Parallel pathways from motor and somatosensory cortex for controlling whisker movements in mice

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Abstract

Mice can gather tactile sensory information by actively moving their whiskers to palpate objects in their immediate surroundings. Whisker sensory perception therefore requires integration of sensory and motor information, which occurs prominently in the neocortex. The signalling pathways from the neocortex for controlling whisker movements are currently poorly understood in mice. Here, we delineate two pathways, one originating from primary whisker somatosensory cortex (wS1) and the other from whisker motor cortex (wM1), that control qualitatively distinct movements of contralateral whiskers. Optogenetic stimulation of wS1 drove retraction of contralateral whiskers while stimulation of wM1 drove rhythmic whisker protraction. To map brainstem pathways connecting these cortical areas to whisker motor neurons, we used a combination of anterograde tracing using adenoassociated virus injected into neocortex and retrograde tracing using monosynaptic rabies virus injected into whisker muscles. Our data are consistent with wS1 driving whisker retraction by exciting glutamatergic premotor neurons in the rostral spinal trigeminal interpolaris nucleus, which in turn activate the motor neurons innervating the extrinsic retractor muscle nasolabialis. The rhythmic whisker protraction evoked by wM1 stimulation might be driven by excitation of excitatory and inhibitory premotor neurons in the brainstem reticular formation innervating both intrinsic and extrinsic muscles. Our data therefore begin to unravel the neuronal circuits linking the neocortex to whisker motor neurons.

Introduction

Mice actively explore their environment using their mystacial whiskers, which they move back and forth at high frequencies (5–20 Hz) to scan the immediate space surrounding their snouts. When a moving whisker encounters an object the whisker bends; this exerts force at its base, driving action potentials in mechanosensitive primary sensory neurons of the trigeminal ganglion. These sensory neurons release glutamate onto postsynaptic neurons in trigeminal brainstem nuclei, from which multiple parallel sensory pathways emerge providing whisker-related tactile sensory information to other brainstem circuits, cerebellar circuits, superior colliculus and the thalamocortical system (Petersen, 2007; Diamond et al., 2008; Bosman et al., 2011). To generate a coherent percept of the environment, the actively acquired sensory information must be processed in the context of motor commands, e.g. to localize an object the mouse must know where the whisker was at the time of whisker–object contact (Curtis & Kleinfeld, 2009). Conversely, sensory signals affect the motor commands controlling whisker movement, e.g. mice rapidly change whisking patterns upon active touch, presumably to enhance information flow (Mitchinson et al., 2007; Crochet et al., 2011). Understanding whisker tactile sensory perception therefore requires the investigation of the interactions between sensory and motor systems (Kleinfeld & Deschenes, 2011). Whereas the pathways for processing whisker sensory information have been the subject of intense investigation, much less is known about the pathways involved in whisker motor control.

The whiskers are moved by both intrinsic muscles (which are entirely located within the mystacial whisker pad) and extrinsic muscles (which attach to the mystacial pad but are anchored externally; Dörr, 1982; Haidarliu et al., 2010). Each intrinsic muscle forms a sling around an individual whisker which, upon contraction, causes the whisker to protract, pivoting around the whisker insertion point in the pad. The rhythmic contraction of intrinsic muscles is thought to be the most important process underlying exploratory whisking (Berg & Kleinfeld, 2003). On the other hand, the extrinsic muscles move the whole whisker pad and are thought to play an important role during whisker retraction (Berg & Kleinfeld, 2003). Intrinsic and extrinsic muscles are both under control of motor neurons located in the lateral facial nucleus (FN) (Klein & Rhoades, 1985; Herfst & Brecht, 2008). Classical retrograde and anterograde tracing revealed a large number of brain regions projecting to (or near to) the lateral FN (Hattox et al., 2002). To specifically label premotor...
neurons, genetically engineered monosynaptic rabies virus (Wickersham et al., 2007) lacking glycoprotein G can be injected into muscle to infect motor neurons expressing rabies G (Stepien et al., 2010). The rabies virus in the motor neurons is thus trans-complemented and can cross one synapse to infect premotor neurons. A previous study has mapped the premotor neurons for whisker muscles using rabies virus expressing fluorescent proteins (Takatoh et al., 2013). Here, we extend these previous findings by analysing the overlap of cortical projections with whisker premotor neurons, revealing distinct circuits for controlling different whisker movements originating from the whisker motor cortex (wM1) and whisker somatosensory cortex (wS1) (Matyas et al., 2010; Petersen, 2014).

Materials and methods

All experiments were performed in accordance with the Swiss Federal Veterinary Office, under authorization 1628 issued by the ‘Service de la consommation et des affaires vétérinaires’ of the Canton de Vaud.

Channelrhodopsin-2 (ChR2) virus injection

Adult (6- to 9-week-old) male and female Emx1-Cre mice [B6.Cg-Emx1<Cre>Flx1(1014)Jae] (RRID: IMSR_JAX:005628); Cre recombinase expressed from the endogenous Emx1 locus] were deeply anaesthetized with isoflurane and the body temperature was maintained at 36 °C by a heating pad. The skull was exposed and the periosteum was removed by gently scraping with a scalpel. The skull was then cleaned with Betadine. A lightweight metal head-post was placed over the craniotomy. The edges were sealed with warm hand cement and dental cement (Matyas et al., 2010). A chamber was made by building a wall with dental cement (Paladur, Heraeus Kulzer, Hanau, Germany) along the edge of the skull. A chamber was made by building a wall with dental cement (Paladur, Heraeus Kulzer, Hanau, Germany) along the edge of the bone covering the left hemisphere. Dental cement was also used to reinforce the attachment of the head-post. Intrinsic signal optical imaging was carried out to map the position of the C2 barrel column in wS1. All whiskers except C2 were trimmed and the chamber over the left hemisphere was filled with warm Ringer’s solution and covered with a glass coverslip. The whisker was deflected at 10 Hz for 4 s with a piezo actuator and the resulting intrinsic signal response was imaged under 630-nm illumination by a CMOS camera (Photon Focus, Lachen, Switzerland; Grinvald et al., 1986). The images were processed with custom routines written in LabVIEW (National Instruments, Austin TX, USA). To express ChR2 in excitatory neurons, we used an AAV2/5 virus expressing double-floxed humanized ChR2 (histidine 134 converted to arginine) fused to enhanced yellow-fluorescent protein (EYFP) under the control of the EF1α promoter [AAV2/5.DIO.EF1α.hChR2(H134R).EYFP, virus made by Penn Vector Core (Philadelphia, PA, USA)]. Injections were targeted either to the C2 barrel column (identified through intrinsic signal optical imaging) or to wM1 at the stereotaxic co-ordinate 1 mm anterior and 1 mm lateral to bregma. A large craniotomy (approximately 3 mm in diameter) was made over wS1 or wM1. The dura was left intact. An outer cladding of one end of the cable (300 and 700 μm below the pia) to infect motor neurons originating from the whisker motor cortex (wM1) and whisker somatosensory cortex (wS1) (Matyas et al., 2010; Petersen, 2014).

Quantification of whisker movement

The contralateral C2 whisker was filmed at 500 Hz with a high-speed camera (Redlake, Tallahassee, FL, USA). Before applying the optogenetic stimulus, a 600-ms baseline period was recorded. All trials where the whisker was moving during this baseline period were discarded. Whisker angle was quantified using custom routines written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA). Power spectral density (PSD) of whisker movements upon 50-Hz blue light stimulation was calculated on a 200-ms time window before the offset of the light stimulus. The PSD was computed by squaring the amplitude spectrum, and then dividing by two times the bin size. The 5- to 20-Hz band (whisking band) power was calculated by integrating the PSD over 5–20 Hz.

Rabies virus injection in the whisker pad

The SAD-AG-Rabies:EGFP or SAD-AG-Rabies:mCherry viruses were produced in B7GG (BIK-B19G2) cells expressing glycoprotein for complementation as described previously (Wickersham et al., 2010; DNA kindly provided by E. Callaway and virus kindly provided by B. Roska). Wild-type mice (male and female) at postnatal day (P)6 were anaesthetized by hyperthermia, by keeping them on ice for 5 min prior to injection. For retrograde tracing of the extrinsic and intrinsic motor neurons in the same animal, one of the SAD-AG-Rabies viruses was injected selectively into the intrinsically nasolabialis muscle (caudal end) and the other was injected into the intrinsic follicular muscle (C2 whisker follicle) on the right side. The volume of virus (titre = 2 × 10⁶ transducing units/μL) injected into both muscles was 1.5 μL. The animals were perfused with 4% paraformaldehyde (PFA) at P8. In a different set of experiments, to confirm the cholinergic identity of labelled motor neurons, 1.5 μL of SAD-AG-Rabies:EGFP was injected into either the extrinsic or intrinsic muscle of ChAT-Cre × (LoxP-Stop-LoxP)-tdTomato animals [B6;129S6-ChATtm1(cres.lov)J] (RRID: IMSR_JAX:006410) × B6;129S6-Gt(Rosa)26Sor<tm1(CAG-tdTomato)Rze> (RRID: IMSR_JAX:007908) Jackson Laboratory, Bar Harbor, ME, USA). For the trans-synaptic labelling experiments, SAD-AG-Rabies was mixed with Herpes Simplex Virus 1-Glycoprotein (HSV1-G) virus [volume ratio of HSV: Rabies; 1 : 2; 1.5 μL of mixture; Biovex (London, UK); Yonchera et al., 2011], a replication-defective virus engineered to express rabies glycoprotein for complementation.
The SAD-ΔG-Rabies plus HSV1-G mix was injected at P6 into intrinsic or extrinsic muscles and the animals were killed at P11. In some experiments, SAD-ΔG-Rabies injections in the whisker pad were coupled with anterograde tracing of cortical axons. An AA2 vector encoding EGFP under the control of the synapsin promoter was used (AAV2/1.hSynapsin.EGFP.WPRE.bGH virus made by Penn Vector Core). The virus (200–500 nL) was injected stereotaxically into either wS1 (1.5 mm posterior and 2.5–3 mm lateral to bregma) or wM1 (1 mm anterior and 1 mm lateral to bregma) of P5 pups (left cortical hemisphere). SAD-ΔG-Rabices: mCherry and HSV1-G were injected unilaterally into the right whisker pad of P6 animals. The animals were killed at P11.

In situ hybridization and immunohistochemistry

Animals were perfused with 4% PFA. For cryostat sections, the tissue was cryoprotected in 10% sucrose (Fluka, St. Louis, MO, USA; diluted in PBS) and embedded in a solution containing 7.5% gelatin (Sigma, St. Louis, MO, USA) and 10% sucrose in PBS before being frozen at -80 °C. Coronal cryostat sections (30 μm) were cut. In situ hybridization against digoxigenin-labelled antisense RNA probes of Gad67 (Lein et al., 2007; Allen Brain Atlas, Gad1-RP_040324_01_F01) or Vglut2 (Lein et al., 2007; Allen Brain Atlas, Slc17a6-RP_050921_01_E03) was carried out as described in the Perkin Elmer (Waltham, MA, USA) TSA Plus fluorescence kit manual. The RNA probes were prepared by polymerase chain reaction amplification of mouse cDNA using primers for Gad67 (forward, TGG GCC CAA ACT GTG CCT; reverse, TGG CGG ATG ATT CTT GGT) and Vglut2 (forward, CCA AAT CTT AGC GTC CTA CCTC; reverse, TAG CCA TCT TCC CTG CTC CACT). The template cDNA was synthesized from total RNA extracted from embryonic day 14.5 mouse brains. The amplicons were then cloned into pCRII-TOPO vector using a TOPO TA cloning kit (Life Technologies). For in situ hybridization, the cryostat sections were post-fixed in 4% PFA in PBS. After washing in PBS, the sections were incubated overnight at 68 °C in a probe solution (diluted in hybridization buffer, 1 : 100) in a humid chamber. Following hybridization and washing, the sections were blocked with 2% blocking reagent (Roche, Basel, Switzerland) for 1.5 h at room temperature and then incubated overnight at 4 °C with anti-digoxigenin antibody conjugated to horseradish peroxidase (1 : 100; Roche Applied Science, cat. no. 1120773910, RRID: AB_514500). After washing, the in situ signal was detected with TSA Plus fluorescence kit (Perkin Elmer). Following the revelation of the in situ signals, the sections were washed in PBS and then blocked with 5% bovine serum albumin in PBS for 1.5 h at room temperature. The sections were then incubated with rabbit anti-RFP antibody (1 : 200; Rockland Immunochemicals Inc., (Limerick, PA, USA) cat. no. 600-401-379, RRID: AB_2209751) overnight at 4 °C. Finally, they were rinsed and incubated with donkey anti-rabbit Alexa 568 (1 : 200, Life Technologies, cat. no. A10042, RRID: AB_11180183) for 2 h at room temperature.

Alignment of brainstem slices with schematic maps

Individual coronal brainstem sections were manually overlaid with the corresponding map from the Paxinos & Franklin Mouse Brain Atlas (2001). The autofluorescence of the brainstem enables the spinal trigeminal tract, the inferior cerebellar peduncle, the medial longitudinal fasciculus and the pyramidal tract to be clearly visible. Other landmarks included the midline, the fourth ventricle and the central canal. Care was taken to ensure that all the landmarks on the sections were aligned with the map. The boundaries of the brainstem nuclei were taken to be at the positions marked on the map.

Whisker pad anatomy

Animals that were injected with SAD-ΔG-Rabies into the extrinsic nasolabialis muscle or the intrinsic follicular muscle (C2 whisker follicle) were transcardially perfused with 4% PFA in PBS. The heads were collected and were post-fixed in 4% PFA in PBS at 4 °C overnight. They were then transferred to 20% sucrose in PBS solution for 2 days, following which they were embedded in OCT compound (Tissue-Tek; Pimos, Sakura, Japan) and allowed to freeze on dry ice. Sagittal sections of 50 μm thickness were made using a cryostat.

Statistical analysis

All data are presented as mean ± SEM. Normality of data distributions was assessed using the Anderson–Darling normality test. For normally distributed data, statistical significance was assessed with Student’s t-test for paired or unpaired observations. For non-normally distributed data, statistical significance was assessed with Wilcoxon’s rank-sum test (unpaired observations) and signed-rank test (paired observations).

Results

wS1 and wM1 drive antagonistic movements of the contralateral whisker

Our first goal was to characterize the differences in whisker movements driven by wS1 and wM1 (Haiss & Schwarz, 2005; Petersen, 2007, 2014; Matyas et al., 2010). To this end, we expressed a Cre-dependent ChR2 virus in wS1 and wM1 of Emx1-Cre mice. In these mice, the expression of Cre recombinase is restricted to excitatory neurons of the neocortex (Chan et al., 2001). Stimulation of wS1 with a 50-Hz light train caused a sustained retraction of the contralateral whisker whereas stimulation of wM1 with a 50-Hz light train caused a dynamic protraction of the contralateral whisker (wS1, −7.3 ± 1.1°, n = 10 mice; wM1, 6.0 ± 0.9°, n = 8 mice; P = 10−7, Student’s t-test for unpaired observations; Fig. 1A–C). The latencies of the movements were not significantly different (wS1, 23.2 ± 6.6 ms, n = 10 mice; wM1, 36.5 ± 5.5 ms, n = 8 mice; P = 0.1, Wilcoxon’s rank-sum test; Fig. 1C). The rhythmicities of the movements were significantly different, with wS1 driving a non-rhythmic whisker movement and wM1 driving a rhythmic whisker movement (5–20 Hz power for wS1, 5.1 ± 1.8 deg2, n = 10 mice; 5–20 Hz power for wM1, 32.5 ± 6.6 deg2, n = 8 mice; P = 1.8 × 10−4, Wilcoxon’s rank-sum test; Fig. 1D).

Long stimulus trains have been shown to elicit complex movements that may involve the recruitment of other cortical areas and complex motor circuits (Graziano et al., 2002; Harrison et al., 2012). We therefore repeated the experiments with single brief light pulses. A single 5-ms light flash delivered to wS1 robustly evoked a small retraction of the contralateral whisker, following which the animal initiated whisking, while a 5-ms light flash delivered to wM1 caused a small protraction (wS1, −1.4 ± 0.4°, n = 6 mice; wM1, 356 V. Sreenivasan et al.
1.9 ± 0.4%, n = 5 mice; P = 10^{-4}, Student’s t-test for unpaired observations; Fig. 1E and F). The latencies of the whisker movements evoked by single 5-ms light flashes delivered to wS1 and wM1 were not significantly different (wS1, 14 ± 1 ms, n = 6 mice; wM1, 34.4 ± 5.7 ms, n = 5 mice; P = 0.06, Wilcoxon’s rank-sum test; Fig. 1F). Thus, wS1 and wM1 drive qualitatively distinct types of whisker movements, with wS1 driving a sustained backward movement and wM1 driving a rhythmic forward movement.

Whisker motor neurons in the lateral FN

In order to understand how wS1 and wM1 cortex can drive whisker movements, we investigated the anatomical pathways involved in generating whisker motion. We began by studying the motor neurons that control forward and backward movements of the whisker. We focused our attention on the upper extrinsic muscle nasolabialis and the intrinsic muscles of the whisker pad. Nasolabialis is anchored outside the pad and extends between the vibrissa rows where it attaches superficially under the skin (Dörfl, 1982; Berg & Kleinfeld, 2003). Contraction of this muscle results in a posterior translation of the entire whisker pad, thus driving retraction of the individual whiskers. On the other hand, the intrinsic follicular muscles are located entirely within the whisker pad. Each intrinsic muscle wraps around the base of a whisker follicle to form a sling and is superficially attached to the skin surrounding the whisker that is immediately posterior. Contraction of the intrinsic muscle causes the base of the follicle to move posteriorly, thereby causing the whisker to pivot and protract (Dörfl, 1982; Berg & Kleinfeld, 2003). In order to retrogradely trace the location of the motor neurons, we injected the glycoprotein-deleted rabies virus (Rabies-ΔG; Wickersham et al., 2007) encoding EGFP or mCherry into muscle of P6 mice and killed them at P8 (Fig. 2A). Analysis of the injections sites revealed that the injections were distinct and were localized to the intrinsic follicular muscle within the whisker pad and the extrinsic nasolabialis muscle anchored externally, respectively (Fig. 2B). The cholinergic identity of the motor neurons was confirmed by injecting Rabies-ΔG-EGFP into either the extrinsic or the intrinsic muscles of ChAT-Cre × LSL-tdTomato mice (which express the red fluorescent protein tdTomato in all cholinergic neurons; Fig. 2C and D). Injection of Rabies-ΔG-mCherry into the extrinsic muscle and Rabies-ΔG-EGFP into the intrinsic muscle (or vice versa) of the same animal revealed an ordered distribution of motor neurons in the FN, where the extrinsic motor neurons were always located dorsally with respect to the intrinsic motor neurons within the lateral FN (Fig. 2E and F) (Klein & Rhoades, 1985; Takatoh et al., 2013). No neurons were labelled in other nuclei of the brainstem.

Intrinsic and extrinsic whisker premotor neurons in the brainstem

We were next interested in mapping the brainstem areas containing premotor neurons that synapse onto these distinct motor neuron pools. Rabies-ΔG injected into muscle is retrogradely transported to motor neurons, but the absence of the gene encoding for the glycoprotein renders it incapable of trans-synaptic spread. To allow the trans-synaptic movement of rabies, an additional retrograde virus encoding the rabies glycoprotein can be co-injected into the muscle, which will then trans-complement Rabies-ΔG in the motor neurons resulting in monosynaptic spread (Stepien et al., 2010). To label the premotor neurons we injected HSV1-G, a Herpes Simplex Virus serotype 1 vector encoding the rabies glycoprotein (Yonehara et al., 2011), along with the Rabies-ΔG into extrinsic muscle (Fig. 3A), intrinsic muscle (Fig. 3A) or both muscles (Fig. 4A) of P6 mice, and killed them at P11.

We found that the premotor neurons of these two muscles were intermingled in many brainstem areas, with very few double-labelled cells (Fig. 4). In agreement with a previous study (Takatoh et al., 2013), we found intermingled premotor neurons for both extrinsic and intrinsic whisker muscles in spinal trigeminal nucleus oralis (Sp5O; Figs 3B and 4B), the vestibular nuclei (Ve; Figs 3B and 4B), the lateral para-gigantocellular nucleus (LPG), the Bötzinger/Pre-Bötzinger complexes, intermediate reticular formation (IRt; Figs 3C and 4C), gigantocellular reticular formation (GIRt), parvocellular reticular formation (PCRs; Figs 3D and 4D) and the medul- lary dorsal reticular formation (MdD; Fig. 3E).

We also found striking differences in the premotor distributions for intrinsic and extrinsic whisker muscles in some brainstem regions. Intrinsic premotor neurons were particularly dense along the mediodorsal to lateroventral axis of the IRt, with some cells also located more medially in GIRt. In contrast, extrinsic premotor neurons were dense in the ventrolateral aspect of spinal trigeminal nucleus rostral interoparals (Sp5Ir), whereas there were very few intrinsic premotor neurons located in Sp5Ir. To quantify, we counted rabies-labelled premotor neurons, on the side of the brainstem ipsilateral to the injection, in coronal sections spanning Sp5O until spinal trigeminal interoparals nucleus (Sp5Ic), and compared the total number of cells counted to the number of cells located in Sp5Ir and IRt for the extrinsic and intrinsic muscles (Fig. 3F). The fraction of extrinsic premotor neurons located in Sp5Ir was significantly higher than the fraction of intrinsic premotor neurons located in Sp5Ir (extrinsic fraction in Sp5Ir, 36 ± 12%, n = 5 mice; intrinsic fraction in Sp5Ir, 8 ± 3%, n = 5 mice; P = 0.005, Student’s t-test for unpaired observations). Conversely, the fraction of intrinsic premotor neurons located in IRt was significantly higher than the fraction of extrinsic premotor neurons located in IRt (extrinsic fraction in IRt, 23 ± 6%, n = 5 mice; intrinsic fraction in IRt, 45 ± 12%, n = 5 mice; P = 0.01, Student’s t-test for unpaired observations; Fig. 3F).

In summary, the premotor neurons for intrinsic and extrinsic whisker muscles were distributed in a largely intermingled manner over a large part of the brainstem. However, there were two major differences: IRt was dominated by intrinsic premotor neurons for controlling whisker protraction, and Sp5Ir was dominated by extrinsic premotor neurons for controlling whisker retraction.

Cortical innervation of the brainstem

Having identified the locations of the motor and premotor neurons for extrinsic and intrinsic whisker muscles, we next investigated the axonal innervation of the brainstem from wS1 and wM1. We injected an AAV virus encoding EGFP into either wS1 or wM1 of adult animals and studied the cortical axons in the contralateral brainstem (Fig. 5A). We found very little overlap in the brainstem regions innervated by wS1 or wM1 (Fig. 5B). In the regions innervated by wM1 and wS1 (Fig. 5A), wS1 projected strongly to Sp5O, IRt, GIRt, IRt, medullary ventral reticul ar formation (MdV) and MdD (Fig. 5B–E). wM1 also weakly innervated a dorsal portion of Sp5Ir (Fig. 5C). In addition, wM1 projected to the ventrolateral portion of the FN. To quantify, we normalized the axon intensity in the spinal trigeminal nuclei (Sp5) and the reticular formation (Rt) to the background fluores-
cence (Fig. 5F). The wS1 innervation of Sp5 was significantly stronger than the innervation of Rt (normalized axon intensity in Sp5, 2.2 ± 0.2; normalized axon intensity in Rt, 1.1 ± 0.04, n = 3 mice; P = 0.03, Student’s t-test for paired observations). Conversely, the wM1 innervation of Rt was significantly stronger than the innervation of Sp5 (normalized axon intensity in Sp5, 1.1 ± 0.04; normalized axon intensity in Rt, 2.2 ± 0.2, n = 3 mice; P = 0.03, Student’s t-test for paired observations).
wS1 axons innervate extrinsic premotor neurons in Sp5Ir

Of the brainstem regions receiving dense wS1 innervation, only the Sp5Ir nucleus contained a major group of premotor neurons, mostly composed of extrinsic premotor neurons and only few intrinsic pre-motor neurons (Figs 3 and 4). The wS1 axons could therefore innervate extrinsic premotor Sp5Ir neurons.

However, our axonal mapping was carried out in adult mice whereas the retrograde rabies labelling of premotor neurons from muscle injections only works well in young animals. We therefore injected AAV-EGFP into wS1 and Rabies-AG-mCherry + HSV1-G into the contralateral extrinsic muscle of P6 mice and analysed the fixed brains at P11. We found a similar axonal innervation of the brainstem in young as compared to adult mice. Coronal sections confirmed that wS1 axons projected into the Sp5Ir region containing extrinsic premotor neurons (Fig. 6A). Imagined at high resolution with confocal microscopy (Fig. 6B), we found cortical axons in close apposition with trans-synaptically labelled extrinsic premotor neurons. We counted the number of appositions at the dendrites of these premotor neurons to be 0.07 ± 0.009 appositions/μm of dendrite.

FIG. 2. The FN contains two distinct whisker motor neuron populations that drive antagonistic musculature. (A) Schematic showing the strategy used to label the motor neurons in the FN. Two different glycoprotein deleted rabies viruses (Rabies-AG and mCherry) were injected unilaterally into the right follicular (intrinsic protractor) and right nasolabialis (extrinsic retractor) muscles. (B) Sagittal section of the whisker pad where Rabies-AG was targeted to the nasolabialis muscle (top) and the C2 follicular muscle (bottom). The top and bottom panels are from different animals. The pad has been counterstained with DAPI. (C) Cholinergic identity of the rabies-labelled extrinsic motor neurons was confirmed by injecting Rabies-AG-EGFP in the extrinsic muscle of ChAT-Cre × LSL-tdTomato animals. Double-labelled extrinsic motor neurons were present in the dorsal aspect of the lateral FN. (D) Cholinergic identity of the rabies-labelled intrinsic motor neurons was confirmed by injecting Rabies-AG-EGFP in the intrinsic muscle of ChAT-Cre × LSL-tdTomato animals. Double labelled intrinsic motor neurons were present in the ventral aspect of the lateral FN. (E) Coronal section through the ipsilateral brainstem at the level of the FN from an animal that received Rabies-AG-EGFP in the intrinsic muscle and Rabies-AG-mCherry in the extrinsic muscle. The red cells are the motor neurons of the extrinsic muscle while the green cells are the motor neurons of the intrinsic muscle. The coronal section is overlaid with a schematic drawing (Paxinos & Franklin, 2001). (F) Group data from seven animals that were injected with Rabies-AG of one colour in the extrinsic muscle and Rabies-AG of the other colour in the intrinsic muscle. Red circles indicate the positions of the extrinsic nasolabialis muscle motor neurons while green circles indicate the positions of the intrinsic follicular muscle motor neurons in the FN. The motor neurons were always located in the lateral part of the FN and the extrinsic motor neurons were located in the dorsal aspect of the lateral FN, while intrinsic motor neurons were located more ventrally.
Fig. 3. Extrinsic and intrinsic premotor neurons are distributed across the ipsilateral brainstem. (A) Schematic drawing showing the strategy used to label the premotor neurons of the extrinsic and intrinsic muscles. Rabies injections were targeted to either the right extrinsic (left) or the right intrinsic (middle) muscle in individual animals. Trans-complementation was achieved by co-injecting the Herpes simplex virus serotype 1 encoding the rabies glycoprotein (HSV-G). Sagittal schematic of the brain showing the rostrocaudal positions (blue dashed lines) corresponding to the coronal sections in the subsequent panels (right). Yellow circle indicates bregma. (B) Coronal section at the level of the Sp5O. Extrinsic premotor neurons (red) were located in the Ve, the Sp5O and the LPG (left). Intrinsic premotor neurons (green) were located in the IRt, the Sp5O and the LPG (middle). (C) Coronal section at the level of the rostral part of the Sp5Ir. Extrinsic premotor neurons were located in the Sp5Ir and an area spanning the GIRt and the LPG nuclei (left). Intrinsic premotor neurons were located in the IRt (middle). The Sp5Ir contained very few intrinsic premotor neurons. (D) Coronal section at the level of the caudal part of the Sp5Ir. Extrinsic premotor neurons were located in the GIRt, IRt and ventral portion of the PCrI (left). Intrinsic premotor neurons were located in the IRt (middle). Some intrinsic premotor cells were also scattered in the PCrI (middle). The Sp5Ir contained very few extrinsic or intrinsic premotor cells. (E) Coronal section at the level of the Sp5Sc. Extrinsic (left) and intrinsic (middle) premotor neurons were located in the MdD and the IRt. The Sp5Sc did not contain either extrinsic or intrinsic premotor cells. The coronal images in panels B–E are overlaid with the corresponding schematic drawings (Paxinos & Franklin, 2001). The premotor neuron distributions observed in double-labelled mice were consistent across four mice.

We next investigated the neurotransmitter phenotype of Sp5Ir premotor neurons by in situ hybridization for GAD67 and Vglut2. The analysis revealed that the large extrinsic premotor neurons labelled in Sp5Ir were glutamatergic (Fig. 6C). A few of the smaller extrinsic premotor neurons were GABAergic (Fig. 6C). As the large glutamatergic extrinsic premotor neurons located in Sp5Ir receive excitatory input from pyramidal neurons of the contralateral wS1, we reason that wS1 → Sp5Ir (extrinsic premotor) → FN (extrinsic motor) constitutes a motor circuit in which stimulation of wS1 could drive retraction of the contralateral whisker (Fig. 6D).

wm1 axons innervate the FN and Irft

We were next interested in the motor circuits downstream of wm1. A previous study reported the presence of monosynaptic connections from wm1 onto motor neurons in the FN of the rat (Grinevich et al., 2005). Accordingly, we observed AAV-EGFP-labelled wm1 axons projecting to the ventrolateral aspect of the contralateral FN labelled in ChAT-Cre × LSL-td-Tomato mice (Fig. 7A). Furthermore, trans-synaptic rabies tracing from the intrinsic muscle also identified intrinsic premotor neurons in frontal and wm1 cortex (Fig. 7B). In contrast, very few cortical neurons were labelled when trans-synaptic rabies virus was injected into extrinsic muscle. Thus,

(11 dendrites from three premotor cells in two mice). These data indicate that wS1 and Sp5Ir extrinsic premotor neurons are likely to be synaptically connected.

Fig. 4. Double-labelling of extrinsic and intrinsic premotor neurons in the ipsilateral brainstem. (A) Schematic showing the strategy used to label premotor neurons of the extrinsic and intrinsic muscles in the same animal. Two different Rabies-ΔG viruses encoding EGFP and mCherry were injected unilaterally on the right side of the same mouse into the intrinsic muscle and extrinsic muscle respectively (left). Trans-complementation was achieved by co-injecting the Herpes simplex virus serotype 1 encoding the rabies glycoprotein (HSV-G). Sagittal schematic of the brain showing the rostrocaudal positions (blue dashed lines) corresponding to the coronal sections in the subsequent panels (right). Yellow circle indicates bregma. (B) Coronal section through the FN and Sp5O in a mouse that received Rabies-ΔG-mCherry + HSV-G in the extrinsic muscle and Rabies-ΔG-EGFP + HSV-G in the intrinsic muscle. Note the intermingled cell distributions in Sp5O and LPG. (C) Coronal section through Sp5Ir of the same mouse. Note the presence of red extrinsic premotor neurons and sparsity of green intrinsic premotor neurons in Sp5Ir. The Irft has a large number of green intrinsic premotor neurons. (D) Coronal section through Sp5Sc of the same mouse. The Sp5Sc contains neither extrinsic nor intrinsic premotor neurons. Also note the presence of green intrinsic premotor neurons in the Irft region near the NA. The coronal images in panels B–D are overlaid with the corresponding schematic drawings (right) (Paxinos & Franklin, 2001). The premotor neuron distributions observed in double-labelled mice were consistent across four mice.
wM1 might contribute to controlling whisker movements by directly synaptic onto the motor neurons innervating intrinsic muscles.

A major innervation site of the wM1 axons in the brainstem was the PCRt (Fig. 5D). However, in our rabies tracing experiments we did not consistently find premotor cells localized in PCRt and, in the cases in which we did, they were few in number and did not display any specific distribution or clustering. Furthermore, lesioning the PCRt has little effect upon whisker movements (Moore et al., 2013).

Next we investigated the wM1 projections in the IRt. It has been suggested that a whisking central pattern generator in the brainstem is localized in the ventral part of the IRt, lying medial to the nucleus ambiguus (NA) (Moore et al., 2013). As the NA contains cholinergic neurons, it is easily distinguishable in the ChAT-Cre × LSL-tdtomato animals (Fig. 7C). We found that wM1 innervated the region of the IRt near to the NA (Fig. 7C, middle). This region was a major hotspot of intrinsic premotor neurons (Fig. 7C, right). To investigate the potential overlap of the wM1 axons with these premotor neurons, we injected AAV-EGFP into wM1 and Rabies-G-mCherry + HSV1-G into the contralateral intrinsic muscle of P6 mice and killed them at P11. Coronal sections showed that, in the IRt, the wM1 axons came into close apposition with the trans-synaptically labelled intrinsic premotor neurons (Fig. 7D). We counted the number of appositions at the dendrites of these premotor neurons to be 0.06 ± 0.008 appositions/μm of dendrite (11 dendrites from three premotor cells in two mice). The rabies-labelled population of premotor neurons in the IRt contained both GAD67+ inhibitory neurons and Vglut2+ excitatory neurons (Fig. 7E). wM1 might thus innervate both excitatory and inhibitory intrinsic premotor neurons. In addition, the same brainstem region also harbours extrinsic premotor neurons. wM1 innervation of IRt (and surrounding brainstem reticular regions) might thus drive rhythmic whisker protraction through a complex network of inhibitory and excitatory premotor neurons for both intrinsic and extrinsic muscles, which might constitute an oscillatory central pattern generator (Fig. 7F) (Moore et al., 2013).

**Discussion**

To further our understanding of the cortical circuits involved in whisker motor control, we characterized the whisker movements di-
ven by wS1 and wM1. Optogenetic stimulation of wS1 evoked whisker retraction while optogenetic stimulation of wM1 evoked whisker protraction that was rhythmic (Fig. 1). We used modified rabies virus to map whisker motor (Fig. 2) and premotor (Figs 3 and 4) neurons, and we investigated the axonal projections from wS1 and wM1 to the brainstem using AAV virus (Fig. 5). We examined the regions with the most prominent overlap between cortical axons and premotor neurons finding that wS1 axons strongly overlap with extrinsic premotor neurons located in the Sp5Ir (Fig. 6) and that wM1 axons overlap with intrinsic premotor neurons in IRt (Fig. 7). In addition we found that wM1 innervates the intrinsic motor neurons in FN (Fig. 7). Together our results suggest that wS1 and wM1 control distinct whisker movements through two parallel motor pathways.

**Distinct whisker premotor neuron subpopulations for intrinsic and extrinsic muscles**

Rabies virus has been used extensively as a retrograde trans-synaptic tracer to map motor circuits in the mammalian brain. Pioneering work used replicating rabies virus to trace motor control circuits across multiple synapses (Ugolini, 1995; Rathelot & Strick, 2006). Monosynaptic inputs can be traced using genetically modified synapses (Ugolini, 1995; Rathelot & Strick, 2006). Monosynaptic rabies virus has been used extensively as a retrograde trans-synaptic tracer to map motor circuits in the mammalian brain. Pioneering work with the replicating rabies virus (Paxinos & Franklin, 2001) at the level of wM1 (middle) with a blue dotted box indicating the area of wM1 shown in the right panel. Intrinsic premotor neurons were present in wM1 with labelled neurons located in layers 5 and 6 (right). The inset shows a labelled cell in wM1 at higher resolution. (C) Coronal schematic drawing (Paxinos & Franklin, 2001) at the level of the NA (left). Blue box indicates the area of the IRt shown in the middle and right panels. The wM1 axons projected to the IRt region near to the NA (middle). This region was also a major hotspot of intrinsic premotor neurons (right). (D) AAV virus encoding EGFP was injected into wM1 of the left hemisphere to label the cortical axons in the brainstem. In the same mouse, Rabies-GFP (right) was injected into the right intrinsic muscle to label the premotor neurons. The right IRt, the wM1 axons innervated the intrinsic premotor neurons near to the NA (left). Orthogonal projections in xz, xz, and yz planes show close apposition between the cortical axon (green) and the premotor neuron (red) located in the IRt (right). (E) The neurotransmitter phenotypes of the labelled neurons in the IRt were determined with fluorescent in situ hybridization. The population of labelled intrinsic premotor neurons in the IRt contained GAD67-positive inhibitory neurons (top) as well as Vglut2-positive excitatory neurons (bottom). (F) Schematic drawing of a possible circuit by which wM1 might drive whisker movement. The “+” indicates excitatory intrinsic premotor neurons and “−” indicates inhibitory intrinsic premotor neurons in the IRt. Together, these might constitute an oscillator that drives rhythmic whisker protraction.

**Distinct motor circuits driven from wS1 and wM1**

The subcortical axonal projections from wS1 and wM1 follow largely parallel pathways innervating neighbouring and largely non-overlapping brain areas (Matyas et al., 2010). In the brainstem we found very little overlap in the regions innervated by wS1 and wM1 (Fig. 5). Projections from wS1 almost exclusively innervated the spinal trigeminal nuclei, including a ventral portion of Sp5O, lateral aspects of both rostral and caudal Sp5I and medial Sp5C. In contrast, wM1 primarily innervated PCRt, IRt, GIRt, MdV and MdD, while largely avoiding the spinal trigeminal nuclei. These distinct patterns of cortical innervation from wS1 and wM1 are likely to differentially activate the whisker premotor neurons in the brainstem.

To try to define the possible downstream motor circuits activated by cortical stimulation, we examined the overlap in the axonal projections from cortex with the locations of whisker premotor neurons. Because wS1 axons specifically innervate the spinal trigeminal nuclei (Fig. 5) and the majority of premotor neurons located in the spinal trigeminal nuclei are from the extrinsic muscle (Figs 3 and 4), we further investigated possible interactions finding close apposition of wS1 axon with extrinsic premotor neurons (Fig. 6). This raises the possibility that wS1 drives whisker retraction through a simple circuit wS1→Sp5Ir (extrinsic premotor)→FN (extrinsic motor; Fig. 6D). In future experiments it will be important to specifically inactivate the neurons in this proposed circuit to test this hypothesis.

Motor circuits in the brainstem downstream of wM1 appear more complex. In part, whisker movements evoked by wM1 might be driven by direct monosynaptic input onto motor neurons in the FN

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The whisker movements evoked by wS1 and wM1 are qualitatively different and probably serve distinct functional roles during behaviour. The whisker retraction driven by wS1 stimulation drives a constant whisker retraction. The greater complexity of the whisker movements evoked by wM1 stimulation may result from activation of more complex central pattern generator circuits in the brainstem, which might also interact with the controllers of other orofacial movements (Moore et al., 2013).

**Functional implications and future perspectives**

The whisker movements evoked by wS1 and wM1 are also more complex than those evoked by wS1. wM1 drives not only protraction but also rhythmic whisking (involving both protraction and retraction), whereas wS1 stimulation drives a constant whisker retraction. The greater complexity of the whisker movements evoked by wM1 stimulation may result from activation of more complex central pattern generator circuits in the brainstem, which might also interact with the controllers of other orofacial movements (Moore et al., 2013).

**Conflict of interests**

The authors have no conflict of interests.

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**Abbreviations**

ChR2, channelrhodopsin-2; FN, facial nucleus; GfRt, gigantocellular reticular formation; HSV1-G, Herpes Simplex Virus 1-Glycoprotein; IRt, intermediate reticular formation; LGP, lateral para-gigantocellular reticular formation; MdD, medullary dorsal reticular formation; MdV, medullary ventral reticular formation; NA, nucleus ambiguus; P, postnatal day; PCRt, parvo-cellular reticular formation; PFA, paraformaldehyde; PSD, power spectral density; Rt, reticular formation; SpSC, spinal trigeminal nucleus caudalis; Sp5f, spinal trigeminal nucleus rostral intermediolateral; Sp5o, spinal trigeminal nucleus oralis; Vc, vestibular nuclei; wM1, whisker motor cortex; wS1, whisker somatosensory cortex.

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The movements evoked by wM1 stimulation are also more complex than those evoked by wS1. wM1 drives not only protraction but also rhythmic whisking (involving both protraction and retraction), whereas wS1 stimulation drives a constant whisker retraction. The greater complexity of the whisker movements evoked by wM1 stimulation may result from activation of more complex central pattern generator circuits in the brainstem, which might also interact with the controllers of other orofacial movements (Moore et al., 2013).

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