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Author

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Olfactory ensheathing glia are required for embryonic olfactory axon targeting and the migration of gonadotropin-releasing hormone neurons

Perrine Barraud¹, James A. St John², C. Claus Stolt³, Michael Wegner³ and Clare V. H. Baker¹,*

¹Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3DY, UK
²Eskitis Institute for Drug Discovery, Griffith University, Brisbane QLD 4111, Australia
³Institut für Biochemie, Emil-Fischer-Zentrum, Friedrich-Alexander-Universität Erlangen, 91054 Erlangen, Germany

*Author for correspondence (cvhb1@cam.ac.uk)

The anosmia and sterility of Kallmann’s syndrome arise when olfactory axons and gonadotropin-releasing hormone (GnRH) neurons to enter the embryonic forebrain, resulting in anosmia and sterility. Sox10 mutations have been associated with Kallmann’s syndrome phenotypes, but their effect on olfactory system development is unknown. We recently showed that Sox10 is expressed by neural crest-derived olfactory ensheathing cells (OECs). Here, we demonstrate that in homozygous Sox10<sup>−/−</sup> mouse embryos, OEC differentiation is disrupted; olfactory axons accumulate in the ventromedial olfactory nerve layer and fewer olfactory receptor neurons express the maturation marker OMP (most likely owing to the failure of axonal targeting). Furthermore, GnRH neurons clump together in the periphery and a smaller proportion enters the forebrain. Our data suggest that human Sox10 mutations cause Kallmann’s syndrome by disrupting the differentiation of OECs, which promote embryonic olfactory axon targeting and hence olfactory receptor neuron maturation, and GnRH neuron migration to the forebrain.

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Key words: Sox10, Olfactory ensheathing glia, GnRH neurons, Kallmann’s syndrome

Introduction

The anosmia and sterility of Kallmann’s syndrome arise when olfactory axons and gonadotropin-releasing hormone (GnRH) neurons, which are needed for pituitary gonadotropin release, fail to enter the embryonic forebrain (Cadman et al., 2007; Cariboni et al., 2007; Hardelin and Dode, 2008). GnRH neurons migrate from the embryonic olfactory epithelium along olfactory and/or vomeronasal nerves into the forebrain (Cariboni et al., 2007; Wray, 2010; Wierman et al., 2011). Recently, spontaneous mutations in the transcription factor gene Sox10 were associated with Kallmann’s syndrome phenotypes: anosmia, hypogonadism and cryptorchidism (Bondurand et al., 2007; Barnett et al., 2009). Sox10 is expressed by migrating neural crest cells and required for the specification and differentiation of neural crest-derived Schwann cells and satellite glia (Herbarth et al., 1998; Southard-Smith et al., 1998; Britsch et al., 2001; Paratore et al., 2002; Finzsch et al., 2010). We recently showed that olfactory ensheathing cells (OECs), which ensheath olfactory axons from the epithelium to their targets in the olfactory bulb (Ekberg et al., 2012), are neural crest-derived and express Sox10 (Barraud et al., 2010). Sox10 expression was subsequently reported in mouse OECs from E10.5 (Forni et al., 2011), when olfactory axons and migratory neurons first emerge from the olfactory epithelium (Valverde et al., 1992; Miller et al., 2010). Here, we test the hypothesis arising from the association of Sox10 mutations with Kallmann’s syndrome, namely that Sox10 is required for OEC differentiation and that OECs are required for the entry of olfactory axons and GnRH neurons into the embryonic forebrain.

Materials and Methods

Embryo collection and sectioning

Sox10<sup>−/−</sup> mutant mice (Britsch et al., 2001) and wild-type litter-mates of C3HeB/FeJ background were obtained from heterozygous crosses. Embryos were immersion-fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Genotypes were determined from tail biopsies as described (Britsch et al., 2001). Embryos were embedded for wax or cryosectioning and sectioned at 5–6 µm (or at 30 µm, for some E16.5 embryos).

Immunohistochemistry

Immunohistochemistry was performed as described (Lassiter et al., 2007). Primary antibodies used were: anti-β-galactosidase (chicken, Abcam; 1:1000); anti-BLB (rabbit, Millipore; 1:1000), anti-GnRH-I (rabbit, Abcam; 1:100), anti-Hu/C/D (mouse IgG2b, Invitrogen; 1:500), anti-laminin (rabbit, Sigma; 1:1000), anti-NCAM (rabbit, Millipore, 2 µg/ml); anti-neuronal NFII tubulin (Tuj1, mouse IgG2a, Covance; 1:500), anti-neuronal NFII tubulin (rabbit, Abcam, 1:100), anti-NPY (rabbit, Abcam, 1:6000), anti-OMP (goat, Wako; 1:500 or 1:1000), anti-p75NTR (rabbit, kind gift of L. Reichardt, University of California at San Francisco, USA; 1:1000), anti-S100 (rabbit, DAKO; 1:50), anti-Sox10 (goat, Santa Cruz Biotechnology; 1:100). Appropriately matched Alexa Fluor 488-, 568- or 594-conjugated secondary antibodies, Alexa Fluor 350-NeutrAvidin and Alexa Fluor 488-streptavidin were obtained from Invitrogen, and biotinylated secondary antibodies from Southern Biotech.

In situ hybridization

Primers against mouse GnRH (GenBank accession number NM_008145.2) were designed using Primer3 Input (Rozen and Skalatkey, 2000). Total RNA was extracted
Fig. 1. See next page for legend.
from the snout and part of the forebrain using Triazol (Invitrogen), and single-strand cDNA generated using Invitrogen’s SuperScript III First-Strand Synthesis System kit. 

GnRH1 was amplified by PCR (forward primer: CTCACCTTCAACAGGAAGC; reverse primer: GGGCCAGTGATCACTGATC). The 344 bp product was cloned into pDrive (Qiagen) using the Qiagen PCR Cloning Kit and sequenced (Biochemistry Department DNA Sequencing Facility, Cambridge, UK). Digoxigenin-labelled antisense riboprobes were generated (Hengsre et al., 1995) and in situ hybridization performed on sections as described (Xu et al., 2008).

Statistical analysis of olfactory receptor neuron maturation and olfactory epithelium thickness

Confocal images covering an optical depth of 15 μm were captured from 30 μm sections through the olfactory mucosa of E16.5 embryos (two wild-type, two Sox10lacZ/+ and three Sox10−/−/− embryos). Adjacent sections were immunostained for OMP and neuronal βIII tubulin. The region of interest covered a 20 μm length of the nasal septum in the middle portion of the dorsal–ventral span of the olfactory mucosa. Three sections were quantified/embryo for each marker, with each section being 240 μm apart (480 μm total rostral–caudal distance); the first section was 300 μm from the most rostral portion of the olfactory bulb. All cells expressing OMP or neuronal βIII tubulin within the imaged regions of interest were counted. For each of the three sections quantified/embryo, the number of OMP-positive and neuronal βIII tubulin-positive cells within the olfactory epithelium on each side of the nasal septum was counted (i.e., 6 measurements/embryo for each marker), and the thickness of the epithelium (from the nasal surface to the basal lamina) measured at three different positions on each side of the septum (i.e., 18 measurements per embryo). The mean/embryo was determined for each measurement, which was converted from pixels to μm and presented as OMP-positive or neuronal βIII tubulin-positive cell count/100 μm of olfactory epithelium, or thickness of olfactory epithelium in μm. GraphPad Prism (GraphPad Software, La Jolla, California, USA) was used to perform one-way ANOVA using Tukey’s multiple comparison test (comparing every mean with every other mean) and unpaired 2-tailed t-tests.

Statistical analysis of GnRH neuron distribution

GnRH1 neurons were counted on 5–6 μm serial sections (10 slides/series: on each slide, each section was collected every 50–60 μm) processed for immunohistochemistry or in situ hybridization to detect GnRH1. At least 100 GnRH1-positive/embryo were counted on serial parasagittal sections of E14.5 embryos from different litters (3 wild-type, 3 heterozygous Sox10+/+ embryos, 3 homozygous Sox10lacZ/lacZ embryos and on serial coronal sections of E16.5 embryos from four different litters (4 wild-type, 4 heterozygous Sox10+/+ embryos, 4 homozygous Sox10lacZ/lacZ embryos). Differences between the means for groups of the same stage (wild-type versus heterozygous Sox10+/+ embryos, and wild-type versus homozygous Sox10lacZ/lacZ embryos) were assessed via one-way ANOVA using Dunnnett’s multiple comparison test, performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA).

Results and Discussion

Sox10 expression in the developing olfactory system is restricted to OECs (and, at later stages, Bowman’s gland/duct cells)

We aimed to understand how Kallmann’s syndrome phenotypes could result from Sox10 mutations (Bondurand et al., 2007; Barnett et al., 2009). We used in situ hybridization (ISH) and immunostaining to examine Sox10 expression during mouse olfactory system development from E10.5 to neonatal stages (Fig. 1A–O1). Our results confirm and extend previous reports (Barraud et al., 2010; Forni et al., 2011) showing that Sox10 expression is restricted to OECs (which are found along the entire length of the olfactory nerve throughout its development), apart from Bowman’s gland/duct cells in the olfactory epithelium at later stages (as we previously described for avian embryos; Barraud et al., 2010). Sox10 expression was not seen (by either ISH or immunostaining) in neurons in the olfactory epithelium at any stage examined (Fig. 1A–O1). Likewise, Sox10 expression was not seen in neurons in the vomeronasal organ epithelium (e.g. Fig. 1C–D2,G–J3), or in the neurons (which include GnRH neurons) migrating along olfactory and/or vomeronasal nerves (e.g. Fig. 1C–J3). At E16.5, non-neuronal Sox10-positive cells were clearly visible within the olfactory epithelium (Fig. 1M–M’). From E17.5 until at least neonatal stages, these were found in large clusters protruding into the mesenchyme (Fig. 1N–O1), and as strands projecting across the width of the epithelium (Fig. 1O1). As we previously reported for avian embryos [figure S8 in Barraud et al. (Barraud et al., 2010)], these non-neuronal Sox10-positive cells in the olfactory epithelium can be identified as developing Bowman’s gland/duct cells, which start to protrude from the mouse olfactory epithelium at E17.5 (Cuschieri and Bannister, 1975). Overall, therefore, while GnRH neurons are migrating (Cariboni et al., 2007) and olfactory axons reach the olfactory bulb, Sox10 expression is restricted to OECs during mouse olfactory system development.

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Fig. 2. See next page for legend.
proximal olfactory nerve and ONL (S100, Fig. 2F,F2; p75NTR, Fig. 2G,G2). However, p75NTR is also expressed by undifferentiated neural crest cells in rodents (Stemple and Anderson, 1992; Rao and Anderson, 1997), and expression of the early glial differentiation marker brain lipid binding protein (BLBP; Fig. 3A–D) (Murdoch and Roskams, 2007), which was also absent from the lamina propria after Sox10 deletion (Fig. 3B,B1), was significantly weaker in the proximal olfactory nerve and ONL (Fig. 3D). This suggests that a glial specification/differentiation defect affects most neural crest cells that colonize the olfactory nerve. Furthermore, we were unable to detect immunoreactivity for the inner ONL-specific OEC marker neuropeptide tyrosine (NPY; Fig. 3E–F2) (Ubink et al., 1994; Ubink and Hökfelt, 2000; Au et al., 2002). Together, these data suggest that in the absence of Sox10, neural crest cells colonize the developing olfactory nerve but normal OEC differentiation fails.

**Sox10 deletion disrupts olfactory axon targeting and olfactory receptor neuron maturation**

The absence of lamina propria OECs at E16.5 in homozygous Sox10lacZ/lacZ embryos was associated with defasciculation of olfactory axon bundles and inappropriate migration of axons within the lamina propria (Fig. 4A–B1). We also noticed an apparent reduction in the number of olfactory receptor neurons (ORNs) expressing the maturation marker olfactory marker protein (OMP) (compare Fig. 4A4,B1; Fig. 4C–E). To investigate this further, we calculated the mean/embryo (± standard error of the mean, s.e.m.) of OMP-positive cells and neuronal III tubulin-positive neurons/100 μm of olfactory epithelium, and the thickness of the olfactory epithelium. One-way analysis of variance (ANOVA) using Tukey’s multiple comparison test showed no significant difference for any measurement between wild-type (n = 2) and heterozygous Sox10lacZ/+ embryos (n = 2), so we compared wild-type and heterozygote data (n = 4) for comparison with homozygotes (n = 3). We confirmed that the mean/embryo (± s.e.m.) of OMP-positive cells/100 μm of epithelium (Fig. 4F) was significantly lower for homozygous Sox10lacZ/lacZ embryos (5.65±0.17; n = 3) than for wild-type/ heterozygote embryos (10.86±0.69; n = 4) (unpaired 2-tailed t-test: P = 0.0014; t = 6.370; 5 degrees of freedom). In contrast, Sox10 deletion did not affect the mean overall number of neurons/100 μm of epithelium (Fig. 4G: wild-type/ heterozygotes: 48.13±1.94; n = 4; homozygotes: 48.71±2.82; n = 3), or the mean thickness of the olfactory epithelium (Fig. 4H: wild-type/heterozygotes: 73.87±1.91 μm; n = 4; homozygotes: 74.60±2.13 μm; n = 3), suggesting that Sox10 deletion specifically affects ORN maturation.

Immunostaining for the axonal marker NCAM also showed that, relative to wild-type, the OLN in dorsal and lateral regions of the olfactory bulb was much thinner after Sox10 deletion, while the ventromedial OLN was much thicker (Fig. 4L). In two homozygous Sox10lacZ/lacZ embryos, we noticed a ventromedial accumulation of olfactory axons so pronounced that axons from both sides of the nasal cavity merged together ventrally, apparently forming whorls/balls (similar to what is observed in Glis3 extra-toes mutant mice, which lack olfactory bulbs; St John et al., 2003) rather than a uniform OLN as in wild-type mice (Fig. 4K–L1).

These data suggest that the disruption of OEC differentiation arising from Sox10 deletion results in olfactory axons failing to find their targets in the lateral and dorsal regions of the olfactory bulb, leading to axon accumulation in the ventromedial region and a significant reduction in the ONL. When combined with the lack of detectable NPY expression in OECs in the ONL of homozygous Sox10lacZ/lacZ embryos (Fig. 3E–F), our results are consistent with the previously proposed hypothesis (based on the timing of onset of NPY expression) that NPY secreted from inner-ONL OECs may be involved in the final stages of olfactory axon outgrowth towards glomerular targets (Ubink and Hökfelt, 2000). The effect on maturation is presumably a consequence of defective axon targeting: the maturation marker OMP is only expressed in ORNs that have already contacted the olfactory bulb (Graziadei et al., 1980).

A significantly smaller proportion of GnRH neurons enters the forebrain after Sox10 deletion

Already at E12.5, immunostaining for neuronal III tubulin and the neuron cell body-specific Elav RNA-binding protein family members HuC/D (Hinman and Lou, 2008) revealed unusually large aggregates containing multiple neuronal cell bodies on the vomeronasal nerve in both heterozygous Sox10lacZ/+ and homozygous Sox10lacZ/lacZ embryos (Fig. 5A). This suggested that defective OEC differentiation was affecting the migration of GnRH neurons. The distribution of GnRH neurons in wild-type, heterozygous Sox10lacZ/+ and homozygous Sox10lacZ/lacZ embryos is illustrated at E14.5 in Fig. 5B (parasagittal sections; GnRH1-positive cells from 3 embryos/genotype, from 3 different litters) and at E16.5 in Fig. 5C (coronal sections at three different rostrocaudal levels; GnRH1-positive cells from 4 embryos/ genotype, from 4 different litters). Each black spot represents a GnRH1-positive cell on a photomicrograph, identified either by ISH or immunostaining. We quantified these data by counting ≥100 GnRH1-positive cells/embryo and calculating the mean percentage/embryo ± s.e.m. of GnRH1-positive cells that had entered the brain (Fig. 5D). One-way ANOVA using Dunnett’s
Fig. 3. After Sox10 deletion, OEC differentiation is defective. All images show E16.5 coronal sections. (A–B') Expression of the early glial differentiation marker BLBP is seen in OECs ensheathing olfactory nerve fascicles (labelled by immunostaining for olfactory marker protein) in the wild-type lamina propria (A,A'), but not after Sox10 deletion (B,B'). (C,D) BLBP expression in the ONL is much stronger in wild-type (C) than homozygous Sox10lacZ/lacZ embryos (D). (E–F') At E16.5, NPY expression is seen in the inner ONL of wild-type embryos (arrows, E,E') but undetectable after Sox10 deletion (F,F'). Abbreviations: epl, external plexiform layer; lp, lamina propria; ob, olfactory bulb; OMP, olfactory marker protein; on, olfactory nerve; onl, olfactory nerve layer; onl-i, inner olfactory nerve layer; onl-o, outer olfactory nerve layer. Scale bars: 100 μm (E,E',F,F'), 50 μm (C,D), 25 μm (E' inset), 10 μm (A,B).
Fig. 4. Sox10 deletion disrupts olfactory axon targeting and ORN maturation. All images show E16.5 coronal sections. (A–E) Olfactory mucosa sections immunostained for the maturation marker OMP plus (A–B') NCAM or (C–E) neuronal βIII tubulin. Relative to wild-type (A,A',C) or heterozygote embryos (D), homozygous Sox10lacZ/lacZ embryos (B,B',E) displayed defasciculated olfactory nerve bundles and inappropriately migrating axons within the lamina propria (asterisk in B,B'), and fewer OMP-positive neurons (compare A',B'; C–E).

(F–H) Bar charts showing the mean/embryo ± s.e.m. for wild-type/heterozygote embryos (2/ genotype) versus homozygous Sox10lacZ/lacZ embryos, of: (F) OMP-positive cells/100 μm of olfactory epithelium (**P<0.0014; 2-tailed unpaired t-test); (G) neuronal βIII tubulin-positive cells/100 μm of epithelium; (H) olfactory epithelial thickness. Sox10 deletion only affects the number of mature (OMP-positive) ORNs.

(I,J) Olfactory bulb sections immunostained for the axonal marker NCAM. Relative to wild-type embryos (I), the ONL in homozygous Sox10lacZ/lacZ embryos (J) is much thinner in dorsal and lateral regions of the bulb while the ventromedial ONL is much thicker. (K–L') NCAM immunostaining showing clearly separated uniform bilateral ONLs in a wild-type embryo (K) versus a merged, ventromedial ONL (asterisk) in a homozygous Sox10lacZ/lacZ embryo (L,L'). Arrowheads in L,L' highlight axonal whorls/balls. Abbreviations as in Fig. 2. Scale bars: 200 μm (K,L'), 100 μm (J,L'), 50 μm (A,B,C).
Fig. 5. A significantly smaller proportion of GnRH neurons enters the forebrain after Sox10 deletion. (A) At E12.5, immunostaining on coronal sections for neuronal βIII tubulin and the neuronal RNA-binding protein HuC/D revealed unusually large aggregates of multiple neuronal cell bodies on the vomeronasal nerve in both heterozygous Sox10lacZ/+ and homozygous Sox10lacZ/lacZ embryos. (B) Schematic representation of the distribution of GnRH neurons (black spots) at E14.5 on parasagittal sections of 3 wild-type, 3 heterozygous Sox10lacZ/+ and 3 homozygous Sox10lacZ/lacZ embryos. (C) Schematic representation of the distribution of GnRH neurons (black spots) at E16.5 on coronal sections at 3 different rostrocaudal levels of 4 wild-type, 4 heterozygous Sox10lacZ/+ and 4 homozygous Sox10lacZ/lacZ embryos. (D) Bar charts showing the mean percentage/embryo + s.e.m. of GnRH1-positive cells that had entered the brain for wild-type, heterozygous Sox10lacZ/+ and homozygous Sox10lacZ/lacZ embryos (3 embryos/genotype at E14.5; 4 embryos/genotype at E16.5; ≥100 GnRH neurons counted/embryo). At E14.5, the mean percentage/embryo of GnRH neurons in the brain was significantly lower than wild-type for both heterozygous Sox10lacZ/+ and homozygous Sox10lacZ/lacZ embryos (**P<0.01; one-way ANOVA using Dunnett’s multiple comparison test). By E16.5, the mean percentage/embryo of GnRH neurons in the brain was no longer significantly different from wild-type for heterozygous Sox10lacZ/+ embryos, but was four-fold lower than wild-type for homozygous Sox10lacZ/lacZ embryos (**P<0.005; one-way ANOVA using Dunnett’s multiple comparison test). (E–F') Examples of GnRH neurons identified after immunostaining E16.5 coronal sections of wild-type hypothalamus (E,E') and homozygous Sox10lacZ/lacZ olfactory mucosa (F,F'). Abbreviations as in Fig. 2. Scale bars: 200 μm (E), 100 μm (A, main panels; E,F), 25 μm (A, insets; F'); 10 μm (E', insets).
multiple comparison test showed that at E14.5, the mean percentage/embryo of GnRH neurons that had entered the brain was significantly lower (P < 0.01) in both heterozygous Sox10loxP/loxP mutants (17.7 ± 5.9%; n = 3) and homozygous Sox10loxP/loxP mutants (13.3 ± 4.8%; n = 3) than in wild-type embryos (37.7 ± 1.2%; n = 3) (Fig. 5D). By E16.5, there was no longer any significant difference between heterozygote (38.8 ± 6.9%; n = 4) and wild-type embryos (51.9 ± 4.7%; n = 4), but four-fold fewer GnRH neurons were present in the brain in homozygous Sox10loxP/loxP mutants (11.9 ± 3.9%; n = 4) than in wild-type embryos (P < 0.005; also see Fig. 5D). Examples of GnRH neurons in the hypolamus of wild-type embryos and the olfactory mucosa of homozygous Sox10loxP/loxP mutants are shown in Fig. 5E–F1. These data suggest that GnRH neuron migration to the forebrain is delayed when one copy of Sox10 is missing, and stalled after Sox10 deletion. A recent report describing a close association between migrating GnRH neurons and OECs (Geller et al., 2013) is consistent with the important role for OECs in GnRH neuron migration that we have demonstrated here.

We conclude that human Sox10 mutations cause Kallmann’s syndrome phenotypes (Bondurand et al., 2007; Barnett et al., 2009) by disrupting the differentiation of OECs, which, as shown here, promote olfactory axon targeting, ORN maturation (most likely because of their importance for olfactory axon targeting) and GnRH neuron migration. A neural crest defect in Kallmann’s syndrome is supported by its inclusion within CHARGE syndrome (Pinto et al., 2005), an autosomal dominant disorder caused by heterozygous mutations in CHD7, encoding a chromatin-remodeling protein that controls neural crest formation (Bajpai et al., 2010), and by the demonstration that anosmin1, loss-of-function mutations in which cause X-linked Kallmann’s syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb function mutations in SOX10 cause Kallmann syndrome with deafness. (Cadman et al., 2007; Hardelin and Dode´, 2008), promotes cranial neural crest cell formation in an autocrine fashion (Endo et al., 2010). Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. J. Neurosci. 31, 635-644.


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The authors have no competing interests to declare.

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Author Contributions

P.B. and C.V.H.B. conceived the project, designed the experiments and wrote the manuscript. P.B. and J.A.S. performed the experiments and discussed and interpreted the data with C.V.H.B. C.C.S. and M.W. provided embryos, reagents and expertise.

Competing Interests

The authors have no competing interests to declare.


