NSCL-1 and -2 control the formation of precerebellar nuclei by orchestrating the migration of neuronal precursor cells

Thomas Schmid, Marcus Krüger\(^1\) and Thomas Braun

Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany

Abstract

During embryonic development post-mitotic neurons of the precerebellar neuroepithelium, migrate from the rhombic lip to the ventral part of the neural tube to form the precerebellar nuclei of the pons and medulla oblongata. In this study, we show that neural basic helix-loop-helix transcription factors NSCL-1 and -2 are expressed in all cells of the anterior extramural migration stream (aes), which forms the precerebellar nuclei. The combined inactivation of NSCL-1 and -2 led to a complete absence of the pontine nuclei and a strong reduction in the reticulotegmental nuclei. We demonstrate that NSCL-1/2 were required for a sustained expression of the netrin receptor and cell guidance molecule Dcc in the aes. Unc5H3, a second netrin receptor, which serves as a stop signal for migratory cells was up-regulated in NSCL-1/2 deficient cells, which ceased migration and accumulated ectopically. Furthermore, we observed a massive increase of apoptosis in cells of the aes in the absence of NSCL-1/2, which together with the arrest of migration might explain the virtually complete loss of aes-derived structures in NSCL-1/2 mutant mice. We conclude that NSCL-1/2 maintain migration and survival of cells in the aes.

Keywords: apoptosis, bHLH genes, development, migration, pons, precerebellar nuclei.

\(^{1}\)The present address of Marcus Krüger is the Max-Planck-Institute for Biochemistry, Am Klopferspitz 18, 82152 Martinsried.

Morphogenetic processes in the central nervous system are characterized by a complicated succession of proliferation, differentiation and migration events, which are directed by various regulatory molecules. The formation of the cerebellum and adjacent nuclei, which feed into the cerebellum is a well-investigated part of this scenario although several aspects of this process are still not understood. A part of the cerebellum and the precerebellar nuclei arise during embryonic development from the rhombencephalon at the so-called rhombic lip (Altman and Bayer 1987a). The rhombic lip represents the rim of the open neural tube and can be divided into an upper (rostral) and a lower (caudal) part, which are brought into close contact when the pontine flexure develops. The rostral lip will form parts of the cerebellum while the caudal lip will generate the highly proliferative precerebellar neuroepithelium (pcn) that will give rise to the precerebellar nuclei which pass on peripheral sensations to the cerebellum and which are also major relays of cortico-cerebellar projections (Kawauchi et al. 2006). Four of the five precerebellar nuclei project mossy fibers to the cerebellar granule neurons. Neurons of the pcn have to follow either of three migration streams before they reach their final destination: (i) the anterior extramural stream (aes), which crosses several rhombomeric boundaries before it gives rise to the reticulotegmental and pontine nuclei (Marin and Puelles 1995); (ii) the posterior extramural stream (pes), which in contrast to the aes crosses the midline before forming the lateral reticular and external cuneate nuclei at a contralateral position (Altman and Bayer 1987a); and (iii) the intramural stream (is) that will constitute the inferior olive (Wang et al. 2005). The pathways that lead to the migration and correct positioning of precerebellar neurons have yet to be defined precisely although some molecules have been identified by gene knockout studies to affect the formation of precerebellar nuclei including Pax6, Math1, Robo3, myosin II-B, CXCR4, DCC and netrin (Serafini et al. 1996; Fazeli et al. 1997; Engelkamp et al. 1999; Yee et al. 1999; Ma et al. 2004; Marillat et al. 2004; vilz et al. 2005; Wang et al. 2005).

Neuronal transcription factors of the basic helix-loop-helix (bHLH) family play important roles in the control of

Received February 24, 2007; revised manuscript received April 15, 2007; accepted April 23, 2007.

Address correspondence and reprint requests to Thomas Braun, Max-Planck-Institute for Heart and Lung Research, Parkstr. 1, 61231 Bad Nauheim, Germany. E-mail: thomas.braun@mpi-bn.mpg.de

Abbreviations: bHLH, basic helix-loop-helix; PBS, phosphate-buffered saline.
cell proliferation, determination, and differentiation in the CNS (Ma et al. 1999). The bHLH transcription factor Math1 is a major determinant of precerebellar nuclei. Disruption of the Math1 gene abolishes formation of mossy-fiber projecting precerebellar nuclei, whereas the inferior olive and locus coeruleus develop normally (Machold and Fishell 2005; Wang et al. 2005). A role of a different subgroup of bHLH genes namely the NSCL-1 and NSCL-2 genes (also known as Nhlh1, (=Hen1) and Nhlh2) (Lipkowtiz et al. 1992) in this process has not been established so far. Both genes are expressed in largely overlapping patterns in different areas of the CNS and PNS (Murdoch et al. 1999; Kruger and Braun 2002). NSCL-2(−/−) mice are hypogonadal and infertile and develop adult-onset obesity, indicating a requirement of NSCL-2 for proper neuroendocrine development (Good et al. 1997; Coyle et al. 2002; Nilaweera et al. 2002). In contrast, mice lacking NSCL-1 develop normally, are fertile and show no apparent morphological abnormality (Cogliati et al. 2002; Kruger and Braun 2002), although a decreased life span of NSCL-1 mutant mice was reported, which was ascribed to a dysfunction of the autonomic nervous system (Cogliati et al. 2002). NSCL-1/2 double mutant animals die at birth and show a virtually complete absence of GnRH-1 neurons in posterior parts of the brain at E18.5 (Kruger et al. 2004). In the current study, we report that the role of NSCL1/2 in the migration of neurons of the aes, which form the pontine and reticulotegmental nuclei that project into the cerebellum.

Materials and methods

Mice
The generation of NSCL-1 (Kruger and Braun 2002) and NSCL-2 (Kruger et al. 2004) knockout mice have been described before. Both strains were maintained on a C57/BL6 genetic background after backcrossing for several generations. To obtain compound mutant embryos, mice were crossed, which were homozygous for the NSCL-1 and heterozygous for the NSCL-2 mutation. Double homozygous embryos were identified by Southern blot analysis using DNA isolated from the yolk sac as described previously (Kruger et al. 2004).

Histology and immunohistochemistry
For immunohistochemistry mice were killed and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were postfixed overnight with the same fixative followed by OCT embedding. Histological examination of brain structures was performed on routine hematoxylin/eosin-stained cryostat sections (16 µm thick) and Nissl-stained paraffin sections (8 µm thick). Bromodesoxyuridine (BrdU) staining was done on cryostat sections (16 µm thick) after injection of BrdU (Sigma, St Louis, MO, USA; 50 mg/kg body weight in PBS) into pregnant animals. To label cell that contribute to aes migration mice were injected at E13.5 and analyzed at E16.5 or E18.5. Brain sections were successively incubated with 3% H2O2/PBS for 10 min at 22°C, with 0.1 N HCl for 30 min on ice and 2 N HCl for 1 h at 37°C, stained with a monoclonal anti-BrdU antibody (Dako, Carpenteria, CA, USA), a biotinylated second antibody, and with peroxidase-conjugated avidin (Vectastain Elite kit, Vector, Burlingame, CA, USA) followed by an incubation in diaminobenzidine. The monoclonal antibodies, 5A5, against PSA-NCAM (polyisialylated neural cell adhesion molecule) (1 : 100) and anti-LacZ (1 : 50) were obtained from the Developmental Studies Hybridoma Bank (DSHB). Rabbit anti-Pax6 (1 : 100) was purchased from Convance Research Products. Polyclonal anti-LacZ (1 : 1000) was acquired from ICN Pharmaceuticals (Aurora, OH, USA). LacZ stainings were performed as described (Zweigerdt et al. 1997).

RNA in situ hybridization
In situ hybridization was performed as reported (Kruger et al. 2002). The Pax6 antisense probe was synthesized from a 0.6 kbp fragment using T3 polymerase (gift from Dr Ahmed Mansouri, MPI für Biophysical Chemistry, Goettingen, Germany), Unc5H3 transcripts were detected using a probe from plasmid pAMP10 that contains a Not-SalI Unc5H3 fragment (600 bp) (gift from Dr Susan Ackerman, Jackson Laboratories, Bar Harbor, USA). A Dec probe was kindly provided by Dr Dieter Engelkamp, MPI für Hirnforschung, Frankfurt, Germany.

Results
Absence of precerebellar nuclei in NSCL-1/2 double homozygous mutant mice
The replacement of major parts of the NSCL-1 and -2 coding sequences by the bacterial lacZ gene allowed us to follow the contribution of NSCL-1 and -2 expressing cells to various parts of the CNS. Despite a strong, specific expression pattern at various stages of neuronal development most parts of the CNS with the exception of the pons seemed to develop normally in NSCL1/2 deficient mice. NSCL-1 and NSCL-2 are both expressed in the developing pons from E14.5 onwards including the pontine and reticulotegmental nuclei (Fig. S1 and data not shown) and display a highly overlapping expression pattern in the pontine nuclei at E18.5 (Fig. 1a and b). No changes in the distribution of LacZ-positive cells and no changes in the expression of different marker molecules were spotted in individual NSCL-1 or NSCL-2 mutants (Fig. 1a and b) as compared to heterozygous animals (Fig. S1 and (Kruger et al. 2006)). However, based on the LacZ-staining it became clear that the pontine nuclei, which belong to the precerebellar nuclei and which are located at either side of midline of the ventral rhombencephalon were absent in NSCL-1/2 double mutant mice (Fig. 1e, f, h and j). The area of rhombencephalon that should harbour the pontine nuclei was devoid of NSCL-1/2 positive cells, which normally contribute the majority of neurons to this structure (dotted line in Fig. 1h). Instead, the stream of NSCL-1/2-lacZ
positive cells that migrated from the pcn via the aes to the reticulotegmental and pontine nuclei came to a hold well before the final destination was reached. Histological examination revealed that double mutant mice exhibited a virtually complete absence of neurons within the region of the pontine nuclei (indicated by a dotted line in Fig. 1j). The missing NSCL-1/2 dependent neurons were not replaced by other neurons thus generating an area with a low number of cells (inlet in Fig. 1j). Only nuclei, which are derived from the aes were affected by the NSCL-1/2 mutation despite a widespread expression of NSCL-1/2 in the pcn. Nuclei, which are derived from the posterior extramural stream and the intramural stream, such as the inferior olive, formed normally (data not shown).

Fig. 1 NSCL-1/2 double mutant embryos lack the pontine nuclei. (a, b) Ventral views of the rhombencephalon of E18.5 homozygous NSCL-1 (a) and NSCL-2 deficient embryos (b) stained for β-Gal activity. LacZ staining of NSCL-1(−/−)/ NSCL-2(+/−) (c, d, g) and NSCL-1(−/−)/ NSCL-2(+/−) embryos (e, f, h) at E18.5. Representative overviews of the ventral side of the brain (c, f). Lateral (d, e) and (g, h) enlarged ventral views of the rhombencephalon. No discernable alteration is evident in single NSCL-1 or NSCL-2 knockouts, while the absence of the pontine nuclei (indicated by dotted lines in (h)) is clearly visible in NSCL-1(−/−)/ NSCL-2(−/−) embryos. Note also the enlargement of the aes in NSCL-1(−/−)/ NSCL-2(−/−) (h) compared to NSCL-1(−/−)/ NSCL-2(+/−) (g). Frontal paraffin sections through the ventral region of the pons of NSCL-1(−/−)/ NSCL-2(+/−) (i, k) and NSCL-1(−/−)/ NSCL-2(−/−) (j, l) embryos at E18.5. The absence of neurons in the region of the pontine nuclei is evident in double homozygous mutant mice after Nissil staining (j). The area in which the pontine nucleus should be located is indicated by a dotted line. Note the general reduction in cell numbers in (j). aes: anterior extramural stream, NP: Nucleus pontis.

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Irregular migration but normal proliferation of precerebellar neurons in *NSCL-1/NSCL-2* double mutant mice

To analyze in more detail the number and fate of proliferating neurons in *NSCL-1/2* mutant mice we performed BrdU labeling experiments. As cells of the precerebellar nuclei are generated around E13.5, we pulse-labeled neurons of the pcn at this developmental stage with BrdU and explored the contribution of marked cells to different rhombencephalic structures at E14.5 and E16.5. As expected no BrdU-labeled neurons were found in the region where the pontine nuclei should be located, further corroborating the complete absence of this structure in *NSCL-1/2* mutant mice (Fig. 2b and d). We also investigated the contribution of BrdU-labeled cells to the formation of the aes-derived reticulotegmental nuclei (RT). The reticulotegmental nuclei of *NSCL-1/2* mutant embryos displayed a reduction of more than 89% of BrdU positive cells (Fig. 2b and c). This finding did also correspond to the contribution of *NSCL-1/NSCL-2* expressing cells to the RT, which were mostly composed of *NSCL-1/NSCL-2-lacZ* positive cells.

In order to investigate whether a general reduction in cell proliferation is responsible for the reduced formation of pontine and reticulotegmental nuclei we assessed the total number of BrdU positive cells in the pons region at E14.5 after labeling at E13.5 (data not shown). We did not detect a major change in the total number of proliferating cells at this developmental stage indicating that a massive alteration in the distribution but not the generation of BrdU-positive cells is responsible for the absence of labeled cells in specific areas of the rhombencephalon of *NSCL-1/2* mutant mice. We also found an enrichment of BrdU positive cells in the lateral parts of the rhombencephalon of *NSCL-1/2* double mutant mice at E16.5 (Fig. 2d, Fig. S2A-C), which was further confirmed by counting of the total number of hematoxylin stained cells in the lateral and medial parts (Fig. S3A) and the posterior and anterior parts (Fig. S2B) of the rhombencephalon. Apparently, cells of the aes were unable to complete their journey through the aes to the precerebellar nuclei of the rhombencephalon regardless of a normal proliferation index.

Aberrant expression of cell guidance molecules in the rhombencephalon of *NSCL-1/2* mutant embryos

Cells of the aes rely on the expression of Ntn1 and its receptor Dcc to generate pontine nuclei. Disruption of the Dcc gene leads to massive changes in the formation of pontine and reticulotegmental nuclei. The number of BrdU positive cells was counted at E16.5 after labeling at E13.5 (Fig. 2d, Fig. S2A-C). We also counted the total number of hematoxylin stained cells in the lateral and medial parts (Fig. S3A) and the posterior and anterior parts (Fig. S2B) of the rhombencephalon. Apparently, cells of the aes were unable to complete their journey through the aes to the precerebellar nuclei of the rhombencephalon regardless of a normal proliferation index.
precerebellar nuclei including a virtually complete loss of pontine nuclei (Fazeli et al. 1997; Yee et al. 1999). We reasoned that the absence of NSCL-1/2 might lead to an aberrant expression of cell guidance molecules responsible for correct placement of aes-derived neurons and decided to study the expression profiles of Ntn1, its receptors Dcc and Unc5H3, and of PSA-NCAM, which is another cell guidance molecule involved in cell migration.

We found a strong expression of Dcc in the lateral and medial parts of the aes of E16.5 NSCL1(−/−)/NSCL-2(+/−) mice (arrows in Fig. 3a and c) reflecting the curved path of cells of the aes, which travel from the pcn ventrolaterally and (arrowhead in d). Inlet in (c) shows the last Dcc positive cells at the boundary to the Np. Please note the lack of Dcc expressing neurons at the rim of the pontine nuclei of NSCL-1/2 mutants (arrows inlet in d, arrowhead in d). The expression of Pax6 is greatly reduced in the region of the aes of NSCL-1/2 mutants, were the last cells arrested of NSCL-1/2 mutants (arrowhead in h) but normally present in the lateral aspects of the aes (f). aes: anterior extramural migration stream, Ce: cerebellum; Np, Nucleus pontis, ie: inner ear, Pg: pituitary gland.

Fig. 3 Aberrant expression of cell guidance molecules and aes markers in NSCL-1/2 mutant embryos. In situ hybridizations of frontal (a,b) and parasagittal sections of the rhombencephalon of NSCL-1(−/−)/NSCL-2(+/−) (c, e, g) and double mutant embryos (d, f, h) at E16.5 using Dcc (a-d) and Pax6 (e-h) probes. Dcc is expressed only in migrating cells of the aes. Pax6 is expressed in the pcn, the aes, and in neurons of the pontine nuclei. In NSCL-1/2 mutants Dcc is only found in the posterior (b) but not in the anterior part of the aes.
The expression of Dcc was turned off when aes-derived cells approached their final position resulting in an absence of Dcc expression in the nucleus pontis (inlets in Fig. 3c). In NSCL1(−/−)/NSCL-2(+−) (a, c, e) and NSCL-1(−/−)/NSCL-2(−/−) (b, d, f) embryos. The image in (e) taken from a control embryo matches the contra-lateral image derived from a double mutant embryo displayed in (f). (a–f) In situ hybridizations with an Unc5H3 probe. In NSCL-1(−/−)/NSCL-2(+−) embryos Unc5H3 is strongly expressed in neurons of the pontine and reticulotegmental nuclei (c, white dashed line in e). Please note the lack of Unc5H3 expressing neurons in the pontine nuclei (d, f) of NSCL1/2 mutants (arrowhead in d, red dashed line in f) but the ectopic expression in the lateral part of the pons (arrow in b, white dashed line in f). NP: nucleus pontis, RT: reticulotegmental nuclei, aes: anterior extramural stream, bp: basal plate neuroepithelium, Ce: Cerebellum, Pg: Pituitary gland.

Fig. 4 Aberrant expression of cell guidance molecule Unc5H3 in NSCL-1/2 mutant embryos. Parasagittal and frontal cryostat sections of E16.5 hindbrains derived from NSCL-1(−/−)/NSCL-2(+−) (a, c, e) and NSCL-1(−/−)/NSCL-2(−/−) (b, d, f) embryos. The image in (e) taken from a control embryo matches the contra-lateral image derived from a double mutant embryo displayed in (f). (a–f) In situ hybridizations with an Unc5H3 probe. In NSCL-1(−/−)/NSCL-2(+−) embryos Unc5H3 is strongly expressed in neurons of the pontine and reticulotegmental nuclei (c, white dashed line in e). Please note the lack of Unc5H3 expressing neurons in the pontine nuclei (d, f) of NSCL1/2 mutants (arrowhead in d, red dashed line in f) but the ectopic expression in the lateral part of the pons (arrow in b, white dashed line in f). NP: nucleus pontis, RT: reticulotegmental nuclei, aes: anterior extramural stream, bp: basal plate neuroepithelium, Ce: Cerebellum, Pg: Pituitary gland.
the absence of NSCL-1/2 instead Unc5H3 was prematurely activated probably causing an untimely stop of migrating cells.

Cells of the aes most likely receive signals via the ligand of Dcc and Unc5H3 and via other pathways from surrounding tissues. Yet, we did not find any obvious change in the expression profile of Netrin during embryonic development (Fig. S3). Similarly, the expression of PSA-NCAM, which delineates the boundaries of the aes but is low in migrating cells, was maintained in NSCL-1/2 mutant embryos (Fig. S4 and data not shown) despite the absence of pontine nuclei. Taken together these findings suggest that the migratory paths of the aes did form independently of incoming cells and was not affected by the loss of NSCL-1/2.

Pax6 is found in a subpopulation of NSCL-1/2 expressing cells in the aes

We also studied the expression of Pax6, which is expressed in migrating cells of the aes and required for the normal formation of the pontine but not the reticulotegmental nuclei. In control animals Pax6 was detected in migrating neurons of the aes both at the transcriptional (Fig. 3e and g) and the protein level (Fig. S5). In NSCL-1/2 double mutant mice the expression of Pax6 was mostly confined to the posterior half of the rhombencephalon (Fig. 3f). No expression was seen in the area of the pontine nuclei, which were strongly positive for Pax6 in NSCL1(−/−)/NSCL-2(+/-) embryos (Fig. S5, Fig. 3h). The most medial expression of Pax6 coincided with the position of the last BrdU-labeled cells of the aes.

To clarify the relation between Pax6 and NSCL-1/2 positive cells of the aes we performed a double staining using antibodies against Pax6 (Engelkamp et al. 1999) (Fig. 5b and c) and β-galactosidase (Fig. 5a and c) and a combined staining for β-galactosidase enzyme activity and Pax6 antibody binding (Fig. 5d) in NSCL1(−/−)/NSCL-2 (+/-) embryos. In this experimental set-up the anti-galactosidase antibody and the β-galactosidase enzyme activity

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**Fig. 5** Pax6 expression in a subpopulation of NSCL-1/2 positive cells constituting the aes. Frontal sections of E16.5 hindbrains derived from NSCL-1(−/−)/NSCL-2(+/-) mice (a–d). Sections were stained with a Pax6 antibody (brown) and subsequently for β-galactosidase activity (blue) (d) or with antibodies against Pax6 (red) and β-Gal (green) (a–c). A co-expression of Pax6 and NSCL-1/2 was only seen in a subset of cells (ventral side of aes) while all cell of the aes seem to express NSCL1/2. Note that Pax6 is predominantly expressed in the proliferative zone, while NSCL-genes are active in migrating postmitotic cells. aes: anterior extramural migration stream.
identify expression of both LacZ-reporter genes driven by the NSCL-1 and NSCL-2 promoters. As shown in Fig. 5 all cells that migrated via the aes to the precerebellar nuclei did express NSCL-1/2. Only a minor subset of cells expressed both Pax6 and NSCL-1/2 (Fig. 5c). The majority of proliferating cells seems to express Pax6 (Engelkamp et al. 1999) while the expression of NSCL-1/2 was mostly confined to non-proliferating cells as already observed at previous occasions (Kruger and Braun 2002). It is interesting to note that cells, which were generated in the pcn initially expressed only Pax6 but not NSCL-1/2. Later, after the onset of migration most cells of the aes initiated expression of NSCL-1/2. Eventually, all cells of the aes expressed NSCL-1/2 and only a minor subset stained positive for Pax6 (Fig. 5, Fig. S5). In the most medial part of aes and in the pontine nucleus itself, however, the majority of neurons expressed Pax6 (Fig. 3g, Fig. S1C, D and Fig. S5). It seems likely that the Pax6 positive neurons specifically accumulated in pontine nucleus and thereby give rise to the characteristic expression pattern of Pax6 in the ventral rhombencephalon. No expression of Pax6 but a strong presence of NSCL-1/2 gene activity was found in neurons that build the reticulotegmental nuclei (Fig. S1, S5), which nicely corresponded to the major reduction in the reticulotegmental nuclei in NSCL-1/2 mutants while these nuclei were not apparently affected in Pax6 mutant mice (Engelkamp et al. 1999).

The absence of NSCL-1/2 leads to an enhanced rate of apoptosis of neurons of the aes

A careful evaluation of the total number of BrdU positive cells in the aes, nucleus pontis and RT at E16.5 (Fig. 2, Fig. S2) indicated a significant reduction in the number of labeled cells in NSCL-1/2 mutant mice, which was in contrast to the normal number of BrdU positive cells at E14.5 (data not shown). Although, the ectopic accumulation of BrdU positive cells of the aes in NSCL-1/2 mutants partially accounted for the loss of cells that normally form the pontine nucleus we calculated a reduction in the total number of labeled neurons of approximately 32% (Fig. 2). To gain further insight into the loss of neurons of aes-derived structures we analyzed the number of apoptotic cells in NSCL-1/2 mutant mice at E16.5. TUNEL staining revealed a massive increase of the number of apoptic cells in NSCL-1/2 mutants compared to control mice (Fig. 6d and f). Since TUNEL assays were performed on sections that had previously been subjected to NSCL-1/2 lacZ staining (Fig. 6a and b) we were able to allocate the increase of apoptotic events precisely to NSCL-1/2 lacZ positive cells of the ventral pons. Interestingly, we only detected an increase of apoptosis in cells that were located within the normal path of the aes. NSCL-1/2 lacZ positive cells that accumulated ectopically in an area that we called accumulation point (= ap, Fig. 6b and d) did not underwent apoptosis but stayed alive, which correlated with the ectopic expression of Unc5H3 in these cells.

Discussion

In the current study, we show that migration of neuronal cells of the anterior extramural stream, which gives rise to the pontine and reticulotegmental precerebellar nuclei depends on the synergistic action of NSCL-1 and NSCL-2. Simultaneous inactivation of both genes led to an arrest of aes migration resulting in an absence of the pontine nuclei and to a severe reduction in the number of cells in the reticulotegmental nuclei, the second nucleus formed by the aes. So far, a growing number of molecules, such as Pax6, Math1, Robo3, myosin II-B, CXCR4/SDF1, DCC and netrin has been shown to affect the generation of precerebellar nuclei to different extents (Serafini et al. 1996; Fazeli et al. 1997; Engelkamp et al. 1999; Yee et al. 1999; Ma et al. 2004; Marillat et al. 2004; Vilz et al. 2005; Wang et al. 2005). NSCL-1/2 seem to act downstream of Math1, which is expressed in the rhombic lip as early as E9.5 and determines the fate of all precerebellar nuclei that project mossy-fibers to the cerebellum (Wang et al. 2005).

The formation of the precerebellar nuclei from the caudal lip-derived pcn is an interesting paradigm demonstrating how a single germinat zone can give rise to several dispersed neuronal subpopulations (Altman and Bayer 1987a). In principle, this complex task is achieved by the co-ordinated action of three distinct migration streams that seem to depend on different transcription factor combinations. Each stream carries neurons from a common point of origin to specific destination sites in the medulla and pons (Altman and Bayer 1987a). NSCL-1/2 are not expressed in the pen that generates all three migration streams but specifically define the aes by enabling its proper migration and path finding. From our data, it seems clear that NSCL-1/2 primarily control the sustained migration of precerebellar neurons and argue against an involvement in the regulation of proliferation although we observed an increase in apoptosis within the aes of NSCL-1/2 mutant mice (Fig. 6). This conclusion is supported by the following arguments: (i) Initially, the total number of BrdU positive cells derived from the pen was not compromised by the absence of NSCL-1/2. Only at later developmental stages (E16.5) we found a reduction in BrdU positive cells in the rhombencephalon. (ii) An increased number of ectopically located neurons cells were detected close to the nucleus pontis as indicated by HE, BrdU and NSCL-1/2-lacZ staining. The lack of apoptosis in these ectopically located neurons indicated that the absence of NSCL-1/2 per se did not affect survival of aes neurons. NSCL-1/2 mutant neuronal precursor cells showed a massive increase of apoptosis when located within the normal migratory path of the aes, which explains the overall reduction in BrdU
positive cells of about 32% in double mutant mice at E16.5. The accumulation of NSCL-1/2 positive cells at an ectopic location did not suffice to level out this numerical loss but clearly demonstrated that the lack of NSCL-1/2 alone does not necessarily result in cell death. It seems likely that the increase of apoptosis is caused by the absence of NSCL-1/2 in combination with additional local cues that arise during aberrant migration.

Since NSCL-1/2 mutant cells responded appropriately to proliferative cues, were correctly sorted out of the pcn, and underwent the initial phase of their journey from the pcn properly, it seems justified to conclude that only specific properties of migrating cells (as f.e. path finding, specific cell–cell interactions, pattern formation, etc.) and not the general migration machinery (as f.e. filopodia or pseudopodia formation, cytoskeleton reorganization, etc.) were

Fig. 6 Increased number of apoptotic cells in the aes of NSCL-1/2 deficient mice. Sections through the rhombencephalon of NSCL-1(+/−)/NSCL-2(+/+) (a, c and e) and NSCL-1(−/−)/NSCL-2(−/−) (b, d and f) embryos at E16.5 after whole mount LacZ staining (a and b) and TUNEL labeling (c and f). NSCL-1/2 LacZ activity is confined to cells of the aes, cerebellum and nucleus pontis. (b) Note that in double mutant mice NSCL-1/2 lacZ positive cell accumulate ectopically at the accumulation point (=ap). TUNEL labeling shows an increased number of apoptotic cells in the aes in NSCL1/2 double mutants (c, d, e and f). The inlets in (e and f) show enlarged pictures from the aes in (a, b). The dashed lines in (a–d) mark the position of the aes. (g) Position of the planes of sections for a, b, c, d is indicated by a red line. The curved dashed line indicates the route taken by precerebellar neurons to the pontine and reticulotegmental nuclei (g. Np: Nucleus pontis, Ce: Cerebellum.

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affected by the lack of NSCL-1/2. Isolation of NSCL-1/2 expressing cells followed by transplantation into ectopic locations and/or in vitro migration assays might help to pinpoint specific migratory defects of these cells. It is also evident that the defect to form precerebellar nuclei was inherent to the migrating cell population itself and not the receiving tissue, which did not express NSCL-1/2 and which still seemed to correctly outline the path taken by cells of the aes as indicated by the normal expression of netrin and PSA-NCAM. The complicated migratory path of progenitors cells for the pontine and reticulotegmental nuclei is probably not directed by a single set of secreted soluble factors and membrane-bound guidance molecules, such as Dcc/Unc5H3, which were aberrantly expressed in NSCL-1/2 mutant mice. In fact, recent evidence suggests that the slit receptor Rig-1/Robo3 is required for a normal migration of pontine neurons. In Rig-1 deficient embryos no Pax6-expressing cells were found near the midline where the pontine nuclei form but accumulated more laterally in marginal stream (Marillat et al. 2004). This phenotype corresponds to the expression of Rig-1 in pontine neurons during the initial phase of migration. At the moment, we cannot exclude that NSCL-1/2 affect the expression Rig-1/Robo3. However, the finding that Rig-1 is down-regulated in pontine neurons that approach the ventral midline does not correlate well with the strong expression of NSCL-1/2 in pontine neurons close to the midline and makes a direct control of Rig-1 expression by NSCLs unlikely. Moreover, Rig-1 has been proposed to control midline crossing of precerebellar neurons (Marillat et al. 2004) while only a minor subset of neurons of the pontine nuclei migrate across the midline and thus have a contralateral origin (Kawauchi et al. 2006). Any analysis of the expression of cell guidance molecules in mutant mice has to distinguish between direct effects of NSCL-1/2-dependent transcription and more indirect effects caused by the mere absence of cells. For example, the lack of Dcc expression in the most medial parts of the aes close to the pontine nuclei is certainly caused by the simple absence of pontine NSCL-1/2 determined neurons in this area whereas the down-regulation of Dcc in the complete medial halve of the aes might indicate a direct regulation of Dcc by NSCL-1/2. At present, we do not have clear evidence for a direct regulation of molecules that effect navigation and migration of neurons of the pontine nuclei, such as DCC, Unc5H1, myosin II-B, CXCR4 and Pax6 by NSCL-1/2. Although we found potential binding sites for NSCL-1/2 in the promoter regions of DCC (four binding sites at $\text{~177, \text{~340, \text{~1000 and \text{~1091}}, Unc5H3 (five bindings sites at \text{~193, \text{~254, \text{~463, \text{~976, \text{~1115})}}, CXCR4 (five bindings sites at \text{~10, \text{~323, \text{~521, \text{~834}), and Pax6 (three bindings sites at \text{~1125, \text{~1280, \text{~1713})}}, the relevance of these sites for the expression of the respective genes is currently unknown.

The NSCL-1/2 phenotype partially resembled defects seen in Pax6 mutant animals although several important differences were evident (Engelkamp et al. 1999). Both mutant strains showed no alteration in the formation of the inferior olive, which forms from the intramural migration stream. Pax6 mutant mice suffered from a severe disorganization of external cuneate and lateral reticular nuclei, which are derived from the pes while the disruption of NSCL-1/2 seemed not to affect pes-derived structures.

Fig. 7 Schematic representation of changes in the formation of precerebellar nuclei in NSCL-1/2 mutant embryos. The aes consists of at least two different cell populations expressing either NSCL-1/2 or Pax6/NSCLs. In wild type mice Unc5H3 is expressed in the cerebellum, in the RT and in the pontine nuclei. In NSCL-1/2 mutants an ectopic Unc5H3 expression occurs within the aes representing the prematurely arrested neurons of the pontine and reticulotegmental nuclei. Neurons that form the RT in control embryos do express NSCL-1/2 but not express Pax6. The dashed lines indicate the lateral border of pontine nuclei in wild type embryos. Pontine and reticulotegmental nuclei are virtually completely missing in NSCL-1/2 mutant embryos indicating a decisive role of NSCL-1/2 to direct correct migration of cells of the aes. Te: tectum, Ce: cerebellum, Np: nucleus pontis, pcn: precerebellar neuroepithelium, Pn: pons, Me: medulla oblongata, RT: reticulotegmental nuclei.

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contrast, Pax6 as well as NSCL-1/2 mutant embryos showed defects of nuclei derived from the aes although the NSCL-1/2 phenotype was more severe: Pax6 mutant embryos are characterized by an incomplete absence of the pontine nuclei when compared to NSCL-1/2 mutants; the reticulotegmental nuclei are present in Pax6 mutants but severely reduced in NSCL-1/2 mutant embryos. We have shown that the aes is composed of two separate cell populations, which express either NSCL-1/2 and Pax6 or only NSCL-1/2. Most likely NSCL-1/2 and Pax6 act in two distinct signaling pathways in NSCL-1/2 and Pax6 co-expressing cells because the expression of Pax6 was not directly affected by the loss of NSCL-1/2. Moreover, the reticulotegmental nuclei, which are massively reduced in NSCL-1/2 mutants formed normally in the absence of Pax6. The regular structure of the reticulotegmental nuclei but the malformation the pontine nuclei in Pax6 mutant mice does indicate that Pax6/NSCL-1/2 positive cells are ear-marked for the pontine nuclei while NSCL-1/2 positive cells form the reticulotegmental nuclei. The latter cells might also give rise to the ectopic accumulation of Unc5H3 positive neurons that are missing in Pax6 mutant mice. At present, it remains unclear whether the ectopic expression of Unc5H3 directly results from the absence of NSCL-1/2 probably via the inability to maintain Dcc expression in the mutants or indirectly by the inability of NSCL-1/2 mutant cell to uphold migration. In any way it seems justified to conclude that the future localization of cells of the aes is determined by a combination of transcription factors that allows cells to respond to different environmental cues and enables them to follow specific paths. It will be important to unveil the downstream targets of NSCL-1/2 and Pax6 in this process to achieve a better understanding of the machinery that drives pattern formation of the rhombencephalon. Fig. 7 summarizes the changes in the expression pattern of some cellular markers and illustrates potential interactions between different cell populations. It will be a challenge for future research to unveil the molecular basis for the interaction of different neuronal subpopulation that leads to an accurate positioning of discrete clusters of neurons in the rhombencephalon.

Acknowledgements
We are indebted to Dr Dieter Engelkamp, Max-Planck-Institut für Hirnforschung, Frankfurt, Germany for kindly supplying a probe for Dcc, to Dr Ahmed Mansouri, MPI für Biophysikalische Chemie, Goettingen, Germany for a Pax6 probe and to Dr Susan Ackerman, Jackson Laboratories, Bar Harbor, USA for an Unc5H3 probe. The authors wish to thank Mario Looso for help with bioinformatics and Eva Bober for helpful discussions and for critically reading the manuscript. This work was supported by the Max-Planck-Society, Deutsche Forschungsgemeinschaft, the ‘Fonds der Chemischen Industrie’ and the European Commission (Myores).

Supplementary material
The following supplementary material is available for this article online:

Fig. S1 Expression of NSCL-2 in the pontine and reticulotegmental nuclei of NSCL-2 (+/−) mutants and normal expression of Unc5H3 in (NSCL-1 (−/−) × NSCL-2 +/-) mice.

Fig. S2 Accumulation of cells in the lateral part of the rhombencephalon of NSCL-1/2 mutant embryos.

Fig. S3 Normal expression of the Unc5H3 and DCC ligand netrin in NSCL-1/2 double mutant mice at E14.5.

Fig. S4 Expression of PSA-NCAM in NSCL-1/2 double mutant mice.

Fig. S5 Aberrant expression Pax6 and Unc5H3 but normal PSA-NCAM expression in NSCL-1/2 mutant embryos.

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