Sim1 and Sim2 are required for the correct targeting of mammillary body axons

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Summary

The mammillary body (MB), and its axonal projections to the thalamus (mamillothalamic tract, MTT) and the tegmentum (mammillotegmental tract, MTEG), are components of a circuit involved in spatial learning. The bHLH-PAS transcription factors SIM1 and SIM2 are co-expressed in the developing MB. We have found that MB neurons are generated and that they survive at least until E18.5 in embryos lacking both Sim1 and Sim2 (Sim1–/–;Sim2–/–). However, the MTT and MTEG are histologically absent in Sim1–/–;Sim2–/– embryos, and are reduced in embryos lacking Sim1 but bearing one or two copies of Sim2, indicating a contribution of the latter to the development of MB axons. We have generated, by homologous recombination, a null allele of Sim1 (Sim1tlz) in which the tau-lacZ fusion gene was introduced, allowing the staining of MB axons. Consistent with the histological studies, lacZ staining showed that the MTT/MTEG is barely detectable in Sim1tlz/tlz;Sim2+/– and Sim1tlz/tlz;Sim2–/– brains. Instead, MB axons are splayed and grow towards the midline. Slit1 and Slit2, which code for secreted molecules that induce the repulsion of ROBO1-producing axons, are expressed in the midline at the level of the MB, whereas Robo1 is expressed in the developing MB. The expression of Rig-1/Robo3, a negative regulator of Slit signalling, is upregulated in the prospective MB of Sim1/Sim2 double mutants, raising the possibility that the growth of mutant MB axons towards the midline is caused by a decreased sensitivity to SLIT. Finally, we found that Sim1 and Sim2 act along compensatory, but not hierarchical, pathways, suggesting that they play similar roles in vivo.

Key words: Hypothalamus, Transcription factor, Mammillary body, Mouse, Sim1, Sim2

Introduction

The mammillary bodies (MBs), which lie at the caudal end of the hypothalamus, are composed of the lateral and medial mammillary nuclei. The MBs participate in two pathways that are essential for processing spatial information (reviewed by Vann and Aggleton, 2004). First, the MBs link two brain regions that are crucial for episodic spatial memory, the hippocampus and the anterior thalamus, by receiving afferents from the former via the fornix, and by sending efferents to the latter via the mammillothalamic tract (MTT) (Van der Kooy et al., 1978; Cauce, 1977; Hayakawa and Zyo, 1989; Allen and Hopkins, 1990). Second, the lateral mammillary nucleus contains head direction cells that aid navigation by firing selectively when an animal is facing in a specific direction in the horizontal plane (Vann and Aggleton, 2003). Reciprocal loops connect the MB and some tegmental centers with the efferent MB axons forming the mammillotegmental tract (MTEG) (Sharp et al., 2001). The lateral mammillary nucleus is likely to be important for transforming the vestibular information provided by the tegmentum to help signal head direction. It has been suggested that the head direction cells are responsible for the spatial memory function of the MB. However, lesions involving both the lateral and medial nuclei result in a more severe learning defect than those restricted to the lateral nuclei, suggesting that the medial nuclei also contribute to the spatial memory process.

Most MB neurons send axonal projections to both the anterior thalamic nuclei and the tegmentum via the MTT and the MTEG, respectively. The MTEG is one of the earliest tracts to develop in the CNS, appearing at about E10.5 (Easter et al., 1993; Mastick and Easter, 1996). Much later, at about E17.5, each axon of the MTEG generates collateral that will contribute to the formation of the MTT (Van der Kooy et al., 1978; Cauce, 1977; Hayakawa and Zyo, 1989; Allen and Hopkins, 1990). A minority of MB neurons appear to contribute only to the MTT (Hayakawa and Zyo, 1989). MTT axons are induced near the boundary between the dorsal and ventral thalam. Recent observations indicate that the transcription factors PAX6 and FOXB1 regulate the expression of signals in this region that induce and/or guide MTT axons (Valverde et al., 2000; Alvarez-Bolado et al., 2000). Both Pax6 and Foxb1 mutant mice are born with an intact MTEG but without a MTT. In Foxb1 mutant embryos, MTT axons are induced but do not grow into the thalamus, whereas branching does not occur at all in Pax6 mutants. Pax6 is produced in a domain surrounding the MTEG, at the level of the bifurcation, as well as along the dorsal border of the ventral thalamus. Foxb1 is expressed along the ventral border of the dorsal thalamus and in the MB. Chimera analysis, however, indicates
that Foxb1 functions in the thalamus to promote MTT formation. The requirements for MTT axon guidance are thus complex, as the signals controlled by Pax6 and Foxb1 are produced by closely located but non-overlapping regions of the thalamus.

The bHLH-PAS transcription factors SIM1 and SIM2 are closely related paralogues, the expression profiles of which overlap in regions of the anterior hypothalamus that will give rise to the paraventricular (PVN), supraoptic (SON) and anterior periventricular (APV) nuclei (Fan et al., 1996). Sim1 is required for the differentiation of virtually all neurons of the PVN/SON/APV, whereas Sim2 controls the differentiation of a subset of PVN and APV neurons (Michaud et al., 1998; Goshu et al., 2004). The interplay between Sim1 and Sim2 is complex; mutant analysis indicates that Sim1 acts upstream of Sim2, but can also compensate for the lack of Sim2, albeit ineffectively. SIM1 and SIM2 belong to a group of proteins that need to heterodimerize with members of another group of bHLH-PAS proteins for which there are only four representatives yet characterized: ARNT (Hoffman et al., 1991), ARNT2 (Hirose et al., 1996), BMAL1/MOP3 (ARNTL – Mouse Genome Informatics) (Hogenesch et al., 1997; Ikeda and Nomura, 1997; Takahata et al., 1998; Wolting and McGlade, 1998) and BMAL2/MOP9 (ARNTL2 – Mouse Genome Informatics) (Hogenesch et al., 2000; Maemura et al., 2000; Okano et al., 2001; Ikeda et al., 2000). Biochemical, expression and mutant analyses indicate that ARNT2 acts as the dimerizing partner of SIM1, and presumably SIM2, for anterior hypothalamus development (Michaud et al., 2000; Hosoya et al., 2001; Keith et al., 2001).

Sim1 and Sim2 are also expressed in the prospective MB. Their function during the development of this structure has not yet been elucidated. Here, we show that MB neurons are generated, but that the MTEG and MTT do not develop, in embryos lacking both Sim1 and Sim2. Instead, MB axons aberrantly cross the midline. The same abnormalities, although less severe, are observed in embryos with reduced dosages of Sim1 or Sim2. Expression and mutant studies indicate that Sim1 and Sim2 act along compensatory pathways that do not require Arnt2 function. We propose that Sim1 and Sim2 regulate the expression of molecules involved in the polarized growth of MB axons.

Materials and methods
Generation of the Sim1\textsuperscript{lacZ} allele
Two and 4.5 kb fragments, corresponding, respectively, to the left and right arms of the targeting vector, were amplified by PCR from a Sim1 BAC clone originating from a 129/Sv mouse genome, and cloned into a Bluescript plasmid. A Pgg-neo cassette was inserted downstream of a Tau-lacZ fusion gene (generous gift of J. B. Thomas, Salk Institute, San Diego) and loxP sites were introduced on both sides of Pgg-neo. The whole Pgg-neo/Tau-lacZ cassette was cloned between the two arms of homologous sequences. Finally, a Pgg-tk cassette was inserted at the 5’ end of the construct.

Twenty micrograms of the construct was linearized at an AscI site located at its 3’ end and electroporated into passage 13 R1 ES cells, which were grown as previously described (Michaud et al., 1998). To obtain negative and positive selection for homologous recombinants, gancyclovir and G418 were added to the culture medium at a final concentration of 0.55 µg/ml and 150 µg/ml, respectively. Double-resistant clones were further analyzed by Southern blotting, using a probe containing Sim1 genomic sequences 5’ of those used in the targeting vector. This probe hybridizes to a 5.2-kb BamHI fragment of the wild-type Sim1 allele and to a 4.5-kb BamHI fragment of the Sim1 mutant allele. Homologous recombinant ES cell clones were microinjected into C57BL/6 blastocysts to produce chimeric mice. The resulting male chimeras were backcrossed to C57BL/6 females.

Genotyping of mice
The production and genotyping of mice and embryos carrying the Sim1\textsuperscript{loxP} or Sim2\textsuperscript{loxP} alleles have been previously described (Michaud et al., 1998; Goshu et al., 2003). Sim1\textsuperscript{lacZ} embryos and mice were genotyped by PCR, using two sets of primers. The first set was designed to detect the mutant allele, and amplifies a 189-bp fragment of the neo gene. The second set was designed to detect the wild-type Sim1 allele, and amplifies a 250-bp fragment that is deleted in the mutant allele. The sequences of these primers are as follows: neo, CTTCGACAGGACAGTTGAGATG and GTAAGACGACC-TGTCCGTTGC; Sim1, CCGAGTTGATCTCTAAATTTA and TAGGCACAGACGCTTACCT. The reaction was carried out at 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 45 seconds, with 10% DMSO for 32 cycles, using Taq polymerase.

Genotyping of double mutants was performed by Southern blot using 5’ external probes. The same probe was used for the detection of the Sim1\textsuperscript{loxP} and Sim2\textsuperscript{loxP} alleles. This probe hybridizes to a 5.2-kb BamHI fragment of the wild-type Sim1 allele, to a 3.4-kb BamHI fragment of the Sim1\textsuperscript{loxP} allele and to a 4.5-kb fragment of the Sim1\textsuperscript{loxP} allele. The Sim2 probe hybridizes to an 11-kb EcoRI fragment of the wild-type Sim2 allele and to a 12-kb EcoRI fragment of the Sim2\textsuperscript{loxP} allele.

C112k mice, which were derived at the Oak Ridge National Laboratory, carry a microdeletion encompassing Arnt2 (Michaud et al., 2000). The anterior hypothalamus defect maps to a 320-350 kb region, of which the Arnt2 structural genes spans 140-170 kb. Wild-type and heterozygous embryos were distinguished from homozygotes by the lack of eye pigmentation in the latter.

Histology, in situ hybridization, \(\beta\)-galactosidase staining and Dil labelling
All analyses were carried out on at least two different embryos of the same stage and with the same genotype. For histology, embryo and newborn brains were fixed in Carnoy’s fluid, embedded in paraffin, sectioned at 6 µm and stained with Haematoxylin. In situ hybridization was performed on paraffin sections, as previously described (Michaud et al., 1998). The following probes were generous gifts: Foxb1 (P. A. Laboski, University of Pennsylvania, Philadelphia); Nkx2.1 (J. L. R. Rubenstein, University of California, San Francisco); Robo1, Robo2, Slit1, Slit2 and Slit3 (M. Tessier-Lavigne; Stanford University, Stanford); Rig-1 (A. Chédat, CNRS/Université de Paris, Paris); Sim1 and Sim2 (C.-M. Fan, Carnegie Institute of Washington, Baltimore). The Lhx1 probe was generated by RT-PCR. Whole brains stained for \(\beta\)-galactosidase activity were sectioned at 100 µm with a vibratome. Dil crystals (Molecular Probes) were inserted into the MB of E14.5 brains fixed with 4% paraformaldehyde. These brains were incubated in paraformaldehyde for one week at room temperature and then sectioned at 100 µm with a vibratome.

Results
Development of mammillary body projections requires Sim1 and Sim2
Extrapolation from birth dating studies performed in rats suggests that MB neurons are born between E10.5 and E13.5 in mice, exiting the cell cycle to migrate from the ventricular layer, which contains their progenitors, into the mantle layer in which they complete their differentiation (Altman and Bayer,
Fig. 1. Co-expression of Sim1 and Sim2 in the developing mammillary body. Adjacent coronal sections through the prospective MB of E10.5 (A,B), E11.5 (C,D), E12.5 (E,F) and E14.5 (G,H) wild-type embryos were hybridized either with Sim1 (A,C,E,G) or Sim2 (B,D,F,H). (A,B) At E10.5, Sim1 is expressed in the lateral aspect of the neuroepithelium, which presumably corresponds to the mantle layer, but is expressed less strongly in the medial aspect (bracket), which corresponds to the ventricular layer (A). Sim2 is mainly expressed in this medial domain (B). (C-F) At E11.5 and E12.5, Sim1 is expressed strongly in the mantle layer, which corresponds to the prospective MB, but also weakly in the ventricular layer (bracket, C). Sim2 is expressed in the ventricular layer and in the medial aspect of the mantle layer of the prospective MB. (G,H) At E14.5, Sim1 shows the same expression pattern. However, Sim2 expression has decreased in intensity and becomes restricted to the ventricular layer. The arrows indicate the domains of Sim1/Sim2 expression in the prospective MB.

1978). Previous studies have shown that Sim1 and Sim2 start to be expressed in the neuroepithelium that will give rise to the MB at E9.5, before the birth of the first neurons (Fan et al., 1996). We performed a detailed comparison of the expression patterns of Sim1 and Sim2 in the prospective MB at latter stages because such a description was lacking. At E10.5, Sim1 is strongly expressed in the mantle layer, which contains the first-born MB neurons, and at weaker levels in the ventricular layer (Fig. 1A). At that stage, Sim2 is co-expressed with Sim1 in the ventricular layer, whereas its expression in the mantle layer is much weaker (Fig. 1B). At E11.5 and E12.5, Sim1 is mainly expressed in the whole mantle layer containing the prospective MB and more discretely in the adjacent ventricular zone. By contrast, Sim2 transcripts are found in abundance in the ventricular zone and in the medial aspect of the mantle layer (Fig. 1C-F). At these stages, Sim1 and Sim2 are thus co-expressed in the ventricular layer and in the medial aspect of the mantle layer, which presumably contains neurons of latter generations. At E14.5, Sim1 shows the same expression pattern, whereas Sim2 expression becomes weak and restricted to the ventricular layer (Fig. 1G,H). At E18.5, Sim1 is expressed in the medial and lateral mammillary nuclei, but we could not detect Sim2 expression in the MB (not shown). Therefore, Sim1 and Sim2 are co-expressed in post-mitotic cells of the MB only during a short period, before E14.5, because Sim2 expression in the mantle zone is transient whereas that of Sim1 is continuous.

We next performed a histological analysis of E18.5 embryos with different dosages of Sim1 and Sim2 in order to determine whether the loss of these genes affects MB development. The MB appears histologically normal in all of these embryos, including those with a loss of both Sim1 and Sim2. Remarkably, the principal mammillary axonal tract (PMT), which gives rise to the MTEG and the MTT, appears less prominent in Sim1−/−;Sim2+/− embryos than in control or Sim1+/−;Sim2−/− embryos (Fig. 2, Fig. 3A-F). However, Sim2 is also required for the development of MB axons, as Sim1/Sim2 double mutants show a thin PMT and no detectable MTT, a more severe phenotype than that observed in Sim1+/−;Sim2+/− mice (Fig. 3L), Sim1−/−;Sim2−/− and Sim1−/−;Sim2+/− embryos have a MB phenotype comparable to that of Sim1+/−;Sim2+/− embryos (Fig. 3G,H,J,K). All together, these results indicate that both Sim1 and Sim2 are required for MB axonal development, with Sim1 having a predominant role over Sim2.

ARNT2, the bHLH-PAS dimerizing partner of SIM1, and presumably of SIM2, for anterior hypothalamus development, is expressed extensively in the CNS, including in the developing MB. We determined whether ARNT2 acts as a dimerizing partner of SIM1 and SIM2 for MB axonal development by comparing histologically the brains of E18.5 wild-type and C112k homozygous embryos, which carry a microdeletion encompassing the Arnt2 locus (Michaud et al., 2000). Surprisingly, we found that the MTT and the MTEG are intact in these C112k mutants. All together, these observations raise the possibility that another dimerizing partner interacts with SIM1 and SIM2 for MB axonal development.

Fig. 2. Organization of the mammillary body projections. The left side of the brain is shown from a sagittal perspective. Rostral is to the right. The principal mammillary tract (PMT) gives rise to the mammillotegmental (MTEG) and mammillothalamic tract (MTT).
A Sim1\textsuperscript{tlz} allele allows staining of mammillary body axons

In order to further characterize the axonal projections originating from Sim1-expressing cells, we generated a new targeted allele of Sim1 (Sim1\textsuperscript{tlz}) in which the initiation codon and the basic and HLH domains were replaced by a Tau-lacZ fusion gene (Fig. 4A). The targeted region overlaps with that of the initial Sim1 mutant allele (Sim1\textsuperscript{–}), in which the initiation codon and the basic domain were deleted. This Sim1\textsuperscript{tlz} allele, predicted to be a null, would allow us to stain the MB axons that express Sim1 and follow their fate in the context of a decrease of Sim1 and/or Sim2. Using a double-selection strategy, we obtained 12/140 (9%) ES cell clones in which the Sim1 locus had undergone homologous recombination. One of these clones was used to generate a male chimera that was crossed to a C57Bl/6 female, resulting in germline transmission of the targeted allele (Fig. 4B). Mice homozygous for this allele show the same phenotypes as those described in mice with the previously described Sim1\textsuperscript{–} allele: Sim1\textsuperscript{tlz/tlz} mice die shortly after birth with a severe defect of the PVN/SON/APV (data not shown). Also, the pattern of lacZ staining in the brain of Sim1\textsuperscript{+/+} embryos and newborn mice was comparable to the distribution of the Sim1 transcript (compare Fig. 1G and Fig. 5F). Finally, histological analysis showed that the MB of newborn Sim1\textsuperscript{tlz/tlz};Sim2\textsuperscript{–/+} mice is preserved, whereas the MTT and PMT are not detectable (Fig. 4C,D). All together, these results indicate that the Sim1\textsuperscript{tlz} allele is suitable to study the impact of Sim1 function during MB development.

**Fig. 3.** MTEG and MTT development affected by Sim1/Sim2 gene dosage. E18.5 brains of various genotypes were sectioned sagittally and stained with Haematoxylin. The upper panels (A-C,G-I) represent medial sections containing the PMT, whereas the lower panels correspond to lateral sections that include the MTT and the PMT. The MTEG is not readily detectable on sagittal sections because of its orientation. The PMT is indicated by arrows, the MTT by arrowheads. The PMT and MTT are well developed in Sim1\textsuperscript{+/–};Sim2\textsuperscript{+/–} (A,D) and Sim1\textsuperscript{+/+};Sim2\textsuperscript{–/–} (B,E) embryos, whereas they are thinner in Sim1\textsuperscript{–/–};Sim2\textsuperscript{+/–} (C,F), Sim1\textsuperscript{+/–};Sim2\textsuperscript{–/–} (G,J) and Sim1\textsuperscript{–/–};Sim2\textsuperscript{–/–} (H,K) embryos. The PMT and MTT were barely detectable in Sim1\textsuperscript{–/–};Sim2\textsuperscript{–/–} embryos (I,L). In all cases, the MB was histologically present.

**Fig. 4.** Creation of a Sim1 allele expressing Tau-lacZ.
(A) Schematic representation of the Sim1 locus (wt), of the targeting vector (HR), and of the Sim1 mutant allele (m). Homologous recombination replaces the initiation codon and the basic HLH domain with a Tau-lacZ fusion gene. The 5′ external probe is indicated. B. BamHI; H, HindIII. (B) Southern blot analysis of genomic DNA from Sim1\textsuperscript{+/+}, Sim1\textsuperscript{tlz/+} and Sim1\textsuperscript{tlz/tlz} mice. The 5′ probe detects a wild-type 5.2-kb BamHI fragment and a mutant 4.5-kb BamHI fragment and a mutant 4.5-kb BamHI fragment. (C,D) Sagittal sections through the MB of Sim1\textsuperscript{+/+};Sim2\textsuperscript{–/–} and Sim1\textsuperscript{tlz/tlz};Sim2\textsuperscript{–/–} E18.5 embryos that have been stained with Haematoxylin. The MTT and PMT are not detectable in the MB of embryos homozygous for the Sim1\textsuperscript{–/–} (C) or the Sim1\textsuperscript{tlz} (D) allele.
Sim1/Sim2 mutant axons are directed towards the midline

We next stained E14.5 brains with variable dosage of the Sim1tlz and Sim2– alleles for β-galactosidase (β-gal) activity. The PMTs of Sim1tlz/+;Sim2+/– and Sim1tlz/+;Sim2–/– brains are clearly recognizable, and their sizes are comparable (Fig. 5A,B,F,G,K,L,P,Q). At the level of the anterior MB, the PMT bundle progresses in a domain dorsal to the MB that produces the β-gal activity. Anteriorly, only one bundle of MB axons appears to develop, whereas more posteriorly, several bundles merge to form the main PMT bundle before it leaves the MB domain (compare Fig. 5A,B and 5P,Q). At the most posterior aspect of the MB, a bundle presumably corresponding to the PMT is recognizable, but is accompanied by additional axons, originating more laterally, which project towards the midline and become splayed on their way to reaching it. These findings are more striking in Sim1tlz/tlz;Sim2+/– and Sim1tlz/tlz;Sim2–/– brains, in which a PMT is barely recognizable and a group of ectopic axons similar to that observed in Sim1tls/+;Sim2–/+ brains, but more prominent, is found (Fig. 5C,H,M,R). At the most posterior level of the MB, these axons cross the midline ventrocaudally to the third ventricle. In embryos lacking two copies of Sim1, the number of bundles arising in the MB appears somewhat decreased. Of note, we did not observe consistent changes of Tau-lacZ expression according to the different genotypes in the region located dorsally to the MB, in which PMT axons progress.

No difference between the pattern of lacZ staining of axonal projections in genetic compound mutants for the Sim1− and Sim1tlz alleles versus that of homozygotes was detected, which was consistent with the assumption that the Tau-lacZ fusion gene does not generally affect axon development in mice (data not shown). In order to further validate the use of the Sim1tlz allele to label MB axons, we inserted crystals of DiI into the MB of E14.5 Sim1+/–;Sim2+/– and Sim1−;Sim2− embryos, and compared their pattern of axonal projections. The PMT was clearly labeled in control embryos and very rare axons could be seen progressing towards the midline (Fig. 6A,B). By contrast, most MB axons are directed towards the midline in Sim1−;Sim2− embryos. The axons become splayed as they reached the midline but a subset of these form a bundle crossing the midline ventrally (Fig. 6C,D). This pattern is similar to that observed with the Sim1tlz allele, indicating that the latter is expressed in most MB axons. This observation...
supports our conclusion that the expression of the Sim1\textsuperscript{tlz} allele per se does not affect MB axon development.

We next used this combination of Sim1\textsuperscript{–} and Sim1\textsuperscript{tlz} alleles to study MB axonal projections in E11.5 embryos, shortly after they appeared. In Sim1\textsuperscript{tlz}/+;Sim2+/– (A) and in Sim1\textsuperscript{tlz}/+;Sim2–/– (B) embryos, but are decreased in Sim1\textsuperscript{tlz}/–;Sim2+/– embryos (C). No bundle was detected in Sim1\textsuperscript{tlz}/–;Sim2–/– embryos (D). Note that axons progress in a domain that is stained. \beta-galactosidase staining in the dorsal domain is weaker in C and D than in A and B because these thick sections (A,B) of early embryos are from a slightly different plane.

**Fig. 7.** \beta-galactosidase staining of mammillary body axonal projections in E11.5 Sim1/Sim2 mutant embryos. E11.5 brains with various genotypes, as indicated, were stained for \beta-galactosidase activity and sectioned coronally. Axonal bundles (arrows) are easily recognizable in Sim1\textsuperscript{tlz}/+;Sim2+/– (A) and in Sim1\textsuperscript{tlz}/+;Sim2–/– (B) embryos, but are decreased in Sim1\textsuperscript{tlz}/–;Sim2+/– embryos (C). No bundle was detected in Sim1\textsuperscript{tlz}/–;Sim2–/– embryos (D). Note that axons progress in a domain that is stained. \beta-galactosidase staining in the dorsal domain is weaker in C and D than in A and B because these thick sections (A,B) of early embryos are from a slightly different plane.

Sim1/Sim2 mutant neurons are generated and survive until E18.5

The MB appears histologically intact in Sim1/Sim2 double mutants. In order to determine whether Sim1/Sim2 affects the differentiation of the MB, we performed marker analysis. The Sim1 mutant allele is a null but this does not interfere with the production and stability of its transcript, which can be used to follow the fate of Sim1 mutant cells (Michaud et al., 1998). We found that the expression of the Sim1 mutant transcript in the MB of E12.5 Sim1\textsuperscript{–}/Sim2\textsuperscript{–} embryos is comparable to that of controls, consistent with the fact that the production of the TAU-\beta-gal fusion protein is maintained in the MB of E14.5 mutant embryos (Fig. 8A,B). Similarly, we found that Lhx1 and Nkx2.1 expression is maintained in the MB of E12.5 double mutants (Fig. 8C-F). Of note, the expression of Sim1, Lhx1 and Nkx2.1 is also maintained in a domain dorsal to the E12.5 MB, in which the PMT progresses. Because Sim1, Lhx1 and Nkx2.1 are expressed in virtually all MB cells, the loss of Sim1 and Sim2 thus does not seem to affect the generation and survival of postmitotic neurons in the developing MB. By contrast, Foxb1 expression is dramatically decreased in the prospective MB and in the dorsal domain of Sim1\textsuperscript{–}/Sim2\textsuperscript{–} embryos, but not in those of embryos with at least one allele of Sim1/Sim2, indicating that Sim1/Sim2 acts upstream of Foxb1 (Fig. 8G,H, Fig. 9E-H). At E18.5, Lhx1 expression remains robust in the MB of double mutants, whereas the expression of the Sim1 mutant transcript is decreased (Fig. 9C,D).

The conservation of Sim1 expression in the MB of double mutants indicates that Sim1 expression does not require the presence of Sim2. Conversely, we found that Sim2 expression
that corresponds to the area of the midline towards which mutant axons grow, whereas Slit2 is expressed in the ventricular layer adjacent to and dorsal to the MB (Fig. 10A,C,E,G; data not shown). Slit1 and Slit2 expression in the caudal hypothalamus was not modified in E11.5 and E12.5 Sim1−/−;Sim2−/− embryos (Fig. 10B,D,F,H; data not shown). Slit3 was not expressed in this region in Sim1−/−;Sim2+/+ or Sim1−/−;Sim2+/− embryos (data not shown). These observations indicate that Sim1 and Sim2 do not regulate the expression of the SLIT genes in the developing MB.

In order to determine whether other components of this molecular system are involved in the genesis of the phenotype, we next compared the expression of Robo1, Robo2 and Rig-1/Robo3 in the MB of E11.5 and E12.5 wild-type and Sim1/Sim2 mutant embryos. Robo1 is expressed almost ubiquitously in the caudal hypothalamus, with higher levels found in the prospective MB (Fig. 10L; data not shown). Robo2 is not expressed in the MB, but it is expressed in more dorsal regions (not shown). The expression of Robo1 and Robo2 in the caudal hypothalamus was unchanged in E11.5 and E12.5 Sim1−/−;Sim2−/− embryos (Fig. 10J,L; data not shown). Rig-1/Robo3 is a distant homolog of Robo1 and Robo2 that appears to function in a cell autonomous fashion to inhibit SLIT signalling by a mechanism that has not yet been resolved (Sabatier et al., 2004; Marillat et al., 2004). At E11.5, Rig-1/Robo3 is expressed in a small patch that is contained within the anterior aspect of the Sim1 domain, but its expression is not found more posteriorly (Fig. 10U,W,Y,A'). In Sim1−/−;Sim2−/−, as well as in Sim1−/−;Sim2−/+ embryos, this anterior domain of expression is dramatically expanded, whereas Rig-1/Robo3 expression becomes detectable in the posterior MB (Fig. 10V,X,Z,B' and data not shown). At E12.5, Rig-1/Robo3 is expressed in a narrow domain that extends obliquely within the mantle layer of the anterior MB (Fig. 10M). Its medial half overlapped with the dorsal aspect of the MB prospective domain, as indicated by comparison with the Sim1 expression pattern, whereas its lateral half is located more dorsally. In Sim1−/−;Sim2−/+ and Sim1−/−;Sim2−/− embryos, Rig-1/Robo3 is expressed ectopically in the ventrolateral aspect of the anterior MB domain, whereas its expression dorsally from this domain is reduced (Fig. 10N and data not shown). At the level of the posterior MB, Rig-1/Robo3 expression is barely detectable in control littermates (Fig. 10O). By contrast, Rig-1/Robo3 expression is clearly detectable in the posterior MB of Sim1−/−;Sim2−/+ and Sim1−/−;Sim2−/− embryos, being restricted to its lateral aspect (Fig. 10P). In summary, Rig-1/Robo3 is expressed ectopically in the developing MB of Sim1/Sim2 double mutants, raising the possibility that it contributes to the axonal defects by decreasing the sensitivity of MB axons to Slit signalling.

Discussion

The loss of Sim1/Sim2 produces a complex axonal phenotype that is characterized by the hypodevelopment of the PMT and the presence of axons either directed towards the midline or crossing it. The severity of these two features increases progressively with the dosage of Sim1/Sim2 mutant alleles. SIM1 and SIM2 are thus novel examples of a growing group of transcription factors that act as critical regulators of axonal morphogenesis and connectivity, and they represent crucial
reference points for further dissection of axonal development in the MB. Interestingly, the optic lobes of sim mutant flies show aberrant axonal projections, whereas the neurons in which sim is expressed are present, raising the possibility that the function of Sim1 in axonal development has been conserved through evolution (Pielage et al., 2002).

**Requirement of Sim1 and Sim2 for MB axonal development**

Axonal growth cones are under the influence of attractive and repulsive signals that guide their growth in the developing brain (reviewed by Giger and Kolodkin, 2001). Recent studies indicate that several groups of longitudinal axons are repulsed from the midline by SLIT during development (Bagri et al., 2002). Slit1 and Slit2 are expressed in the ventricular layer adjacent to the MB, as well as dorsally, whereas Robo1 is expressed in the MB. These observations suggest that SLIT signalling might also repulse MB axonal cones from the midline, a possibility that needs to be validated through gain- or loss-of-function experiments in mice. Interestingly, we have

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**Fig. 10.** Sim1 and Sim2 repress Rig-1/Robo3 expression in the developing mammillary body. (A-T) Coronal sections through the MB of E12.5 Sim1+/–;Sim2+/+ and Sim1–/–;Sim2–/– embryos were hybridized either with a Slit1 (A-D), Slit2 (E-H), Robo1 (I-L), Rig-1/Robo3 (M-P) or Sim1 (Q-T) probe. Sections correspond either to the anterior or posterior aspect of the MB, as indicated. The sections hybridized with the Rig-1/Robo3 probe (M-P) are adjacent to those hybridized with the Sim1 probe (Q-T). Expression of Slit1, Slit2 and Robo1 is similar in Sim1+/–;Sim2+/+ and Sim1–/–;Sim2–/– embryos. In the anterior MB of Sim1+/–;Sim2+/+ embryos, Rig-1/Robo3 is expressed in a narrow region that includes a medial domain (yellow arrowhead) that is contained within the dorsal aspect of the MB Sim1 expression domain (M,Q). In Sim1–/–;Sim2–/– embryos, Rig-1/Robo3 expression in the prospective MB (arrow) occupies a larger area extending ventrally and laterally (N,R). Rig-1/Robo3 expression in the region dorsal to the MB (blue arrowhead) is decreased in Sim1–/–;Sim2–/– embryos (M,N). In the posterior MB, Rig-1/Robo3 expression is upregulated in Sim1–/–;Sim2–/– embryos (arrow) (O,P). (U–B’/H11032) Coronal sections through the MB of E11.5 Sim1+/–;Sim2+/+ and Sim1–/–;Sim2–/– embryos were hybridized either with a Rig-1/Robo3 (U–X) or a Sim1 (Y–B’/H11032) probe. Sections correspond either to the anterior or posterior aspect of the MB, as indicated. The sections hybridized with the Rig-1/Robo3 probe (U–X) are adjacent to those hybridized with the Sim1 probe (Y–B’). (U,V) Rig-1/Robo3 is ectopically expressed (V) in the MB (arrows). (W,X) Asterisks indicate a second region in which Rig-1/Robo3 expression is upregulated (X).
found that Rig-1/Robo3 expression is upregulated in the prospective MB domain of Sim1\textsuperscript{+/+};Sim2\textsuperscript{+/+} and Sim1\textsuperscript{+/+};Sim2\textsuperscript{−/−} embryos. Rig-1/Robo3 functions in a cell autonomous fashion to inhibit SLIT signalling in neurons of the spinal cord and hindbrain (Sabatier et al., 2004; Marillat et al., 2004). We propose that a loss of repression of Rig-1/Robo3 in Sim1/Sim2 mutant MB neurons decreases the responsiveness of their axons to SLIT, resulting in their growth towards the midline. Consistent with this possibility, E14.5 Sim1/Sim2 mutant axons originate from the anterior and posterior MB, whereas ectopic Rig-1/Robo3 expression is detected at E12.5. Of note, whereas Rig-1/Robo3 expression is upregulated in the MB domain of mutant embryos, we found that it is decreased in a domain located immediately dorsal to that of the MB. The decrease of Rig-1/Robo3 expression in this dorsal domain could reflect either distinct regulatory interactions or impaired development of these cells.

Ectopic expression of Rig-1/Robo3 in the developing MB, however, does not readily explain other aspects of the axonal phenotype of Sim1/Sim2 embryos. At E11.5, mutant axons do not form clearly recognizable bundles, suggesting a decrease of MB projections, while there are not yet axons directed towards the midline. At E14.5, bundles projecting towards the midline are present in the posterior MB of mutant embryos, but there are no visible axons originating from the anterior MB of these mutants. These observations suggest a decrease of axonal growth in Sim1/Sim2 mutants that cannot be simply explained by an abnormal interaction with the midline. One possibility would be that SLIT2, produced by the ventricular layer that lies ventromedially to the MB, repulses the axons dorsally, contributing to their polarized growth.

Other explanations can be proposed to account for the decrease of axonal growth in the double mutants. For instance, Sim1/Sim2 could function in a cell-autonomous fashion to regulate the expression of signalling components required for response to an attracting signal. Alternatively, Sim1 and Sim2 would be required in the environment in which the MB axons progress to control the expression of an attractive or a permissive signal. Indeed, Sim1 is expressed in a domain dorsal to the MB, which contains the PMT from the time of its appearance. Before E11.5, Sim1 and Sim2 expression overlaps in a region of the ventricular layer that presumably gives rise to MB and the dorsal domain. At later stages, they are co-expressed in the lateral ventricular layer and in the medial aspect of the mantle layer of the prospective MB, but do not overlap in the dorsal domain. If Sim1 and Sim2 are indeed required in the dorsal domain for the correct development of MB axons, one might postulate that they function at an early stage in precursors of the cells of the dorsal domain. The fact that Foxb1 expression in this domain is downregulated in the Sim1/Sim2 double mutant, but not in Sim1\textsuperscript{+/+};Sim2\textsuperscript{+/+} embryos, indicates that Sim2 can influence expression in these dorsal cells. Finally, Sim1 could function in both the axons and their surrounding tissues, as was shown for the transcription factor Lola in the developing fly (Crowner et al., 2002).

**Cascade of transcription factors controlling MB development**

Signals produced by axial mesodermal structures, such as Shh and Bmp7, are required to induce Nkx2.1 expression in the neuroepithelium that will give rise to ventral regions of the developing hypothalamus, including the MB (Kimura et al., 1996; Ericson et al., 1995; Pabst et al., 2000). The MB and several ventromedial nuclei of the caudal hypothalamus do not develop in embryos with a loss of Nkx2.1, suggesting that it is required to specify the whole ventrocaudal hypothalamic anlage (Kimura et al., 1996; Marin et al., 2002). The fact that the MB domain of Sim1 expression is dramatically reduced in Nkx2.1 mutant embryos as early as E11.5 indicates that Nkx2.1 functions upstream of Sim1 for MB development (Marin et al., 2002). Consistent with this conclusion are our observations that Sim1 and Sim2 are not required for the generation and initial differentiation of MB neurons, and that Nkx2.1 expression is not affected by the loss of both Sim1 and Sim2. Similarly, Nkx2.2, a close homolog of Nkx2.1, is required for Sim1 expression in the developing ventral spinal cord, whereas Nkx2.2 expression in this region is not affected by the loss of Sim1 (Briscoe et al., 1999; Briscoe et al., 2000). Collectively, these observations suggest the existence of homologous pathways in these two ventral regions of the CNS, along which the NKK2 and SIM genes would act.

We have found that Foxb1 expression is greatly reduced in the MB of Sim1/Sim2 double mutants. This observation raises the possibility that Foxb1 mediates the effect of a decrease of Sim1/Sim2 on MB axonal guidance. However, Foxb1 mutant analysis does not support this possibility, as the loss of Foxb1 function only affects MTT development (Alvarez-Bolado et al., 2000). Consistently, chimera analyses suggest that Foxb1 is required in the dorsal thalamus for MTT formation. Moreover, we did not observe a decrease of Foxb1 expression in embryos with at least one allele of Sim1/Sim2, despite the fact that axonal guidance abnormalities are observed in these embryos. The loss of Foxb1, however, might suggest that Sim1 and Sim2 are required to control aspects of MB differentiation other than axonal growth that were not revealed by our analysis.

**Respective functions of bHLH-PAS proteins during MB development**

The basic HLH and PAS domains of SIM1 and SIM2 share high identity, whereas their carboxy-terminal domains are poorly conserved. Consistent with the low identity of their carboxy termini, SIM1 and SIM2 display distinct transcriptional properties in cultured cell systems. The SIM1:ARNT(2) heterodimer transactivates reporter constructs via the ARNT carboxy terminus (Moffett and Pelletier, 2000; Woods and Whitelaw, 2002). SIM1 has neither activation nor repression activity in this context. By contrast, SIM2:ARNT(2) activates transcription only when the carboxy terminus of SIM2 is deleted. The carboxy terminus of SIM2 appears to have a repressive function, which quenches the transactivating activity of ARNT (Moffett and Pelletier, 2000; Woods and Whitelaw, 2002). Because SIM1 and SIM2 compete for binding to ARNT(2) and to the DNA-binding site, these different properties of SIM1 and SIM2 result in some transcriptional antagonism, at least in vitro (Moffett and Pelletier, 2000).

Our study indicates, however, that Sim1 and Sim2 can compensate for the absence of each other, the former playing a predominant role over the latter during MB development. We did not observe a reduction of Sim2 expression in the MB of Sim1\textsuperscript{−/−} embryos, or vice versa, suggesting that the interaction
between Sim1 and Sim2 is not hierarchical. All together, these results indicate that Sim1 and Sim2 can play similar roles in vivo, even though their C termini have diverged considerably. There are other lines of evidence supporting this conclusion. Overexpression of Sim1 or Sim2 using a Wnt1 enhancer activates Shh expression in the mouse midbrain, demonstrating that Sim1 and Sim2 can act similarly in a given embryonic context (Epstein et al., 2000). Moreover, Sim1 can compensate for the absence of Sim2, albeit ineffectively, during differentiation of the PVN. The interplay between Sim1 and Sim2 is, however, complex in the developing PVN, as mutant analysis indicates that they also control different aspects of differentiation of the PVN. The interplay between during development. For instance, dysfusion downregulates tracheaeless expression in the developing trachea of the fly, and NXF competes with Sim2 for binding to elements that regulate the expression of a gene engaged in dendritic-cytoskeleton modulation at synapses (Jiang and Crews, 2003; Ooe et al., 2004). It will be interesting to determine whether these or other bHLH-PAS proteins interact with Sim1/Sim2 during the development of the MB.

Biochemical, expression and mutant studies indicate that ARNT2 is required for PVN development by acting as the dimerizing partner of SIM1. It appears likely that SIM2 also heterodimerizes with ARNT2 in the PVN, as they can physically interact (Goshu et al., 2004). However, because the PVN phenotype of Sim2−/− mice is identical to that of Sim1−/− mice, and is more severe than that of Sim2+/− mice, it has not been formally shown that SIM2 controls PVN neuronal differentiation through this interaction. Surprisingly, histological analysis suggests that MB axonal tracts can develop in the absence of Arnt2. A homologue, Arnt, could compensate for the absence of Arnt2, but its expression level is particularly low in the MB of wild-type and Arnt2 mutant embryos (A.C. and J.L.M., unpublished). Alternatively, Sim1 and Sim2 could dimerize with a member of another subgroup of partners, such as BMAL1 or BMAL2, raising the possibility that the use of different partners could influence the function of SIM1/SIM2. Such heterogeneity in the composition of the SIM1 and SIM2 complexes could account for the discrepancy between their respective in vivo and in vitro transcriptional activities.

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