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Whisker-related neural patterns develop normally despite severe whisker defects in *Msx2* knockout mice

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Abstract

In mice, whiskers on the snout form a highly specialized tactile organ with exquisitely patterned neural representations in the brain. Targeted deletion of the *Msx2* gene leads to severe craniofacial defects, and stubby, curly whiskers. We examined the whisker pad histology, innervation, and whisker-related pattern formation along the trigeminal pathway in *Msx2* −/− mice. Although the whiskers are severely deformed, whisker follicle structure, pattern and density of innervation, as well as central neural patterns in the brainstem, thalamus, and cortex appeared normal. We conclude that whisker-related neural patterns can form in the absence of normal whiskers, as long as whisker follicle innervation is intact.

Keywords

Barrel cortex; Barrellet; Whisker follicle innervation; Cytochrome oxidase histochemistry; Homeobox gene

Mouse *Msx2* gene is a member of the homeobox gene superfamily, and is highly conserved among species from sea urchins to humans [2-4]. It is a transcription factor widely expressed throughout early development and organogenesis, as revealed by expression of a LacZ containing transgene under control of *Msx2* promoter [17], and has important roles in epithelia–mesenchyme interactions [4]. During early development, *Msx2* expression is detected in primitive streak ectoderm and mesoderm [17], and later it is expressed in dorsal neuroepithelium, neural crest cells [6], retina [7], hindbrain, maxillary pad, apical ectodermal ridge of developing limb buds, mammary gland epithelium [17], and the matrix epithelial cells of hair follicles [11]. Targeted disruption of the *Msx2* gene causes defects in eye development [7], bone growth, craniofacial development, tooth development, cerebellar development, mammary gland development, as well as loss of pelage, abnormal hair cycling [21], and curly, stubby whiskers. Transgenic mice over expressing *Msx2* under the control of a cytomegalovirus promoter have epidermal dysplasia, smaller and irregularly shaped hair follicles, accompanied by shrunken matrix regions, fewer matrix epithelial cells, thinner outer root sheath, and poorly developed bulb regions [11].

Observation that whiskers are defective in *Msx2* knockout (*Msx2* −/−) mice led us to investigate whisker-related neural pattern formation along the trigeminal pathway. Trigeminal ganglion (TG) cell axons form the first link between the whiskerpad and the central nervous system. Central TG axon terminals replicate the patterned distribution of

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whisker follicles on the snout, and their postsynaptic partners in the brainstem trigeminal nuclei detect these patterns and orient their dendrites towards these arbors. Collectively, pre and postsynaptic elements organize into whisker-specific modules, the 'barrelettes' [18,19]. In the brainstem trigeminal complex barrelette patterns are seen within the principal sensory nucleus (PrV) and the spinal trigeminal nucleus [13]. Tri-geminothalamic projection cells of the PrV then convey these patterns to the ventroposteromedial nucleus of the dorsal thalamus (VPM) where 'barreloids' form; barreloid cells in turn, convey these patterns to the layer IV of the somatosensory cortex where 'barrels' form [22]. Damage to the whisker follicles or to the infraorbital branch of the trigeminal nerve during a critical period in development (up to postnatal day 4 in mice) irreversibly alters the morphological organization of whisker representations in the brain [1,20]. In adult animals, complete or partial trimming of whiskers also leads to plastic changes in functional properties of neurons in the barrel cortex [23]. In recent years, neural activity, in particular *N*-methyl-D-Aspartate (NMDA) receptor mediated neural transmission along the whisker-barrel pathway has been implicated as a major player in patterning of the brainstem, and upstream somatosensory centers in mice [5,8,9,15,16]. If neural activity plays a role in whisker-specific patterning of the somatosensory pathways, to what extent do whiskers and their activation participate in this process? In the present study, we examined the cytological organization and innervation of the whisker pad and whisker-specific patterns along the trigeminal pathway of *Msx2* −/− mice.

Generation of *Msx2* −/− mice was reported previously [21]. Although knockout animals are viable when kept on a protein rich liquid diet, pups from matings of heterozygous animals were used in experiments described in this study. Knockout pups could easily be distinguished from their wild type littermates due to abnormal whiskers on the snout. All protocols used were in agreement with NIH rules and regulations for animal housing and treatment. *Msx2* −/− and wild type P5 littermates (n=7 each) were killed by overdose of sodium pentobarbital, perfused with saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains were removed and the right cerebral cortex was flattened and sectioned parallel to the pial surface, and the rest of the brain was sectioned in the coronal plane. Cortex and brainstem sections from wild type and *Msx2* −/− animals (n=7 each) were processed for cytochrome oxidase histochemistry as described previously [8,9,13]. Sections were mounted on glass slides, coverslipped and photographed under the light microscope using the CoolSNAP documentation system. Whisker pad sections from *Msx2* −/− pups and wild type littermates (n=2 each) were used for hematoxylin and eosin (HE) staining to visualize cytological organization. Subsequently, 60-μm thick sections from whisker pads of *Msx2* −/− and wild type animals (n=2 each) were processed for immunohistochemistry. Briefly, after blocking in 10% normal goat serum in PBS containing 0.3% Triton X-100, sections were incubated overnight in 1:500 rabbit anti neurofilament M (Chemicon AB 1987) antibody at 4°C. Sections were washed, and incubated in 1:200 dilution of either FITC (Sigma F0382) or CY3 (Chemicon AP 132C) conjugated goat anti rabbit antibodies. Sections were then mounted, coverslipped and observed under epifluorescence using appropriate filters. Photomicrographs were taken with the CoolSNAP camera, and adjusted for brightness and contrast using the Adobe Photoshop Program.

Msx2 −/− animals show severe defects in craniofacial development including various phenotypes like delayed suture closure, cleft palate, and defective whiskers. Close examination of the whisker pad reveals a full set of whisker follicles, although the whiskers emerging from follicles are curly and stubby (Fig. 1). We have not examined if these deformed whiskers could still be used for exploratory behavior once the whisking behavior starts at 2 weeks of age. Apart from the defective whiskers, there was no major difference in gross appearance, size, or organization of whisker pads between *Msx2* −/− mice and wild type littermates.

Hematoxylin and eosin stained tangential and coronal sections through the whisker pad did not reveal any major differences between the *Msx2* −/− animals, and their wild type littermates (Fig. 2A, B). Size, organization, and distribution of whisker follicles in the whisker pad appeared to be normal in knockouts. Major structural components, including dermal papilla, looked normal in the *Msx2* −/− animals. NF immunohistochemistry also showed clear innervation patterns around the follicles with defective whiskers (Fig. 2C, D). Density and organization of labeled fibers and the extent of whisker follicle innervation appeared similar between the *Msx2* −/− and control animals. Both deep vibrissal nerves and superficial vibrissal nerves could easily be detected in coronal sections of *Msx2* −/− animals, indicating normal sensory innervation of the individual whisker follicles.

In both wild type, and *Msx2* −/− P5 animals, cytochrome oxidase staining revealed a similar pattern at all levels of the trigeminal pathway (Fig. 3). Five rows of cytochrome-oxidase dense patches (barrels), corresponding to the five rows of whiskers, were clearly visible in flattened cortex sections, and in coronal sections through the thalamus (barreloids) and brainstem trigeminal nuclei (barrelettes) from both knockout and wild type animals. Cortical barrel area measurements (n=7 each) were not significantly different (P>0.5, data not shown) between *Msx2* −/− animals and wild type littermates.

Msx2 expression has been reported in neural crest cells, developing hindbrain [6], and whisker pad [17]. In the hair follicle, *Msx2* expression is confined to the matrix cells [11], which are keratinized to form the hair shaft inside the follicle. This expression pattern is in agreement with our observation that there are no defects in whisker follicles or whisker pad aside from curly and sturdy whiskers growing inside the follicles. Although *Msx2* expression is detectable in the whisker pad during embryonic development [17], lack of it does not seem to cause any gross defects in whisker pad development and organization.

Previous observations from animal models similar to *Msx2* knockouts are in agreement with our findings. Studies on *activinβA* and *follistatin* knockout mice [10], as well as vibrissaeless mutant rats [14] revealed the following phenotypes: *Activin* knockouts have a normal array of whisker follicles, but no whiskers, and the follicles are atrophied. *Follistatin* knockouts have thin, curly whiskers resembling the *Msx2* knockout phenotype, however the follicles are misoriented. Vibrissaeless mutant rats on the other hand, appear to have normal whisker follicles, although they lacked vibrissae due to defects in hair growth, but not in follicle development. Innervation of follicles was normal in both *follistatin* knockouts, and vibrissaeless mutants, resembling the case in *Msx2* −/−. On the other hand, follicle innervation density was decreased in *activin* knockouts, accompanied by a smaller TG, decreased physiological responses, and lack of patterns in the brainstem trigeminal nuclei. *Follistatin* knockouts could not be kept alive for longer than 6 h after birth, so pattern development could not be studied at later stages, but they still displayed some patterns in the brainstem at that age, although it was more diffuse and less organized compared to wild type littermates, which might be due to misoriented whisker follicles.Vibrissaeless mutants survive into adulthood, and have normal follicular innervation had normal cortical patterns. Collectively these studies and present observations indicate that a full set of intact whisker follicles and their innervation is sufficient for whisker specific pattern formation in the central nervous system.

While specific defects of whiskers that do not affect follicles themselves or their innervation do not interfere with the development of central patterns, these deformities could have other consequences. Sensory deprivation by whisker clipping during the critical period or even in adult rodents has been reported to cause severe impairments in tactile discrimination abilities, alterations in GABAergic inhibition within the barrel cortex, response properties of barrel cortex neurons, changes in the number and size of dendritic spines, and altered

intracortical connection patterns [12]. Thus examination of intracortical connectivity, peripherally driven response characteristics of barrel cortex cells, and the performance of these animals in whisker dependent tactile tasks could unveil defects.

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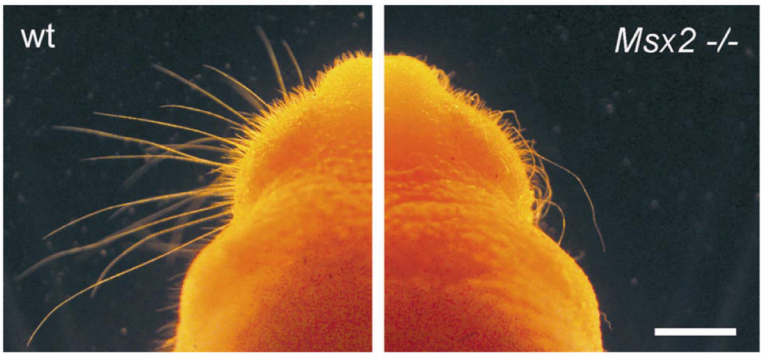

Fig. 1.

Appearance of whiskers on the snout of *Msx2* knockout pups. Compare short, stubby whiskers of knockouts (right) to the normal appearance in wild type littermates (left) at postnatal day 5. (Scale bar=2.0 mm).

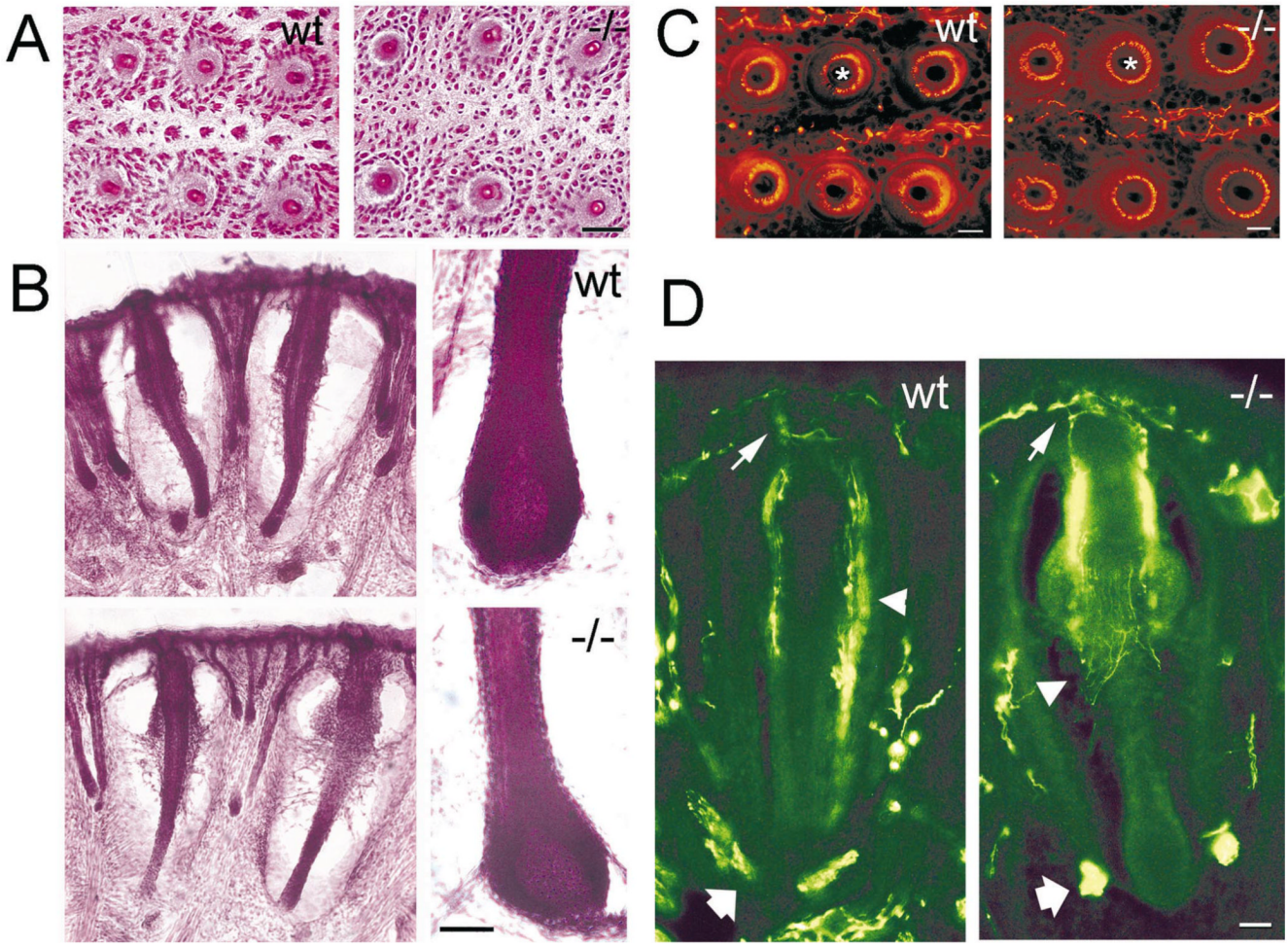

Fig. 2.

Structure and innervation of whisker follicles. (A) H and E stained tangential sections of the whisker pad of control (left) and *Msx2* −/− (right) animals. There is no major difference in the structure, organization or size of whisker follicles. (B) H and E stained coronal sections parallel to the whisker follicles in wild type (top) and knockout (bottom) animals. Structure and orientation of follicles appear normal in the knockouts. Higher magnification photos of dermal papilla are shown on the right panel. (C) Neurofilament immunohistochemistry showing innervation of wild type (left) and knockout (right) whisker pads. There is no obvious difference in the pattern and density of innervation of whisker pad between or around individual follicles (asterisk depicts the center of a representative follicle). (D) Innervation of individual whisker follicles of control (left) and knockout (right) animals shown in coronal sections. Both superficial (thin arrows) and deep (thick arrows) vibrissal nerves appear to display normal density and innervation pattern. Note nerve branches surrounding follicles (arrowheads) revealing normal innervation of individual follicles. (Scale bar=0.2 mm in (A and C), 0.04 mm in (B and D)).

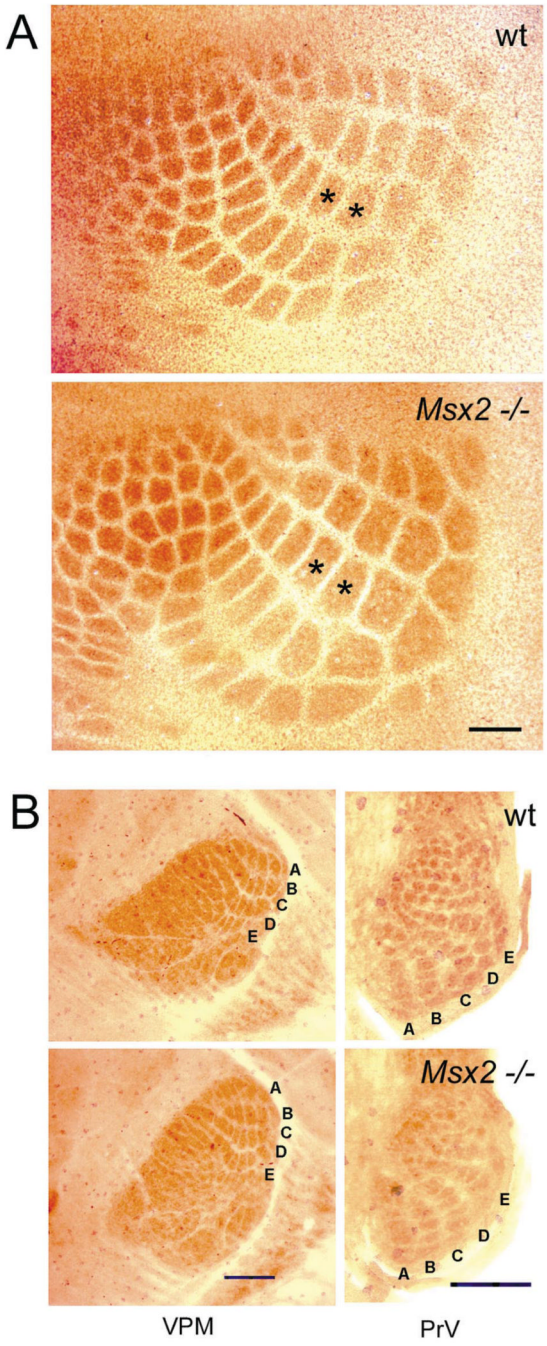

Fig. 3.

Cortical and subcortical pattern formation revealed by cytochrome oxidase staining. (A) Tangential cortical sections from control (top) and *Msx2* knockout animals (bottom). A normal whisker-related neural pattern of barrels (asterisk) develops in the knockout animals. (B) Subcortical pattern formation in the VPM (left) and PrV (right) of wild type (top) and knockout (bottom) animals. Five rows of barrelettes and barreloids (labeled A through E) develop normally in knockouts. (Scale bar=0.2 mm).