Facial whisker pattern is not sufficient to instruct a whisker-related topographic map in the mouse somatosensory brainstem

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ABSTRACT

Facial somatosensory input is relayed by trigeminal ganglion (TG) neurons and serially wired to brainstem, thalamus, and cortex. Spatially ordered sets of target neurons generate central topographic maps reproducing the spatial arrangement of peripheral receptors on the face. Facial pattern provides a template for map formation, but whether it is sufficient to impose a brain somatotopic pattern is unclear. In the mouse, lower jaw sensory information is relayed by the trigeminal nerve mandibular branch, whose axons target the brainstem dorsal principal sensory trigeminal nucleus (dPrV). Input from mystacial whiskers on the snout is relayed by the maxillary branch and form a topographic representation of rows and whiskers in the ventral principal trigeminal nucleus (vPrV). To investigate the importance of peripheral organisation in imposing a brain topographic pattern, we analysed the Edn1 mutant mice, in which lower-to-upper jaw transformation results in ectopic whisker rows on the lower jaw. In Edn1 mice, the lower jaw ectopic whiskers were innervated by mandibular TG neurons which initially targeted dPrV. Unlike maxillary TG neurons, the ectopic whisker- innervating mandibular neuron cell bodies and pre-target central axons did not segregate into a row-specific pattern nor targeted the dPrV with a topographic pattern. Following periphery-driven molecular repatterning to a maxillary-like identity, mandibular neurons redirected partially their central projections from dorsal to ventral PrV. Thus, a spatially ordered ectopic whisker pattern on the lower jaw is not sufficient to impose row-specific pre-target organization of the central mandibular tract nor a whisker-related matching pattern of afferents in dPrV, albeit still able to induce maxillary-like molecular features resulting in vPrV final targeting. These results provide novel insights into the relative importance of periphery-dependent versus periphery-independent mechanisms of trigeminal ganglion and brainstem patterning in matching facial whisker topography in the brainstem.
INTRODUCTION

Relay of somatosensory stimuli from the body surface to higher brain centers is highly organized allowing the sensing of positional origin of an input. Facial somatosensory inputs are serially relayed through the trigeminal circuit to the brainstem, thalamus and neocortex. The trigeminal circuit is somatotopically organised, such that topographic maps of connectivity matching the distribution and density of sensory receptors of facial dermatomes are generated at all levels of the pathway (Erzurumlu and Killackey, 1983; Erzurumlu et al., 2010; Ma, 1991; Ma, 1993; Ma and Woolsey, 1984; Schlaggar and O'Leary, 1993; Van Der Loos, 1976; Woolsey and Van der Loos, 1970).

Distinct facial dermatomes are innervated by the peripheral axonal processes of trigeminal ganglion (TG) primary sensory neurons, whose central axons form the trigeminal nerve (nV) and project to innervate second order neurons in the brainstem trigeminal column, composed of the rostral principal (PrV) and the caudal spinal (SpV) sensory nuclei. TG neurons bridge the facial sensory periphery and the brainstem where facial maps are first formed. During prenatal development, somatotopic segregation of TG cell bodies contributes to the segregation of the trigeminal nerve into its three main divisions - the mandibular, maxillary, and ophtalmic branches, which peripherally innervate the corresponding facial dermatomes (Arvidsson and Rice, 1991; Erzurumlu and Jhaveri, 1992; Erzurumlu and Killackey, 1983; Erzurumlu et al., 2010; Hodge et al., 2007).
In mouse, the largest portion of the facial somatosensory map is devoted to the representation of mystacial whiskers which are organised into five rows of four to seven follicles at invariant positions on the snout. Whisker inputs are somatotopically mapped at each level of the pathway as spatially ordered neuronal modules, called barrelettes (brainstem), barreloids (thalamus), and barrels (cortex) (Ma and Woolsey, 1984; Van Der Loos, 1976; Woolsey and Van der Loos, 1970) reproducing facial whisker distribution.

The central axons of the mouse trigeminal nerve divisions start sending radially oriented collaterals at about E14.5 to innervate the PrV and SpV brainstem nuclei, and at about E16.5 begin to arborize forming dense terminals (Erzurumlu et al., 2006; Ozdinler and Erzurumlu, 2002). In the developing PrV, mandibular axon collaterals selectively target the dorsal portion (dPrV) whereas whisker-related afferent collaterals preferentially target the ventral portion (vPrV) with a dorso-ventral row-specific organisation (Erzurumlu and Killackey, 1983; Erzurumlu et al., 2010; Hodge et al., 2007; Oury et al., 2006; Xiang et al., 2010; this study). Thus, the spatial segregation of collateral targeting by distinct trigeminal divisions in PrV provides an early template to build topographic equivalence between the face and the brainstem.

To what extent peripheral signals and/or patterns are sufficient to impose a central somatotopic pattern is still debated. One approach to understand a potential instructive role of the periphery in imposing a central somatotopic pattern has been to manipulate the number and/or spatial organization of whiskers within the whisker pad. Such peripheral changes were reflected on the somatotopy of the barrel map (Ohsaki et al., 2002; Van der Loos et al., 1984). Moreover, retrograde signalling from the developing face was shown to be involved in establishing spatial patterns of gene expression in the TG and transferring somatotopic information to the brainstem (da Silva et al., 2011; Hodge et al., 2007). Such experiments seemed to indicate an instructive role of signalling from facial receptors to establish ordered
connectivity at the peripheral and central level for somatotopic map generation.

On the other hand, the early steps of cortical development and arealization rely on intrinsic patterning mechanisms that are independent of sub-cortical input relaying information from the periphery (Cohen-Tannoudji et al., 1994; Grove and Fukuchi-Shimogori, 2003; Lopez-Bendito and Molnar, 2003; O'Leary et al., 2007; Sur and Rubenstein, 2005). Furthermore, recent studies have begun to uncover some of the intrinsic molecular mechanisms underlying the establishment of somatotopic organization at brainstem level (Erzurumlu et al., 2010; Oury et al., 2006). Thus, while facial pattern provides a template for map formation, it is still unclear whether it is sufficient to impose central somatotopic pattern. Moreover, the relative importance of facial pattern to organise TG and central topography of connectivity at pre-natal stages, before facial maps are apparent at sub-cortical and cortical levels, is still poorly understood.

By using multicolor tracing methods, we first determined that a coarse row- and single whisker-specific somatotopic map is built in somatosensory brainstem nuclei already from the onset of the targeting process at pre-natal stages, with a relative degree of precision and peripheral positional discrimination. To investigate the importance of peripheral organisation in imposing a topographic pattern we took advantage of mutant mice exhibiting an ectopic whisker pattern on the lower jaw. In Edn1 null mutant mice, the lower jaw is morphologically transformed into an upper jaw-like structure, including a partial duplication of the whisker pad with a second set of ordered whisker rows located in ectopic, mandibular position (supplementary material Fig. S1A) (Clouthier et al., 1998; Kurihara et al., 1994; Ozeki et al., 2004).
The Edn1 mutation is lethal at birth, thus preventing the analysis at postnatal stages. Nonetheless, we found that, in Edn1 mutants, the ectopic whisker pad is innervated by mandibular, though not maxillary, TG neurons whose central axon collaterals initially targeted the dPrV. However, unlike maxillary neurons, ectopic whisker-innervating mandibular neurons and pre-target axons did not segregate into a row-specific pattern nor targeted the dPrV with a topographic pattern. Following periphery-driven molecular re-patterning to a maxillary-like identity, mandibular neurons redirected their central projections from dorsal to ventral PrV. Thus, a spatially ordered lower jaw ectopic whisker pattern is not sufficient to impose pre-target organization of the mandibular tract nor a whisker-related matching pattern in dPrV, but it is still able to induce maxillary-like molecular features resulting in vPrV final targeting.
MATERIAL AND METHODS

Mouse strains
Edn1\textsuperscript{tm1Utj} (referred as Edn1\textsuperscript{+/−}) homozygous mutant mice were described (Kurihara et al., 1994). Each in situ hybridisation and tracing experiment at any given stage was carried out on at least $n=3$ Edn1\textsuperscript{−−} mutant and control embryos and fetuses; some tracing experiments were carried out on $n=4$ or $n=6$ mutant and control fetuses, respectively, as indicated in the main text. All animal experiments were approved by local veterinary authorities and conducted in accordance with the Guide for Care and Use of Laboratory Animals.

Simple and double in situ hybridization
Embryos were dissected and fixed in 4% paraformaldehyde/ PBS overnight at 4°C. Tissues were cryo-protected in 20% sucrose and embedded in gelatine 7.5%/10% sucrose/PBS. Cryostat sections (20-25 μm) in coronal and sagittal orientations were frozen at -80°C until processing. For chromogenic in situ hybridization, the OC1, Tbx3, and Hmx1 (Hodge et al., 2007) probes were a gift from F. Wang. Cdhl3 probe: nucleotide 1139-2145 from coding sequence was cloned into pCRII-TOPO vector using a TOPO TA cloning kit (Life Technologies, Carlsbad, CA, USA. Fluorescent in situ hybridisations were performed using RNAscope manufacturer’s protocol (Advanced Cell Diagnostics, Inc., Hayward, CA) In brief, sections were air dried for 30min at RT, and a hydrophobic barrier was drawn around section with an Immedge hydrophobic barrier pen. Sections were incubated 20min with pre-treated solution 4 at RT in a humid chamber, washed twice with PBS and then incubated with the mixture of probes Tbx3 and Hmx1, 2H at 40°C in an oven. cDNA probe sets were designed and generated by ACD company. Targeted sequences were: Mm-Tbx3-C1: nucleotides 460-1597 of accession number NM_011535.3 and Mm-Hmx1-C2: nucleotide 109-1483 of accession number NM_010445.2. POL2RA, a protein expressed in mammalian cell was used as positive control, and probe against E. coli DapB (not expressed in mammalian cells), was
used as negative control (Data not shown). Sections were then incubated with preamplifier and amplifier probes by applying AMP1 (40°C for 30 min), AMP2 (40°C for 15 min), and AMP3 (40°C for 30 min), followed by incubation with AMP4 AltA (40°C, 15 min), containing fluorescently labelled probes to detect respectively Tbx3 RNA in green (Alexa 488 nm), and Hmx1 RNA in orange (Alexa 550 nm). A final incubation of slides with Dapi for 30s was performed before mounting them with Prolong Gold antifade reagent. Imaging of fluorescent signals was performed using an Axio imager Z2 upright microscope coupled to a LSM700 Zeiss laser scanning confocal at 40x. Maximum intensity projections and stitching of double fluorescent in situs were performed using Zen Software.

**Immunohistochemistry**

Cryo-section were immuno-labelled with a rabbit primary antibody against Active Caspase-3 (Promega; A-11011, 1:500 dilution), and a Goat Alexa 568 conjugated secondary antibody, counterstained with Dapi and mounted with Prolong Gold antifade reagent.

**Cytochrome oxidase staining**

Cytochrome oxidase (CO) histochemical staining, 40 μm cryostat sections were cut in the coronal plane to visualize barrelettes at brainstem levels. The CO staining was performed according to the procedure described in (Wong-Riley, 1979).

**NeuroVue® dye-coated filters labeling**

NV Red (FS-1002), NV Maroon (FS-1001), NV Jade (FS-1006) NeuroVue® dye-coated filters were from Molecular Targeting Technologies, Inc. (West Chester, PA). Embryos were fixed in PBS–PFA4% overnight at 4°C. Small pieces (<1mm²) NeuroVue® filter dyes were cut and placed into the specimen at specific locations. Embryos were incubated at 37°C for 2 weeks (E10.5, E11.5), 3 weeks (E12.5), 4 weeks (E14.0, E14.5) and 6 weeks (E16.5, E17.5) in PBS–PFA4%. Diffusion of staining around the injection sites was monitored using a MacroFluo Z6 APO (Leica Microsystems). Whole-mount brains were dissected keeping
trigeminal ganglion attached to the brain and imaged. Flat-mount preparations of E10.5 and E11.5 embryos were obtained by dissecting the neural tube, mounted in PBS between a slide and a cover slip and imaged. Older embryos were embedded into 4% agarose. 50-100 μm vibratome sections were mounted in Aqua-Poly/mount (Polysciences, Inc.) and analyzed under MacroFluo Z16 APO and confocal microscope (LSM700, ZEISS).

**Retrograde labeling of single whisker with dextran**

Biotin-conjugated lysine-fixable dextran (Invitrogen, D-7135) was employed for retrograde labelling of specific whisker rows in E13.5 mouse embryos. Dextran crystals were prepared according to (Stirling et al., 1995). E13.5 embryos were dissected and labelled with Dextran crystals in given whisker follicle, cultured for 6 hours as described in (Oury et al., 2006; Stirling et al., 1995) then fixed in PBS–PFA4% and prepared for cryo-section as previously described. 25μm sagittal sections were collected and processed for chromogenic in situ hybridization against Cdh13. Biotin was revealed afterward using fluorescent conjugated streptavidin, mounted and analyzed under fluorescent microscope (Olympus).

**Image analysis**

Brightness, contrast and gamma (not for ISH) were adjusted in Adobe Photoshop 2015 for better visualization and figures assembled in Adobe illustrator 2015. Quantification of double fluorescent in situ in Fig. 2 was realized using Slidebook 6. Briefly, after background substraction of the image, an automatic threshold was used to determine the percentage of positive surface area (i.e. pixels above threshold) belonging to the hand-drawn mandibular area. Average and T-Test statistics were made using Excel.
RESULTS

Whisker-related patterns of axon targeting are established prenatally in the hindbrain trigeminal nuclei

Distinct rows in the whisker pad are innervated by neuronal populations with segregated latero-medial distribution within the TG maxillary portion (da Silva et al., 2011; Erzurumlu and Jhaveri, 1992; Erzurumlu and Killackey, 1983; Hodge et al., 2007), whereas barrelette topography is not prefigured by a pre-ordered position of neurons within row-specific TG sub-populations (da Silva et al., 2011). We therefore investigated the establishment of whisker-specific afferent pattern in the developing brainstem and carried out row- and whisker-specific neuronal tracings with multicolour lipophylic NeuroVue® fluorescent dyes, that allow controlled diffusion by progressive dye release from a filter membrane (Jensen-Smith et al., 2007).

By applying tiny pieces of NeuroVue® coated filters we simultaneously traced selected subsets of nerve endings innervating individual rows at E14.0. Single row-innervating primary neuron cell bodies segregated along the latero-medial axis of the maxillary TG portion (Figure S2E). Furthermore, each set of single row-innervating nerve fascicles remained segregated in their central projections with a row-specific topography along the dorso-ventral axis of the trigeminal tract maxillary division (Figure S2A-E). These results confirmed a high degree of order in the trigeminal peripheral system during prenatal development (Erzurumlu and Killackey, 1983) and supported the validity of our tracing procedure.

To investigate the spatial arrangement of whisker-specific central collateral targeting, we simultaneously labelled distinct antero-posterior whisker follicle positions in different rows of E14.5 and E17.5 whisker pads (Figure S2F-I). The insertion of NeuroVue® filters at single follicles allowed targeting of the follicle terminals and just their close neighbours. To
improve tracing precision, we selectively labelled whiskers at distinct positions where they are bigger and less densely spaced (position 1-5) (Figure S2J). Along the dorso-ventral axes of the vPrV and SpVi nuclei, collaterals maintained the row-specific somatotopy observed in the trigeminal tract (Figure S2F,H and not shown; Xiang et al., 2010). Notably, collaterals from afferents innervating anterior whiskers projected deeper medially into the vPrV nucleus than collaterals of posterior whisker positions (colour bars, Figure S2F,H). The early somatotopic pattern of whisker-specific collateral targeting was maintained and further refined by E17.5 (Figure S2G,I). At this stage, collaterals showed dense arborization prefiguring maturation of the final whisker map and barrelette formation as observed by cytochrome oxidase staining at post-natal stages (P6, Figure S2K).

In summary, these results further extend previous work. They show that the topographic mapping of row-specific and single whisker-specific afferent targeting in brainstem nuclei is established at the onset of collateralization with a relative degree of precision and peripheral positional discrimination and refined throughout late prenatal and early postnatal stages.

**Ectopic whisker arrays in Edn1 mutants are innervated by trigeminal mandibular primary neurons**

We first asked whether the ectopic whisker pad of Edn1 null mutants is innervated and by which TG division. We carried out triple retrograde labelling at E14.5 and applied NeuroVue® at the ectopic whisker pad on the mutant lower jaw and at two dorsoventral positions in the maxillary whisker pad (Figure 1C,D). The retrogradely traced neurons innervating the ectopic whiskers were located in the mandibular portion of TG and were segregated from those in the maxillary division wiring the normal whisker pad (Figure 1E,F). Thus, the ectopic whisker pad on the lower jaw of Edn1 mutants is not able to attract and redirect the peripheral projections of maxillary primary sensory neurons.
Furthermore, the central axon of neurons innervating the duplicated whisker pad did not intermingle with the axons of the neurons innervating the maxillary whisker pad and maintained a dorsally segregated position in the trigeminal tract of Edn1⁻/⁻ fetuses as do mandibular axons in WT (Figure 1G,H).

**Induction of maxillary-like molecular features in Edn1 mutant mandibular primary neurons upon ectopic whisker pad innervation**

BMP4 and TGF-β retrograde signalling from the whisker pad/follicles has been suggested to be involved in organising topographic patterns of TG neuron central connectivity (da Silva et al., 2011; Hodge et al., 2007). In particular, BMP4-dependent signalling regulates early positional differences of Tbx3 and Hmx1 transcription factor expression in distinct divisions of the TG (Hodge et al., 2007). Bmp4 expression is readily detected in the duplicate arrays of whisker primordia on the Edn1 mutant lower jaw (Ozeki et al., 2004). We therefore asked whether maxillary-like spatial patterns of transcription factor expression were ectopically induced in the mandibular division of Edn1 mutants upon innervation of the duplicated whisker pad.

In wild-type (WT) E11.5 and E13.5 TG, Hmx1 and OC1 are selectively expressed in the mandibular division, whereas Tbx3 is highly expressed in ophthalmic and maxillary neurons with only sparse expression in mandibular neurons (Figure 2B-D; Hodge et al., 2007). In Edn1⁻/⁻ mutants, expression of the general marker Drg11 was normally maintained throughout the TG (Figure 2A,E). In contrast, Tbx3 was induced in a larger number of mandibular neurons than in WT, while Hmx1⁺ and OC1⁺ cells were concomitantly reduced (Figure 2A-H). This was further confirmed with Hmx1/Tbx3 double fluorescent in situ hybridisation (FISH) and quantified (Figure 2I-K). This effect was not due to increased cell death in the mandibular TG component, as assessed by activated caspase-3 immunostaining (Figure S3A,B). Moreover, no Tbx3 expression differences were observed at E10.25 between
WT and Edn1−/− TG neurons (Figure S3G,L), indicating that the molecular changes observed in E11.5 Edn1−/− mandibular neurons are induced as their axons grow into the duplicated whisker pad.

To further support a periphery-induced re-patterning of mandibular neurons in Edn1 mutants, we next searched for additional TG maxillary-specific expression markers in the Allen Developing Mouse Brain Atlas (http://developingmouse.brain-map.org). At E13.5, cadherin-13 (Cdh13) is selectively expressed in the maxillary TG division, while it is excluded from the mandibular division (Figure 2M). By combining retrograde dextran tracing of row-specific afferents and Cdh13 in situ hybridisation at E13.5, we further assessed that Cdh13 is preferentially expressed in TG neurons innervating row C-E (Figure S3C-F). As these rows are duplicated in a mirror pattern in Edn1 mutants (Ozeki et al., 2004) (Figure S1), we assessed Cdh13 expression in E13.5 mutant TG. Notably, Cdh13 was ectopically induced in the mandibular division of Edn1 mutants (Figure 2Q).

In summary, these findings demonstrate that positional molecular differences are induced in Edn1−/− mutant TG, leading a subset of mandibular neurons to acquire molecular features normally expressed by maxillary primary sensory neurons innervating mystacial whiskers.

The ectopic whisker pad is not sufficient to impose maxillary-like topography to mandibular central axons in Edn1 mutants

We next asked whether the observed molecular changes were sufficient to induce corresponding maxillary-like changes in the central topography of TG mandibular neurons in Edn1−/− fetuses. We assessed whether the tract innervating the duplicated arrays of whiskers in Edn1−/− mutants also become somatotopically organized, similar to the maxillary tract organization (Figure S1). In WT fetuses (n=4/stage), NeuroVue® labelling of two distinct positions at E14.5 and E16.5 on the lower jaw revealed intermingled non-segregated primary
neuron cell bodies in the mandibular TG division (Figures 3C, S4B). This was further depicted in the lack of spatial segregation of mandibular afferent central axons (Figures 3E, S4C-D), contrasting with the strict somatotopic organisation of the maxillary tract (n=4/stage) (Figures 1C, S2E).

Similarly, in E14.5 and E16.5 Edn1 mutant fetuses (n=4/stage), despite the ectopic whisker arrays on the lower jaw, NeuroVue® labelling of two distinct ectopic row positions (Figures 3B, S4E) also resulted in a non-segregated distribution of retrogradely labelled cell bodies in the TG (Figures 3D, S4F) and a lack of somatotopic organisation of central axons (Figures 3F, S4G,H), similar to the WT mandibular branch organisation. Thus, the maxillary-like molecular changes induced in neurons innervating the duplicated whisker rows in Edn1 mutants (Fig. 2) are not sufficient to impose a maxillary-like topography to their central afferents.

**Ectopic whisker-innervating afferents initially project in dPrV without generating ordered collateral patterns but redirect their projection to vPrV at late prenatal stages**

We then investigated the central pattern of collateral targeting in E14.5 Edn1 mutants. In WT fetuses, mandibular TG axons project radially oriented collaterals into rhombomere (r)2-derived dPrV, whereas r3-derived vPrV selectively receives collateral input from whisker-related afferents (Oury et al., 2006). These distinct populations of targeting collaterals remain spatially segregated within the PrV and never cross each other (Figures 1H, 3G, 4C-D,I-J) (Oury et al., 2006). In Edn1 mutants, axon afferents from the duplicated whisker arrays did not project collaterals into the vPrV area normally hosting the whisker map but targeted the dPrV area normally generating the lower jaw map (Figure 1J). Moreover, labelling of distinct peripheral positions on the lower jaw did not result in spatially segregated collaterals along either the row-specific dorso-ventral or whisker-specific latero-medial axes.
in the dPrV (Figure 3H), unlike what observed with the whisker-related collateral topographic mapping in the vPrV (Figure 1I, S2F,G,H,I).

Remarkably, unlike at E14.5, at E16.5 ectopic whisker-innervating Md* collaterals turned from dPrV (and dorsal SpV), and navigated into vPrV (and ventral SpV) (arrows, Fig. 4G,H,O,P). Upon labelling of two distinct ectopic row positions no clear sorting of collaterals navigating into vPrV or ventral SpV was observed (arrows, Figure S4G-H). The fraction of collaterals attracted ventrally was variable (roughly 5-40%; see Figures 4G-H, S4G-H). This variability likely depended on the precision/amount of labeling of the ectopic whisker follicles vs. the surrounding lower jaw tissue in each experiment and/or on the extent of mandibular neuron repatterning among distinct Edn1−/− mutant individuals. Nonetheless, such a mis-targeting behaviour of Md* collaterals was observed in all mutants (n=6/6) and never in wild type (n=6/6) (quantified in Figure 4I,J).

Thus, the Md* primary neurons innervating the duplicated whisker pad in Edn1 mutants initially project central axon collaterals into dPrV, similar to normal lower jaw input. Despite the observed maxillary-like molecular changes as early as E11.5 (Figure 2), these Md* incoming afferents are unable to generate ordered ectopic whisker-related patterns of collateral targeting in dPrV, unlike the maxillary whisker afferents in vPrV. However, the molecular changes in Md* TG neurons may enable ectopic whisker afferents to respond to late vPrV-specific targeting cues prior to barrelette formation (Figure 4K-L).
DISCUSSION

Brainstem whisker maps develop through an interplay between peripheral signals, intrinsic pre-patterning of TG neurons with their axonal processes, and brainstem target neurons, establishing a prenatal coarse topographic connectivity map. Further refinement by whisker-related activity and/or retrograde molecular signalling from periphery allows to achieve final one-to-one topography at postnatal stages. Recent studies addressed the importance of facial signals to refine central projections (da Silva et al., 2011; Hodge et al., 2007) while others highlighted the importance of brainstem patterning to receive appropriate and spatially restricted peripheral projections and allow topographic representation (Erzurumlu et al., 2010; Oury et al., 2006). It is still unclear however, the relative importance of facial signals vs. TG pre-target axon sorting vs. central pre-patterning in the building of topographic equivalence between the face and the brainstem. In this study, we asked whether an ordered array of whisker receptors on the face is sufficient to impose a matching topographic connectivity into the brainstem, in order to unveil the relative contributions of specific TG neuron pre-sorting events and/or central pre-patterning.

A few main conclusions can be drawn from our study. Firstly, we provide evidence that, in addition to a row-specific somatotopy of maxillary pre-targeting axons, a single-whisker map of afferent collateral targeting is set out in vPrV from the outset of the collateralization process with a significant degree of precision and peripheral positional discrimination which is progressively refined through late prenatal and postnatal stages (Figure S2). Thus, postnatal activity-dependent refinement processes may only act locally to stabilize pre- and post-synaptic elements but they are not the main contributors to build an ordered whisker-specific topography within the brainstem sensory nuclei. This is supported by the analysis of NMDA receptor knockout mice, in which barrelette neuron dendrite
remodelling and pattern is impaired but the general topographic organization of afferents is maintained in mutant PrV (Lee et al., 2005).

Secondly, we show that the central axons of TG mandibular neurons innervating distinct lower jaw surface positions display poor spatial segregation within the mandibular tract, as do their collateral patterns in dorsal PrV. Our analysis of Edn1 mutants further demonstrate that the ectopic whisker arrays are selectively targeted by the mandibular branch, according to their position on the lower jaw, and that this innervation in turn induces significant maxillary-like molecular changes in mandibular TG neurons, supporting whisker-specific retrograde signalling (da Silva et al., 2011; Hodge et al., 2007). However, such molecular changes are not sufficient to instruct a maxillary-like, row-specific, pre-target axon sorting of the mandibular tract; nor to instruct the establishment of row- and/or whisker-related patterns of collateral targeting in the dorsal ‘mandibular’ area of PrV. Together, these findings highlight a fundamental intrinsic pre-ordering difference between TG maxillary and mandibular primary axons which will be important to address in future studies. Pre-ordering of somatosensory thalamocortical axons is essential for the transfer of precise topographic equivalence between thalamic and cortical whisker maps (Lokmane et al., 2013). Our current results suggest that maxillary neuron pre-target axon pre-ordering (Erzurumlu and Jhaveri, 1992; Erzurumlu and Killackey, 1983; reviewed in Erzurumlu et al., 2010) may be intrinsically organized, independently of facial or brainstem influences, and may be an important spatial requisite to match intrinsic positional information in the vPrV (see summary diagram in Figure 4).

Lastly, even though ectopic whisker-innervating mandibular axons initially target the dPrV, following periphery-driven repatterning their collaterals turn and navigate from dorsal to vPrV converging into the whisker-related area already occupied by the processes of maxillary neurons innervating the normal whisker pad on the snout (Figure 4). These latter
findings unveil a temporal sequence of events underlying the transfer of peripherally-induced whisker-specific information to the brainstem and, on the other hand, suggest the importance of a vPrV-specific intrinsic patterning program to match whisker-specific input. Further studies will be required to address the molecular determinants of such a program. In this respect, it is however noteworthy that Hoxa2 displays a differential expression pattern between dPrV and vPrV, and has an important role in prenatal PrV patterning (Oury et al., 2006). Hoxa2 is expressed in all PrV mitotic progenitors but differentially maintained in the post-mitotic neurons of vPrV, though not dPrV (Oury et al., 2006). Moreover, Hoxa2 is not expressed in TG neurons or facial mesenchyme (Oury et al., 2006). The early dorso-ventral differential expression of Hoxa2 regulates the specific targeting of mandibular versus maxillary trigeminal axon collaterals between the dorsal and ventral components of the developing PrV (Erzurumlu et al., 2010; Oury et al., 2006). In particular, conditional Hoxa2 inactivation selectively impaired whisker-related afferent targeting in the vPrV (Oury et al., 2006), indicating that Hoxa2 may regulate the expression of whisker afferent collateralization factor(s) in vPrV. Brainstem maturation is a critical regulator of the onset of arborization of trigeminal ganglion afferents in brainstem nuclei (Erzurumlu et al., 2010; Ozdinler and Erzurumlu, 2002). Prenatal row- and whisker-specific afferent topographic collateralization could be under the control of collateral-target interactions mediated by differentially expressed surface-bound and/or secreted guidance molecules (Erzurumlu et al., 2010). In this regard, Hoxa2 has been shown to positively regulate Epha4 and Epha7 expression in vPrV (Oury et al., 2006). Moreover, our finding that Cdh13 displays row-related expression in TG neurons suggests that matching adhesive cues between central afferent axons and brainstem target neurons might also contribute to topographic collateralization into vPrV. Lastly, dPrV is devoid of Hoxa2 expression at the stage of collateral formation (Oury et al., 2006). Thus, the lack of topographic patterns of collateral targeting observed in Edn1 mutant dPrV, which
receives selective input from the duplicated whisker arrays (Fig. 4), might be indeed in part accounted for the lack of a Hoxa2-dependent patterning program that normally instructs prenatal afferent targeting topography and establishment of whisker-related patterns in vPrV.

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COMPETING INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

CL performed most of the experimental work, quantifications, and documentation. AB carried out several tracing experiments, identified the Cdh13 marker, some quantification experiments, and additional unpublished results relevant for the study. NV carried out several single and double in situ hybridisations, and relative documentation. CL, AB, NV, and FMR designed and analyzed the experiments. YK and HK provided extensive access to and production of essential biological samples, discussions throughout the work, and comments on draft. FMR conceived the study, and CL and FMR wrote the manuscript.
REFERENCES


Figure 1 Ectopic whisker pad innervation in Edn1 null mutant fetuses

(A-J) E14.5 wild type (A,C,E,G,I) and Edn1⁻/⁻ (B,D,F,H,J) fetuses showing labeling of mandibular (Md) (blue), lower (green), or upper (red) maxillary (Max) whisker row positions.
(B,C). (C, inset), Edn1<sup>−/−</sup> ectopic whisker pad (Md*) innervation, summarized in (A,B). (E,F) Trigeminal ganglion (TG) coronal section showing mandibular neurons retrograde tracing innervating wild-type mandible (Md) (E) and ectopic Edn1<sup>−/−</sup> whisker pad (Md*) (F). (G) Lateral view showing wild type dorsoventral segregation of central trigeminal axon bundles relaying distinct Max and Md positions. (H) Ectopic whisker pad (Md*) innervation in Edn1<sup>−/−</sup> fetuses does not change pretarget central axon ordering within the tract. (I,J) Principal nucleus (PrV) coronal sections showing similar dorsoventral distribution of central afferent collaterals in WT (I) and Edn1<sup>−/−</sup> (J) embryos. Note segregation of mandibular (blue) and maxillary (green and red) inputs in dorsal and ventral PrV, respectively. Scale bar in C is 200µm, E and G is 100µm, I is 50µm and represents (C,D), (E,F), (G,H), and (I,J).
Figure 2 Induction of maxillary-like molecular features in Edn1 mutant mandibular primary neurons (A-H, L-O) Sagittal sections through trigeminal ganglion (TG) mandibular (md), maxillary (max), and ophthamic (oph) divisions at E11.5 and E13.5 in wild-type (A-D,L-M) and Edn1⁻/⁻ (E-H,N-O) littermates hybridized with Drg11 (A,E,L,N), Tbx3 (B,F),
Hmx1 (C,G), OC1 (D,H), and Cdh13 (M,O) probes. In Edn1 mutant md (md*) (F-H, O), Tbx3 expression is increased (black arrowheads), Hmx1 and OC1 (white arrowheads) decreased, and Cdh13 ectopically induced (black arrowheads). (I-K) Hmx1 and Tbx3 double fluorescent in situ hybridization and quantification of positive area showing significant Hmx1 signal reduction and Tbx3 increase in wild-type (I) and Edn1<sup>+/−</sup> (J) littermates at E11.5. Note that the yellow/orange bright spots correspond to aspecific background due to blood vessel autofluorescence (e.g. asterisk in I). (K) Result quantification. The graphs represent the average of percentage of positive pixels above threshold within the mandibular area with standard deviation (wild type, n=33 sections; Edn1<sup>+/−</sup> n=29 sections; from 5 embryos each). Scale bar in A, I is 50µm and L is 100µm and represents A-H, I-J and L-O respectively.
Figure 3 Poor topographic organisation of mandibular trigeminal nerve branch and central axon projections in wild-type and Edn1⁻/⁻ fetuses

(A-H) Labelling of distinct positions of mandibular process in E14.5 wild type (A,C,E,G) and Edn1⁻/⁻ (B,D,F,H) fetuses. (A,B) Lateral face view with row E (blue), anterior-dorsal (green)
and posterior-ventral (red) mandibular position labeling in wild-type (A) and Edn1<sup>−/−</sup> (B). (C-F) Trigeminal ganglion (TG) coronal sections (C,D) and whole-mount trigeminal tract lateral views (E,F). In wild-type (C,E) and Edn1<sup>−/−</sup> (D,F) fetuses, neurons innervating distinct lower jaw positions show no clear spatial segregation of cell bodies in TG mandibular division (Md and Md*, respectively) nor of their central axons within the mandibular tract. (G,H) Principal nucleus (PrV) coronal sections of wild-type (G) and Edn1<sup>−/−</sup> (H) fetuses showing lack of targeting collateral topographic organisation of sensory neurons innervating the two distinct positions labeled on the mandibular process in (A) and (B). Scale bar: A is 200 µm, C and E are 100 µm, G is 50 µm and represents A-B, C-D, E-F and G-H.
Figure 4 Late redirection of mandibular central axon projections from dorsal to ventral PrV and SpV in Edn1<sup>−/−</sup> fetuses

(A-H) Distinct position labelling of maxillary (Max) and mandibular (Md) facial processes in E16.5 wild-type (B-D) and Edn1<sup>−/−</sup> (F-H) fetuses. (A,E) Diagram of dye labelling of lower maxillary (red) and mandibular (green) positions corresponding to sections (B-D,F-H).
Trigeminal ganglion (TG) (B,F), principal nucleus (PrV) (C,G) and spinal nucleus (SpV) (D,H) coronal sections. In Edn1<sup>-/-</sup> (G-H), ectopic whisker-innervating mandibular (Md*) axon collaterals targeting dPrV and dorsal SpV turn ventrally into vPrV and ventral SpV (arrows).

(I) Method for phenotype quantification. An axis was set parallel to the lateral border of the PrV (black dashed line). The dorsal-most position of maxillary collaterals (red dashed line) was set as 0. The ventral-most position reached by mandibular collaterals (green dashed line) was measured on the axis, and counted as negative for a gap or as positive if invading the maxillary collateral domain (considering the furthest axon invading the inappropriate area).

(J) Quantification graph (wild type, n=19 sections; Edn1<sup>-/-</sup>, n=15 sections; from 5 embryos each). (K) Diagram of trigeminal pathway somatotopy at E14.5-E16.5. The trigeminal nerve is dorsoventrally sorted in ophthalmic (Oph), maxillary (Max) and mandibular (Md) branches. In trigeminal ganglion (TG), Max neuron cell bodies additionally display whisker row-specific lateromedial spatial segregation. Row- and whisker position-specific information is coarsely topographically mapped by Max neuron collaterals emerging from central sensory tract along dorsoventral and mediolateral axes of ventral PrV, respectively, as early as E14.5 and progressively refined. In contrast, TG Md neurons innervating distinct mandible positions, their central axons, and dorsal PrV collateral targeting display poor somatotopic segregation. C1 (orange dot) and C7 (red dot) whiskers illustrate whisker-specific distribution of afferent collaterals targeting the latero-medial axis of PrV. (L) Schematic depicting Edn1<sup>-/-</sup> phenotype and molecular changes. In Edn1<sup>-/-</sup> fetuses, spatially ordered ectopic whiskers are not sufficient to induce row-specific axon sorting in the TG mandibular branch nor of its central projections that initially target dorsal PrV. Nevertheless, upon innervation of ectopic whiskers, Md neurons acquire molecular features of Max neurons (Md*) that, at later stages, lead Md* collaterals to navigate out of the ‘lower jaw map’ area in dorsal PrV and enter the ‘whisker map’ area in ventral PrV. Scale bar in B is 50 µm and represents B-D, F-H.
Supplementary Figure S1. Morphology and innervation of ectopic whisker pad in Edn1-/- embryo

(A) Face of Edn1-/- mouse at E18.5 showing homeotic transformation of distal mandibular process into maxillary-like structure, including the partial duplication of the whisker pad and three ectopic rows (blue arrows). (B) Transverse section through the ectopic whisker pad of Edn1-/- embryo at E14.5, which mandibular branch of the trigeminal nerve was labelled with NeuroVue®, showing innervation (red) around the ectopic whisker follicles (asterisks). Scale bar 100 and 50µm in A and B respectively.
Supplementary Figure S2. Topographic segregation of facial sensory inputs during development

(A-B) Ventral view of a flatted neural tube after labelling of the face in mandibular (Md-green) and maxillary (Max-red) presumptive domain at E10.5 (A) and labelling of the mandibular process (Md-green), and in lower (Max-red) and upper (Oph-blue) positions of the upper jaw at E11.5 (B). Central projections of trigeminal mandibular neurons are the first to reach posterior hindbrain at E10.5 quickly followed by the rest of trigeminal neurons projections at E11.5 showing a differential timing of elongation, summarized in (C). (D) Whole mount view of E12.5 embryo after labelling in mandibular (Md-green), lower (Max low-red) and upper (Max up-blue) whisker pad areas showing maintenance of sensory inputs segregation from the periphery to the brain. (E) Sagittal view of E14 embryo after labelling of rows A, C and E in the whisker pad showing a
ventro-dorsal segregation of maxillary inputs within the brainstem somatosensory tract and also latero-medial segregation of trigeminal neurons within TG (inset). (F-K) Coronal sections of the hindbrain at PrV level after labelling in positions A1-C3-E5 (green-red-blue) (F, G) and positions A4-C4-E4 (green-red-blue) (H, I) on the whisker pad at E14.5 (F, H) and E17.5 (G, I) respectively. Collaterals sent by sensory axons reach different latero-medial positions in the nucleus according to their peripheral antero-posterior location within a row (J). This collateralization process take place in respect of the whisker position in the mature map of the PrV nucleus revealed at P6 with cytochrome oxidase staining (K). Scale bar: A is 25µm, B is 50µm, D is 200µm, E, F, G and K are 100µm. F and G represents F and H and G and I respectively.
Supplementary Figure S3. Supporting information for molecular changes observed in TG of Edn1−/− embryos

(A-B) Immunochemistry against Caspase-3 of sagittal sections through TG of control (Ctrl) (A) and Edn1−/− embryos (B). No increase in staining was observed at E11.5 in the mandibular portion of the TG (dash line) in Edn1−/− following innervation of ectopic array of whiskers. (C-F) in situ hybridisation for Cdh13 on sagittal section through TG of Ctrl embryos at E13.5 previously labelled with Biotin-conjugated Dextran in row A (C,E) and E (D,F). Overlay of in situ and Dextran show that Cdh13 expression mostly collocalize with TG cell bodies retrogradely labelled from row E (F) compare to row A (E). (G-L) in situ hybridisation on sagittal section through TG of Ctrl (G-I) and Edn1−/− (J-L) embryos at E10.25. Drg11, Tbx3 and OC1 do not display noticeable difference of expression between Ctrl and Edn1−/− embryos. Scale bar: A is 50µm, C is 100µm and G is 25µm and applies for A-B, C-F and G-L respectively.
**Supplementary Figure S4. Lack of segregation of mandibular central axon projections after redirection from dorsal to ventral PrV and SpV in Edn1⁻/⁻ foetuses**

*(A-H)* Labelling of distinct positions of the maxillary and mandibular process at E16.5 in wild type *(B-D)* and *Edn1⁻/⁻* *(F-H)* embryos. *(A,E)* Schematic depiction of peripheral dye labelling of upper(*green*) and lower(*red*) mandibular positions corresponding to sections *(B-D,F-H)*. Coronal sections through TG *(B,F)*, PrV *(C,G)* and SpV *(D,H)*. Ectopic whisker-innervating Md* collaterals turn from dPrV and dorsal SpV, and navigate into vPrV and ventral SpV (arrows, *G,H*). Labelling of two distinct dorso-ventral positions on the ectopic whisker pad shows that Md* neuron cell bodies *(F)* do not acquire a segregated organisation but remain intermingled as wild type Md neuron cell bodies *(B)*. No clear sorting of collaterals navigating into vPrV or ventral SpV is observed (red and green arrows, *G,H*). Scale bar in *B* is 50 µm and represents *(B-D, F-H)*.