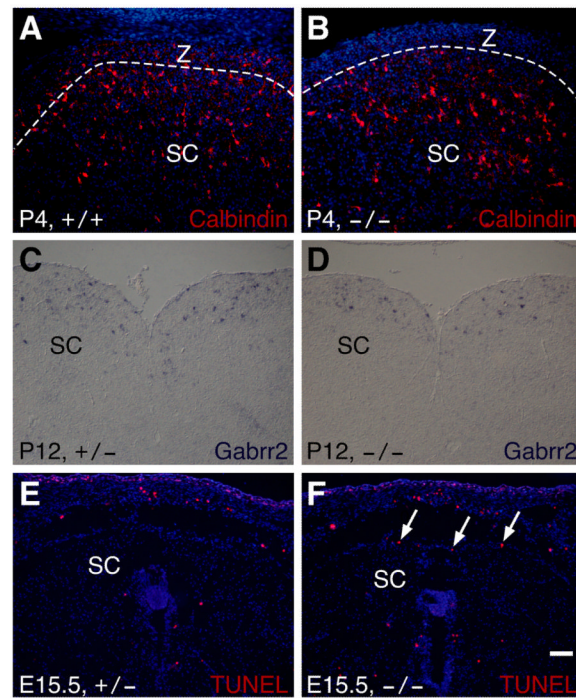


Fig. 3. Neuronal loss in the zonal layer of *Barhl1*^{-/-} superior colliculi. **A,B:** Immunoreactivity for calbindin D28K in P4 superior collicular sections (counterstained with nuclear DAPI). Note the near complete absence of calbindin-immunoreactive neurons from the zonal layer (Z) of the mutant superior colliculus (SC) but their normal presence in other layers. **C,D:** In situ hybridization of P12 superior collicular sections with a riboprobe for *Gabrr2*. There were fewer *Gabrr2*-expressing cells in the mutant. **E,F:** TUNEL labeling of apoptotic cells revealed an increased cell death (indicated by arrows) in the superficial layer of the mutant superior colliculus at E15.5. Scale bar = 50 μm (A,B), and 100 μm (C-F).

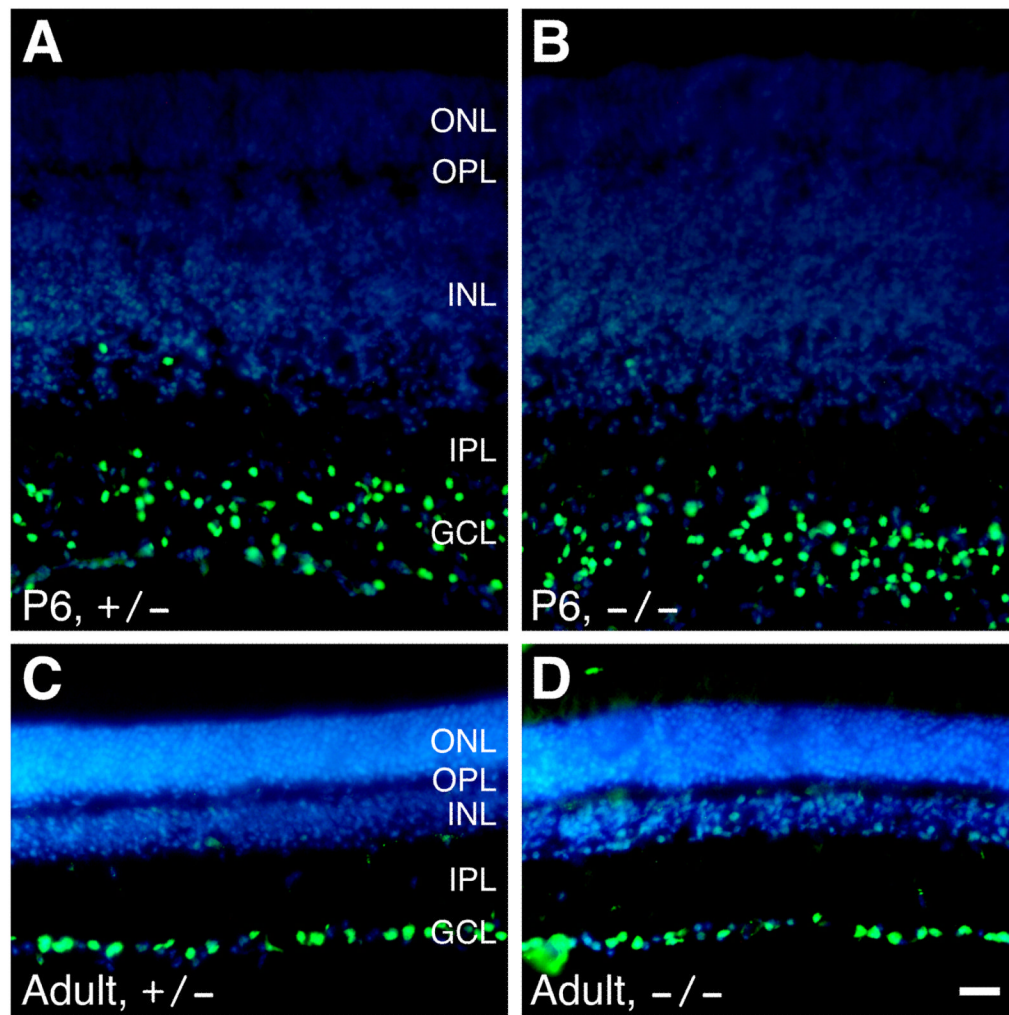


Fig. 4. Ganglion cells survive properly in *Barhl1*^{-/-} retinas. **A–D:** Immunoreactivity for Brn3a in P6 and adult retinal sections (counterstained with nuclear DAPI). The number of Brn3a-immunoreactive ganglion cells (green) did not alter in mutant retinas. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar = 25 μ m (A–D).