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RESEARCH ARTICLE

The evolution of active vibrissal sensing in mammals: evidence from vibrissal musculature and function in the marsupial opossum *Monodelphis domestica*

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SUMMARY

Facial vibrissae, or whiskers, are found in nearly all extant mammal species and are likely to have been present in early mammalian ancestors. A sub-set of modern mammals, including many rodents, move their long mystacial whiskers back and forth at high speed whilst exploring in a behaviour known as 'whisking'. It is not known whether the vibrissae of early mammals moved in this way. The grey short-tailed opossum, *Monodelphis domestica*, is considered a useful species from the perspective of tracing the evolution of modern mammals. Interestingly, these marsupials engage in whisking bouts similar to those seen in rodents. To better assess the likelihood that active vibrissal sensing was present in ancestral mammals, we examined the vibrissal musculature of the opossum using digital miscroscopy to see whether this resembles that of rodents. Although opossums have fewer whiskers than rats, our investigation found that they have a similar vibrissal musculature. In particular, in both rats and opossums, the musculature includes both intrinsic and extrinsic muscles with the intrinsic muscles positioned as slings linking pairs of large vibrissae within rows. We identified some differences in the extrinsic musculature which, interestingly, matched with behavioural data obtained through high-speed video recording, and indicated additional degrees of freedom for positioning the vibrissae in rats. These data show that the whisker movements of opossum and rat exploit similar underlying mechanisms. Paired with earlier results suggesting similar patterns of vibrissal movement, this strongly implies that early therian (marsupial and placental) mammals were whisking animals that actively controlled their vibrissae.

Key words: rat, whiskers, vibrissae, whisking, active sensing, mammalian evolution.

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INTRODUCTION

The early evolution of mammals is associated with a number of significant milestones such as the onset of thermoregulation, restructuring of the inner ear and the emergence of a six-layered cortex. Hair also appears in mammals for the first time, probably having a role in tactile sensing prior to the appearance of pelage hair as a means of maintaining body temperature (Maderson, 1972; Maderson, 2003). Indeed, some of the first hairs may well have been facial vibrissae, or whiskers, similar to those found in nearly all extant mammal species (Pocock, 1914; Ahl, 1986). Many terrestrial mammals that have well-developed mystacial whiskers, termed macrovibrissae, move them back and forth at high speed during exploration, a behaviour known as 'whisking' (Wineski, 1985; Mitchinson et al., 2011). Whisking is a form of active tactile sensing (Prescott et al., 2011) and, in rodents such as rats and mice, is regulated by sensory feedback in a manner that appears to boost the sensory information obtained by the animal (Mitchinson et al., 2007). We recently showed (Mitchinson et al., 2011) that similar patterns of rhythmic macrovibrissal movement, also under sensory guidance, are present in *Monodelphis domestica* (Wagner, 1842), a small ground-dwelling marsupial native to South America, whose last common ancestor with modern rodents was an early mammal of the Triassic period living more than 160 million years ago (Luo et al., 2011). The presence of whisking in both rodents and marsupials therefore presents the intriguing possibility that a common ancestor to all modern therians (marsupial and placental mammals) may have employed active vibrissal sensing. However, by observing behaviour alone it is not possible to rule out the possibility of convergent evolution of whisking in rodents and the opossum. That is, both lines may have evolved the capacity to actuate their whiskers at a later time, as a result of similar evolutionary pressures. Fossil evidence is not able to resolve this issue as the few fossilised skulls of early mammals that have so far been found have not preserved evidence of facial musculature (Ji et al., 2002; Luo, 2007; Luo et al., 2011). The alternative strategy, pursued here, is to explore this question *via* comparative physiology. Specifically, the hypothesis of convergent evolution of active vibrissal sensing in both rodents and marsupials would appear much less likely if we can obtain evidence that the underlying mechanisms are similar in these two groups of whisking animals. To that end, in the current study we studied the anatomy of the opossum mystacial pad, making direct comparisons between the vibrissal musculature of this animal and that of the common rat.

Whereas the vibrissal musculature of the rat has recently been described in detail (Haidarliu et al., 2010; Haidarliu et al., 2012), that of the opossum had not hitherto been mapped. We therefore replicated the methodology of our earlier study of the rat facial musculature using cytochrome oxidase staining (Haidarliu et al., 2010) to obtain digital microscope images of the opossum mystacial pad that could be directly compared with those of rodents. This

method identifies interesting similarities and differences in the vibrissal muscles of the two species. Most importantly, we investigated here whether the large facial whiskers are moved by an intrinsic sling-like musculature that connects each whisker follicle to the adjacent one along each whisker row. There are many ways in which muscles might be configured in order to provide whisker protraction; if this specific mechanism is present in both rodents and marsupials it strongly indicates that whisking in rats and opossum is not convergent but represents a shared inherited trait.

As well as controlling back and forth whisking movements, recently described extrinsic muscles also control more complex whisker behaviours that can occur in both the protraction and retraction phases of the whisk (Haidarliu et al., 2010). We have previously shown (Mitchinson et al., 2011) that the periodic whisker movements of rats, mice and opossums become bilaterally asymmetric (i.e. left and right whisker fields protract by different amounts) under conditions where the animal is making a head-turn (head-turning asymmetry) or making contact with an object at an oblique angle (contact-induced asymmetry). This modulation is consistent with active sensing strategies that promote exploration in the direction of movement, in the case of head-turning asymmetry, or increase the likelihood of further whisker–object contacts in the case of contact-induced asymmetry. In an earlier study (Grant et al., 2009), we also showed that when rats are exploring a vertical surface they also reduce the angular separation, or 'spread', of their whiskers by differentially controlling the velocity of movement of the more rostral and more dorsal whiskers (and possibly also by reshaping the mystacial pad) (see Haidarliu et al., 2010). Spread reduction also results in an increased number of contacts with the surface that is being investigated and thus can be understood as a further element of active sensing control. In this investigation, we explored whether the corresponding extrinsic musculature is present in the opossum and similar to that of the rat. In addition, we obtained new videographic data for the opossum to determine whether it is able to differentially control the velocities of rostral and caudal vibrissae so as to modify angular spread and retraction velocities.

Comparative analysis of the vibrissal musculature in these two distantly related mammalian groups therefore both casts new light on the sensory capabilities of early mammals and, at the same time, helps us to understand how modification of the facial musculature in vibrissal specialists such as the rat has enabled modern rodents to better exploit this uniquely mammalian sense.

MATERIALS AND METHODS

Muscle staining

Animals

Four, 1 year old opossums, *M. domestica*, were used in this study: one female and three males. The animals were obtained from a colony maintained by the University of Trieste, and all procedures required for animal care were approved by the local Ethics Committee and communicated to the Ministerial Office, Trieste. For the purpose of the current study the animals were anaesthetised with urethane (25%, 0.65 ml 100 g^{-1} body mass) and perfused transcardially (100 ml of phosphate buffer, PB, pH 7.4 followed by a mixture of 2.5% glutaraldehyde, 0.5% paraformaldehyde and 5% sucrose in 0.1 mol l^{-1} PB). They were then decapitated, placed in the perfusion solution to which an additional 25% sucrose was added and refrigerated overnight. The mystacial pads were then removed bilaterally, by cutting down the sagittal plane and cutting around each pad (about 2 mm each side of the pad). Any pieces of bone were removed from the pads and they were placed flat between stainless steel grids in perforated plastic histology cases (Medex Supply, Monsey, NY, USA) to prevent curling. The histology cases were then put into a solution of 20% sucrose in 0.1 mol l^{-1} PB pH 7.4 for 2 days and refrigerated. In addition to facial vibrissae, the opossum also has prominent genal (cheek) vibrissae. In one male and one female we therefore also removed the genal whisker area and stored them in the same way.

Staining of the mystacial pads

After fixing, each of the pads was sectioned with a Microm cryostar (Walldorf, Germany) cryostat into 50 μm slices. As there were eight pads (from four animals), six pads were sliced tangentially and two pads were sliced coronally. The four genal areas were all sliced tangentially. All slices were stained for cytochrome oxidase activity (see Haidarliu et al., 2010) as follows. The slices were floated in a solution of 10 ml of 0.1 mol l^{-1} PB containing 0.75 mg cytochrome *c*, 40 μl catalase solution and 5 mg diaminobenzidine (DAB) and 0.5 ml distilled water. They were then placed in an incubator at 37°C on a shaking platform for 1–2 h until the stain developed (i.e. until there was a strong difference between reactive and unreactive tissues). The slices were then rinsed in 0.05 mol l^{-1} PB, mounted in distilled water and left to air dry briefly before coverslipping with Entellan.

Figures of the stained musculature were prepared from digital images. A Nikon light microscope with 1×, 2×, 20× and 40× objectives was used to obtain the images which were collected in SPOT and exported as .tif images. Some figures used a Leica fluorescence microscope with a blue filter. Only small adjustments in contrast and brightness were made to the figures.

Xylene clearing of skin

One male 2 year old opossum was used to get a clear picture of the pad layout, by clearing with xylene as follows. The mystacial pads and genal areas were removed from each side, as above. The samples were then shaved, the fatty layers removed from the bottom with a scalpel, pressed flat and dehydrated with ethyl alcohol (50%, 70%, 95% and 100%), then immersed in xylene until they became transparent. The prepared samples were then lit from below with a spotlight and photographed using a Canon digital camera.

Behaviour filming

Animals

Behavioural clips were collected for a previous study (Mitchinson et al., 2011), but re-analysed for the current study. Twenty-six opossums, *M. domestica*, each around 1 year old, were filmed using high-speed videography at the University of Trieste animal facility. The experimental setup was as described previously (Mitchinson et al., 2011). Specifically, a high-speed, high-resolution (1024×1024 pixel) digital video camera (Photron Fastcam, San Diego, CA, USA) was suspended above a transparent viewing arena illuminated from below with a custom-built light box. Animals were placed into the arena, one at a time, and allowed to freely explore. In half the trials, a Perspex block was placed in the arena beneath the camera field of view. Video data were collected in near-darkness using an infrared light box for illumination. Multiple 1.6 s video clips, at 500 frames s^{-1}, were collected opportunistically (by manual trigger) when the animal moved beneath the field of view of the camera. Comparison data from 19 rats [12 Royal College of Surgeons (RCS) dystrophic rats, 7 Hooded Listers] were collected under similar circumstances, at the University of Sheffield animal facilities, with a light-box operating in the visible light spectrum. Previous investigations have found that the whisker movements of genetically

blind (dystrophic) and sighted animals are similar in these conditions with both strains displaying modulation of whisker movement indicating the use of active touch sensing strategies (Mitchinson et al., 2007; Mitchinson et al., 2011; Grant et al., 2009).

Clip selection and whisker tracking

Clip selection was based on the criteria given previously (Mitchinson et al., 2011). Specific clips were chosen to include (i) episodes of 'regular whisking', when the animal was moving its whiskers periodically without contacting anything but the smooth floor; and/or (ii) episodes of 'contact whisking', where the animal was contacting one corner of the Perspex block with its whiskers, with no nearby obstructions other than the floor. The animal's snout and whiskers were tracked in each clip episode, using the BIOTACT whisker tracking tool (Mitchinson et al., 2011; Perkon et al., 2011). The output of the tracker, for each video frame, is the orientation and position of the snout, and a set of 'whisker curves', from which whisker angular position (relative to the midline of the head), for each identified whisker, can be derived. For the opossum, tracking parameters were set such that only the mystacial vibrissae were tracked and not the genal vibrissae (but see below for measurements of genal whisker motion). Tracking was validated by manually inspecting the tracking overlaid on to the video frames.

Our analyses focus on the movement of the entire whisker field on each side of the snout using a measure of mean angular position calculated as the unsmoothed mean of all the tracked whisker angular positions on each side and in each frame. We also computed a measure of horizontal dispersion of the whiskers in each field termed the mean angular spread and calculated as the standard deviation of all the tracked whisker angular positions in each frame. Finally, we computed mean angular retraction and protraction velocities, calculated from the angular position, as the average velocity of all the backward (negative) whisker movements, and forward (positive) whisker movements, respectively. These measurements were all averaged to give a mean value per clip. Note that there was no tracking of whiskers across frames; therefore, the number of identified whiskers varied across frames and clips. Our previous investigation (Mitchinson et al., 2011) demonstrated that these automated estimates of whisker motion provide a good proxy for estimates calculated by tracking by eye. In the current study, both the mean angular position and angular spread measurements were further validated by examining video tracking and plots of each clip. To reduce noise, we also included clips only if there were four or more whiskers tracked on each side of the face, for the entire episode of interest. This led to the inclusion of 30 and 51 regular whisking clips, and 23 and 43 contact whisking clips, respectively, for rat and opossum. Identical tracking and analysis routines were used with both opossum and rat clips which should ensure similar levels of noise in the tracking data.

To examine the capacity for movement in the genal whiskers we also tracked, by visual inspection using a manual whisker tracking tool as described previously (Mitchinson et al., 2007), one genal whisker and two macrovibrissae (one in each of the left and right whisker fields) in 12 clips of regular whisking, between 0.55 and 1.3 s in length.

RESULTS

Whisker layout and general appearance

Opossum whiskers are arranged in a grid-like pattern of rows and arcs (Fig. 1A,C), visually somewhat similar to that seen in rats (compare Fig. 1B and 1D), but with 23 whiskers in total on each side compared with 30 (typically) in the rodent species. In the opossum, the whiskers form four rows, labelled A–D (Fig. 1C), with three to seven large whiskers in each, whereas the rat has five rows of up to seven whiskers each. The dorsal-most row A of the opossum contains just three whiskers (A0, A1 and A2), row B contains four whiskers (B1–B4), and each of the two ventral rows (C and D) has seven whiskers (C0–C6, and D0–D6). The overall grid-like pattern is disturbed by the two 'straddler' whiskers, α and β, at the caudal edge of the pad, whose horizontal alignment is offset relative to the main whisker rows such that α is positioned between the rows A and B, and β between rows B and C (Fig. 1C). Vertically, these whiskers are reasonably well aligned with the most caudal whiskers in rows A, C and D, such that they form part of the first whisker arc (A0, α, β, C0, D0) (Fig. 1G). For comparison, the rat whisker array has four straddlers offset vertically relative to the five whisker rows (Fig. 1D) and appearing to form their own arc. Overall, the mystacial pad is generally less prominent in the opossum compared with the rat (compare Fig. 1A and 1B); the opossum also lacks the prominent microvibrissal array – the short non-actuated whiskers on the chin and upper lip – that is seen in rodents such as rats and mice. The opossum does, however, have a few nasal whiskers arranged in a horizontal row (Fig. 1C, labelled NV), which have rudimentary sling-like muscles (Fig. 1E, on the dorsal-most follicles, compare positions with NV in Fig. 1C). In addition, there is a furry buccal pad (FBP, the area of the pad containing follicles ventral and caudal to the whisker pad), with hair follicles that are not arranged into rows (Fig. 1C). The whiskers of the furry buccal pad differ from the whiskers of the mystacial pad in that they are smaller and have no intrinsic muscles. Part of the buccal pad is positioned more caudally than the mystacial whiskers, and the ventral fascicles of the musculus (m.) nasolabialis and m. maxillolabialis separate the furry buccal pad from row D (Fig. 1E, note the FBP is below the dark muscle staining in the ventral area of the pad). Opossum whisker follicles have a well-defined capsule, and can contain both cavernous and ring sinuses. Sometimes the ringwulst (a doughnut-shaped structure) can be observed in large whiskers (see for example Fig. 2B). Thus, structurally they have many similarities to the vibrissal follicles of the rat.

Intrinsic muscles

In tangential slices cut through the entire opossum snout and stained for cytochrome oxidase activity, intrinsic muscles are easily defined as short, dark-brown strips connecting the large neighbouring whiskers in each row (Fig. 1E, Fig. 2). A key finding is that within each of the rows, the whiskers are connected to each other by sling-like intrinsic muscles as previously described for a variety of whisking rodents (see Discussion). Although there is substantial similarity in this overall arrangement, there are some interesting differences between the opossum and rat musculature that we detail next.

In the rat, intrinsic muscles connect the straddler whiskers β to both rows B and C, and γ with both rows C and D (Haidarliu and Ahissar, 1997) (Fig. 1F). However, in the opossum, the situation is inverted, as whisker B1 connects to, or 'straddles' the two large caudal whiskers (α and β), such that the dorsal-most part of the B1 intrinsic muscle attaches to the ventral part of α, and the ventral-most part of the B1 intrinsic muscle attaches to the dorsal part of β (Fig. 2B,C). Because of these differences, henceforth we will refer to the α and β whiskers as inverted straddlers.

In rows A and B, in the opossum, in addition to intrinsic muscles composed of a sling and two caudally directed horizontal muscle fascicles (HF in Fig. 2C), each pair of whiskers is also joined by a supplementary intrinsic muscle fascicle that connects the ventral

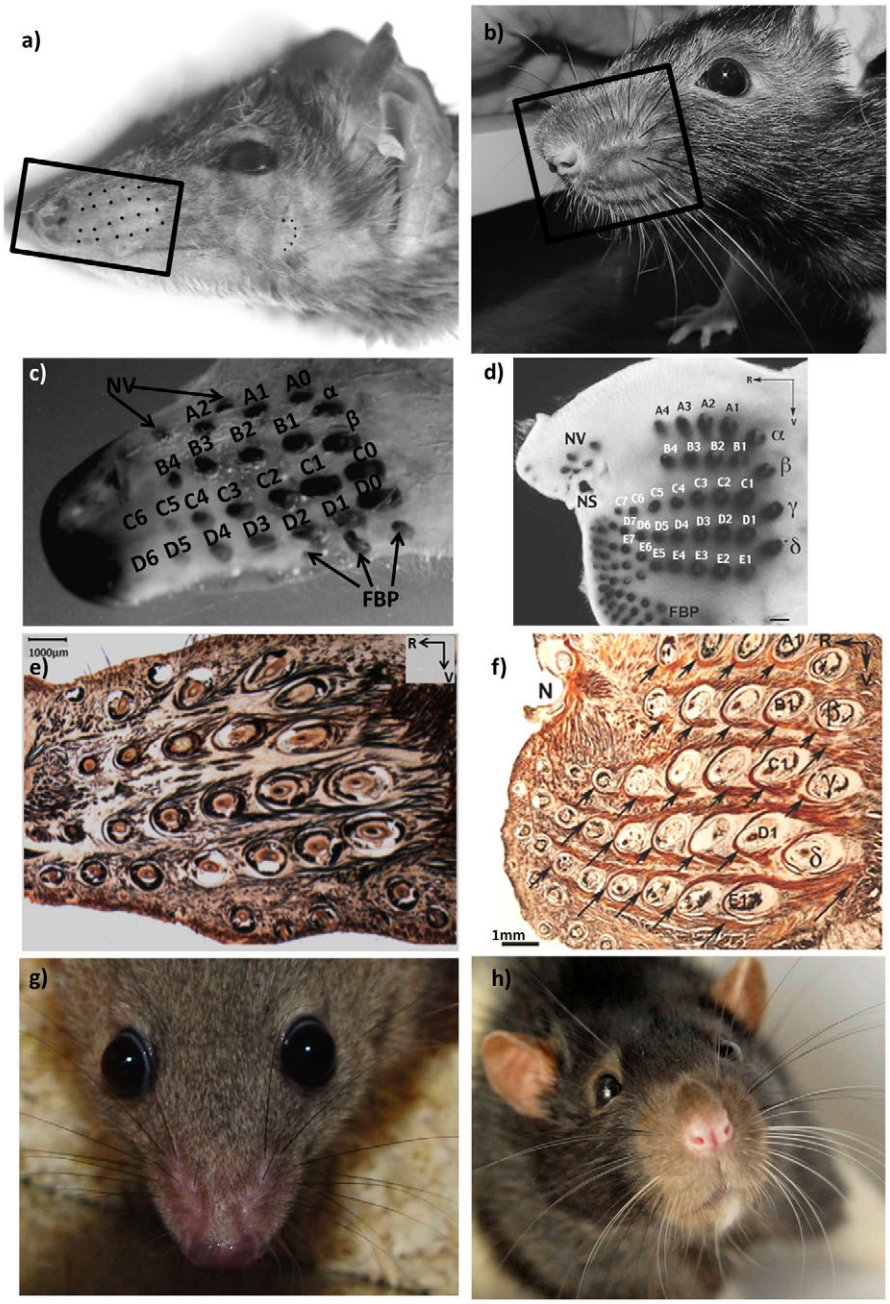

Fig. 1. The layout of the *Monodelphis domestica* mystacial pad in comparison to that of the rat. (A) Photograph of the possum head; the mystacial pad (whiskers removed) is indicated by the black box. (B) Photograph of the rat head; mystacial whiskers and pad are indicated by the black box. (C,D) A map of the macrovibrissal follicles revealed in ethanol/xylene-cleared entire snout preparation in the opossum (C) and the rat (adapted from Haidarliu et al., 2010) (D). The furry buccal pad (FBP) is indicated. NV is nasal vibrissae, NS is nostril. (E,F) A slice of the mystacial pad, stained for cytochrome oxidase activity in the opossum, revealing the sling-like intrinsic musculature (E), and in the rat (adapted from Haidarliu et al., 2010). N is nostril. (G,H) Close-up of the opossum (G) and rat (H) whisker layout from the front. R, rostral; V, ventral.

capsular surface of the more rostral whisker follicle with the dorsal capsular surface of a more caudal whisker follicle in the same row (OF in Fig. 2C). From the capsule of the caudal-most whisker in opossum row A (A0), a third muscle strip also originates. It is directed dorsocaudal and inserted into the corium, the base layer of the mystacial pad. The location of the origin and insertion sites of these oblique muscle strips suggests that they might serve for torsional rotation of the whiskers in rows A and B during protraction, and simultaneously for ventral whisker shaft deflection that may result in whisker palpation of the objects from above. Interestingly, the presence of such oblique muscle strips, termed here oblique intrinsic muscles, has not been reported in any other whisking species.

The fact that the dorsal two whisker rows in the opossum are notably different to the ventral two rows in their intrinsic musculature suggests that there might be a compartmentalisation of the whisker pad. If so, the layout in the opossum could be considered as having a nasal compartment containing two rows of whiskers (A and B) and the two inverted straddlers, and a maxilliary compartment containing two rows of seven whiskers without straddlers (Fig. 1C). In the nasal compartment, the two inverted straddlers fortify the dorsocaudal section of the mystacial pad, which is well aligned with the position of the eye (Fig. 1A,G); indeed, Fig. 1G shows that the inverted straddler whiskers are arranged directly in front of the eyes and might help to protect this sensitive area.

Extrinsic muscles

We next compare the extrinsic muscles of the *M. domestica* mystacial pad with those of the rat. The extrinsic muscles in the rodent mystacial pad have been called a variety of names; the terminology used here agrees with the nomenclature used previously (Haidarliu et al., 2010), and also conforms to *Terminologia Anatomica* (Federative Committee on Anatomical Terminology, 1998).

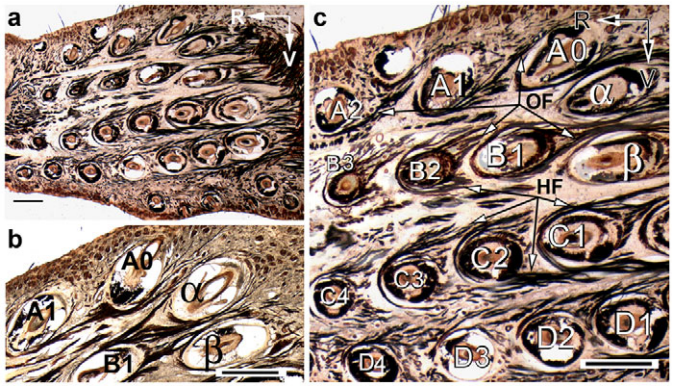

Fig. 2. Opossum intrinsic musculature. (A) General layout of the intrinsic muscles. (B) A close-up of the α and β 'inverted straddlers'. Whisker B1 can be seen to be straddling α and β (see also C). (C) Oblique intrinsic muscles. As well as the horizontal fascicles (HF) of the intrinsic muscles, there are also the oblique fascicles (OF) that connect, for example, the ventral capsular surface of A1 with the dorsal capsular surface of A0. Scale bars are 1 mm.

Superficial retracting muscles

In the opossum, the most superficial muscles of the mystacial pad are the m. nasolabialis and m. maxillolabialis (Fig. 3), which both attach caudal to the mystacial pad. M. nasolabialis is a striated muscle (Fig. 3A,B) that originates from the maxilla and os nasale, rostral to the orbit. It runs between the caudal part of the whisker rows. The m. maxillolabialis is also a striated muscle (Fig. 3C,D) that is slightly deeper than the m. nasolabialis. It originates from the ventrocaudal part of the maxilla, and its fibres pass under the m. nasolabialis (Fig. 3C), and then run rostrally, between the vibrissae rows, from B to D. Contraction of these muscles pulls the corium of the mystacial pad caudally, causing a retraction of the vibrissae. This is similar to what has been observed in the rat (Haidarliu et al., 2010).

Deep retracting muscles

In the rat, the deep retracting muscles originate deep and rostral to the pad and then run most of the way down the entire pad, whereupon they insert deeply into the mystacial fibrous plate (Haidarliu et al., 2010). The three muscles have been named, in dorsoventral order, as different parts of the m. nasolabialis profundus – the pars interna profunda, pars maxillaris superficialis and pars maxillaris profunda. The opossum similarily has three muscles also arranged dorsoventrally that have very similar origins (Fig. 4A). As these appear to correspond to the same muscles as those of the rat it is appropriate to use the same nomenclature. All three m. nasolabialis profundus muscles in opossum lie in a rostral-to-caudal direction and are connected to fibrous collagenous bundles situated between the vibrissal rows (Fig. 4A, left). These bundles, which have been identified as collagen as they possess a blue autofluorescence at a typical wavelength (Fig. 4B), have rosette-like ends where they attach to the muscle fibres rostrally (Fig. 4A,B) and belong to the three parts of the m. nasolabialis profundus (see also Fig. 10B). Pars interna profunda attaches to a collagen bundle that runs between rows A and B, pars maxillaris superficialis attaches to a collagen bundle that runs between rows B and C, and pars maxillaris profunda attaches to a collagen bundle that runs between rows C and D (Fig. 4B, left). Deep in the pad, the bundles contact the fibrous mat beneath the mystacial pad (Fig. 4C). If these muscles contract, the collagenous bundles pull the mystacial fibrous plate rostrally, which should retract the vibrissae in much the same way as in the rat. In terms of their general appearance, these muscles in the opossum appear to have a similar function to those of the rat; however, they are much shorter and actuate the pad *via* collagen bundles, unlike in the rodent species where the muscles run the full length of the pad. This is therefore an important difference between the two species.

Extrinsic protraction muscles

A further set of mystacial muscles comprises the extrinsic protracting muscles, which are also different parts of the m. nasolabialis profundus. In opossum, the pars media superior (PMS) and the pars media inferior (PMI) originate rostral to the pars maxillaris profunda origin from the septum intermusculare and rostral tip of the premaxilla (os incisivum). These attachment points are indicated by the black arrows in Fig. 5 (left). These muscles are relatively short dorsoventrally (Wible, 2003) compared with those in the rat.

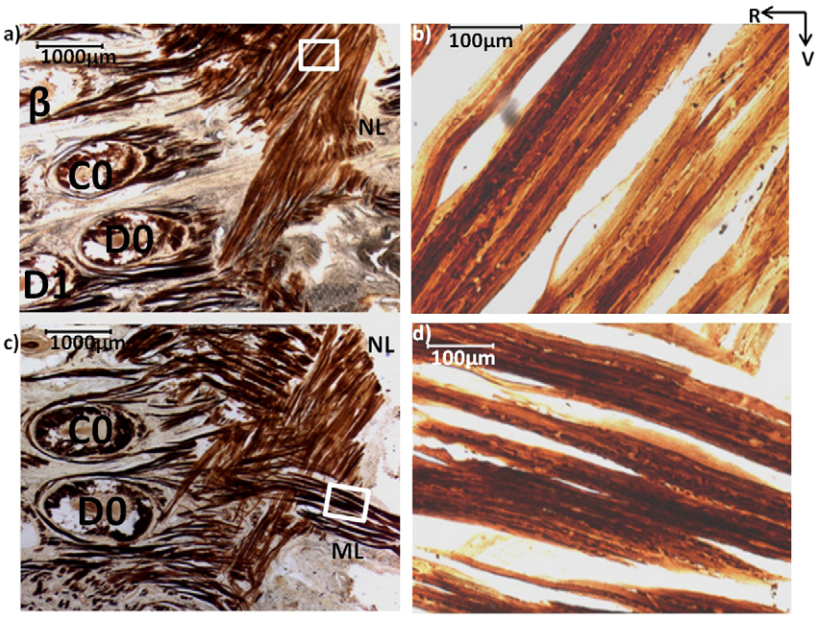

Fig. 3. Opossum superficial muscles m. nasolabialis (NL) and m. maxillolabialis (ML). (A) Attachment of NL to the mystacial pad. (B) Close-up of the striated NL muscle. (C) NL and ML can both be seen in a more superficial slice than that in B. (D) Close-up of the striated ML muscle fibres.

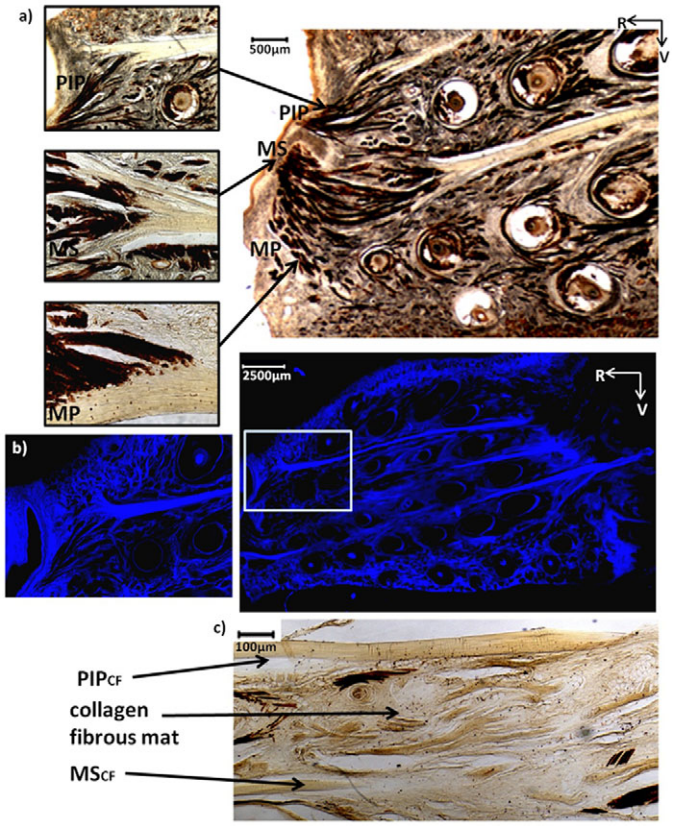

Fig. 4. Deep retracting vibrissal muscles in the opossum. (A, right) View of the the mystacial pad around the nose, showing the deep retracting muscles pars interna profunda (PIP), pars maxillaris profunda (MP) and pars maxillaris superficialis (MS). Close-ups at different levels through the pad of PIP, MS and MP, respectively, attached to their corresponding collagen fibres are shown on the left. (B, right) Close-up of the collagen bundle attaching to the PIP muscle by rosette-like endings prepared using blue collagen autofluorescence. Muscle attachment to collagen bundles between rows A–B, B–C and C–D shown using blue collagen autofluorescence is presented on the left. (C) PIP collagen fibres (PIP_{CF}) and the MS collagen fibres (MS_{CF}) inserting into the collagen mat. These fibres attach to the muscles and insert into the deep collagen fibrous mat so that the whole pad is moved when the muscles protract.

Specifically, from their rostral attachment points, both muscle parts run caudally, between whisker rows A–D, until vibrissal arc 2. Fig. 5 shows fragments of transected PMI and PMS, which continue caudolaterally and are inserted into the corium of the mystacial pad between all vibrissal rows and ventral to row D. In summary then, whereas in the rat the extrinsic protraction muscles are strongly inserted into the corium throughout every whisker row, in the opossum these muscles have a weaker presence throughout the pad and are absent from the more caudal sections.

Genal whiskers

As well as the mystacial whiskers, the opossum also has genal whiskers. These are six whiskers that are arranged in a horseshoe shape in the cheek area (Fig. 6A). The genal whiskers have striated intrinsic muscles, but rather than being attached to each other, they are attached mostly to the skin superficial collagenous layer. Genal whisker follicles have a strong capsule, and can contain both cavernous (with mesh and trabeculae) and ring sinuses; sometimes the ringwulst can be observed.

Behavioural consequences of differences in mystacial musculature

We have described above several interesting differences in mystacial muscle groups in the opossum compared with those of the rat – in the intrinsic muscles, the deep retracting muscles and the extrinsic protracting muscles. We next compare rat and opossum behavioural data between regular (non-contact) whisking and contact episodes in order to establish whether there is any identifiable consequence of the differences in mystacial musculature on observable whisking and active sensing behaviour. In particular, we hypothesised that the different extrinsic muscle organisation in the two species might impact on their capacity to modify relative whisker movement velocities following contact, and so cause measurable differences in the angular spread of the whiskers. As described in Materials and methods, we calculated the mean angular whisker positions, mean angular spread, mean retraction velocity and mean protraction velocity for 30 and 51 regular (non-contacting) whisking clips, and 23 and 43 contact whisking clips for rat and opossum, respectively. Traces from the raw behavioural data, shown in Fig. 7A,B, illustrate that during regular whisking bouts opossums move their whiskers in a sinusoidal, back-and-forth, pattern similar to that in the rat. In our previous analysis (Mitchinson et al., 2011), we found that rats whisk at a slightly higher frequency, during regular whisking episodes, compared with opossum (mean 8.7 Hz compared with 7.3 Hz) and with somewhat larger amplitudes (mean 43 deg compared with 36 deg). In both species, the whiskers spread out more as they protract forward and bunch together as they retract (Fig. 7C,D); this is likely to be largely as a consequence of the morphology of the system rather than due to active control (Grant et al., 2009).

Whereas the general form of whisking is similar in the two species during regular whisking bouts, in rat there is a substantial decrease in angular spread of the whiskers following and during contact with an object (between ANOVA: $F_{1,52}$=5.473, P=0.023), as shown in Fig. 8A. This is consistent with our earlier study (Grant et al., 2009) and has also been noted in our laboratory to be a bilateral effect in this species (R.A.G. and T.J.P., unpublished data). In comparison (see Fig. 8A), there is no evidence of any change in spread in the opossum following contact with an object (between ANOVA: $F_{1,93}$=0.013, P=0.910). Similarly, as shown in Fig. 8B, there is also a large decrease in retraction velocity following contact with a vertical surface in rat (between ANOVA: $F_{1,54}$=4.813, P=0.033), which is not matched in opossums (between ANOVA: $F_{1,95}$=0.316, P=0.575). There are no significant differences in whisker protraction velocity (Fig. 8C) and set-point (the average mean angular position, see Fig. 8D) between regular whisking and contact whisking in either species. Example digital video frames, shown in Fig. 9, further illustrate that whilst the rats reduce their whisker spread following a contact, in opossums, whisker spread remains largely unchanged during object exploration.

As shown in Fig. 6, the genal whiskers, which can be clearly seen at the caudal edge of the cheek in Fig. 9B,D, themselves have sling-like muscles with some similarities to those that move the macrovibrissae. To evaluate the capacity for independent movement in these whiskers, we examined 12 exemplar clips of opossum regular whisking. During bouts of whisking with the mystacial vibrissae, we typically also saw some lower amplitude oscillatory motion in the tracked genal whisker as illustrated in Fig. 10. Genal whisker motion was largely synchronised with movement of the mystacial vibrissae; however, macrovibrissal movement does not appear to entail movement of the genal whiskers, as illustrated in Fig. 10 where there are whisk cycles during which the macrovibrissae move sinusoidally but the tracked genal whisker

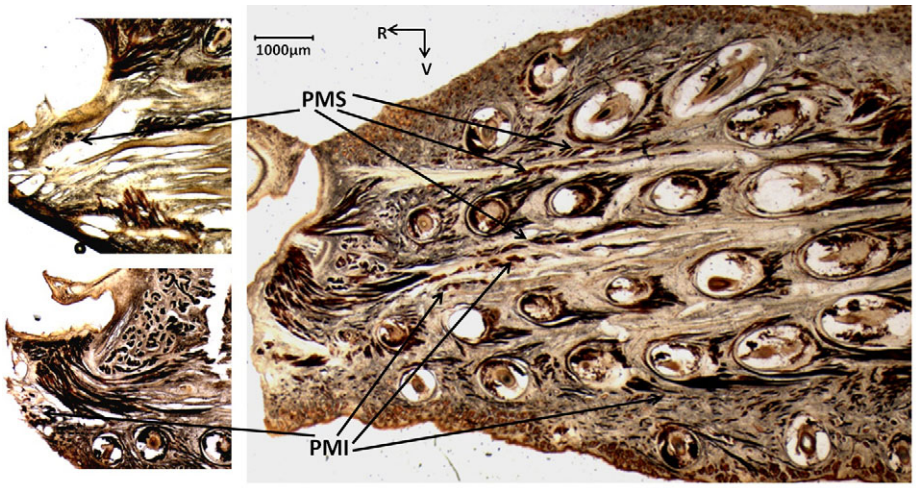

Fig. 5. Opossum extrinsic protracting muscles. An intermediate tangential layer of the pad showing pars media inferior (PMI) and pars media superior (PMS). PMS intercepts between rows A–B and B–C. PMI intercepts between rows B–C and C–D. PMS and PMI muscle attachment is shown on the left.

does not. This finding is consistent with the genal whisker motion being driven, at least in part, by its own intrinsic muscle.

DISCUSSION

The opossum mystacial pad has a grid-like follicle layout, with nasal and maxilliary compartments. There are fewer whisker rows than in rat; however, in both species the whiskers are coupled together within rows by horizontal intrinsic muscles, a distinctive pattern suggesting that a similar arrangement may have existed in an early mammalian common ancestor. Similarly, opossums and rats also have generally similar extrinsic musculature that can reshape the mystacial pad and drive whisker retraction and protraction movements.

We also noted some interesting differences between the rat and opossum vibrissal musculature. First, we found oblique intrinsic muscles in the opossum that have not been described in any other species. Second, we found whiskers that are connected to each other by a distinctive arrangement of intrinsic muscles, that we term inverted straddlers, as this reverses (rostrocaudally) the pattern seen in rats. The superficial extrinsic muscles were generally similar to those observed in rats, but the deep retracting and extrinsic protracting muscles appeared to be considerably less well developed in the opossum, contributing to the overall appearance of the mystacial pad as less substantial in opossum than in rat. Indeed, within the opossum mystacial pad, muscle territory is reduced, whilst collagen representation is relatively enriched compared with the whisker pad in the rodent species.

Some of these differences in mystacial musculature seem likely to relate to qualitative differences in the capacity of the animals to control their whiskers during active sensing behaviours. In particular, analyses of rat whisking during exploration of objects, here and in our previous study (Grant et al., 2009), have shown that these animals have the capacity to reduce the angular spread of their whiskers in a manner that increases whisker tip contact with surfaces of interest. In the analysis of opossum whisking behaviour reported above we found no evidence of a similar capacity in this species, suggesting that this may have evolved separately within the lineage of modern rodents.

Comparisons with other species

Fig. 11 summarises the arrangement of the opossum snout muscle system. We next discuss some similarities and differences to other mammalian species in which the whiskers and facial musculature have been investigated.

Organisation of the facial vibrissae

The layout of the opossum mystacial pad is quite similar to that of the wallaby (Waite et al., 2006) as there seem to be four rows

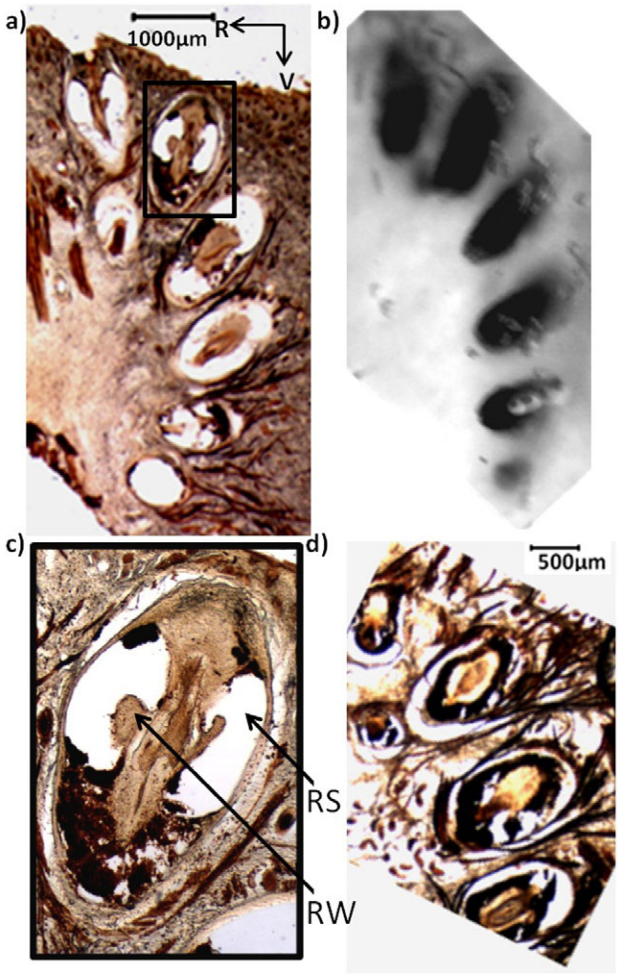

Fig. 6. Opossum genal whiskers. (A) Genal whisker arrangement from tangential slices. The genal whisker area can be seen clearly in Fig. 1A. (B) Ethanol/xylene-cleared preparation showing genal whiskers arranged in an approximately vertical row. (C) Close-up of the genal follicle showing a ringwulst (RW) and ring sinus (RS). (D) Sling muscles on the genal whiskers, the extremities of which are inserted dorsocaudally into the corium.

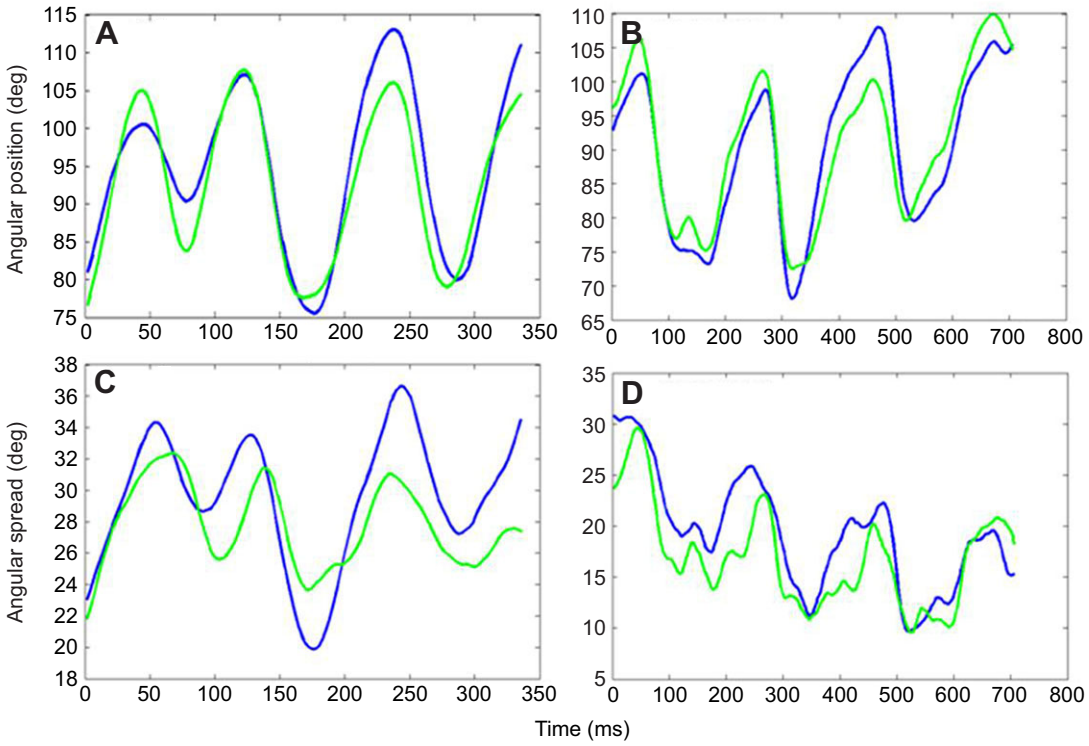

Fig. 7. Examples of rat (A,C) and opossum (B,D) whisker movements during movement across a smooth surface (regular whisking). (A,B) Mean angular position of the left (blue) and right (green) whisker fields over the course of three whisk cycles. (C,D) Changes in angular spread of the whiskers over the course of the same three whisk cycles. In both species, spread is strongly correlated to angular position such that whiskers spread out as they are protracted forward.

of vibrissae in both species, with two nasal whiskers present in the dorsal-most row. Weller (Weller, 1993) also found two dorsal-most whiskers in brush-tailed opossums; however, that study reported six rows of whiskers in total (or five if their row A is labelled as nasal whiskers). It seems possible that the relatively large size of the furry buccal pad whiskers might lead them to be confused with the mystacial whiskers; indeed, Weller (Weller, 1993) described the whiskers in the lower rows as 'numerous and difficult to count'. By looking at the muscles of the pad it is possible to clearly distinguish the mystacial whiskers from the nasal whiskers and furry buccal pad. If these whiskers have been mislabelled as mystacial pad whiskers in the Weller study then it would appear that the opossum does have a very similar whisker layout to other marsupials in which the whiskers have been investigated.

Compared with other mammals in which whisking has been recorded (see Mitchinson et al., 2011), the opossum has relatively few whiskers, indeed far fewer than hamsters (Wineski, 1985; Haidarliu and Ahissar, 1997), rats (Haidarliu et al., 2010), mice (Dörfl, 1982) and shrews (Kulikov, 2011). Amongst rodents, the layout of the guinea pig mystacial pad is perhaps more similar to that of the opossum (Haidarliu and Ahissar, 1997), containing a row of nasal whiskers and only two whiskers in row A.

Intrinsic musculature

Intrinsic muscles that couple rostral and caudal whiskers within the same row have also been described in mice (Dörfl, 1982), hamsters (Wineski, 1985), guinea pigs (Haidarliu and Ahissar, 1997), rats (Haidarliu et al., 2010) and shrews (Yohro, 1977), lending confidence to the view that this may be a primitive mammalian trait. A new finding in the opossum is the layout of the inverted straddlers in the dorsocaudal area of the pad, and the presence of oblique intrinsic muscles in the two dorsal whisker rows. In a study of big-clawed shrew, Yohro (Yohro, 1977) also found differences in the dorsal and ventral whisker rows but with the ventral whiskers having more muscles 'straddling' between whisker rows. We might expect to find variations in this part of the pad in other species, and in the future it might be interesting to attempt to relate these differences to behavioural specialisations. Here, we also found differences between the dorsal and ventral whisker layouts suggesting compartmentalisation of the pad. Compartmentalisation in mice was described by Yamakado and Yohro (Yamakado and Yohro, 1979), who identified a nasal compartment (rows A and B) and a maxilliary compartment (ventral rows) of whiskers that develop from different growth centres in embryo. Different growth centres are also likely to be responsible for the nasal and maxillary divisions in the opossum pad, and so potentially this might be a primitive mammalian feature.

A further novel finding in opossum are the bundles of intrinsic muscles in row A and B that originate from the ventral surface of the rostral whisker capsule and are inserted into the dorsal surface of the neighbouring caudal whisker. The position and attachment of these muscles suggest that they cause a torsional rotation of whisker rows A and B. Torsional rotation of whiskers in rat was first described by Knutsen and colleagues (Knutsen et al., 2008) who proposed that torsional movements could allow ventral whisker rows to scan downwards whilst dorsal whisker rows scan upwards. As we were only filming from above in the current study we are unable to make estimates of the torsional rotation of whiskers; however, based on the muscle layout in opossum it seems possible that the dorsal two rows may rotate more than the two more ventral rows.

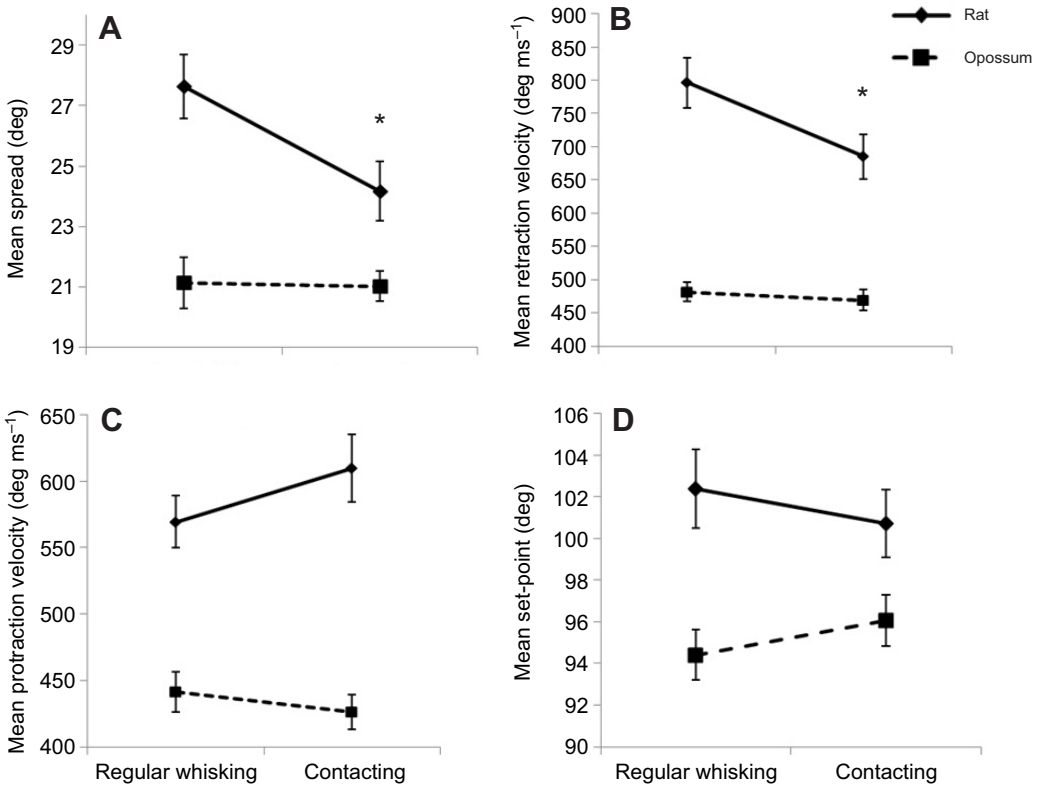

Fig. 8. Comparing whisking behaviour in regular and contacting whisking bouts in rats and opossums. (A) Mean angular spread (estimated by standard deviation of whisker angular positions) of the rat and opossum whiskers, in episodes of regular whisking and contact whisking. Rats significantly reduce their spread when they contact a surface (*P<0.05), whereas there is no significant difference in possums. (B) Mean retraction velocity is also significantly decreased during contact in rat (*P<0.05) but not in opossum, suggesting that differential control of whisker velocity may underlie the change in whisker spread. (C,D) Mean whisker protraction velocity and set-point (average of the mean whisker position) are not altered significantly between regular and contact whisking in either species.

Extrinsic musculature

Superficial extrinsic muscles that drive retraction movements of the vibrissae, similar to those seen in opossum, have been described in hamsters (Wineski, 1985), mice (Dörfl, 1982; Klingener, 1964), rats (Haidarliu et al., 2010), jerboas (Klingener, 1964), didelphid opossums (Minkoff et al., 1979) and shrews (Yohro, 1977). However, in the big-clawed shrew, the striated m. nasolabialis superficialis is also associated with smooth muscle fibres just beneath the corium (Yohro, 1977), unlike in the rat and opossum, suggesting that there are variations in extrinsic musculature between different species.

The different components of the m. nasolabialis profundus muscle group have been described in mouse (Dörfl, 1982; Klingener, 1964; Rinker, 1954), hamster (Wineski, 1985) and rat

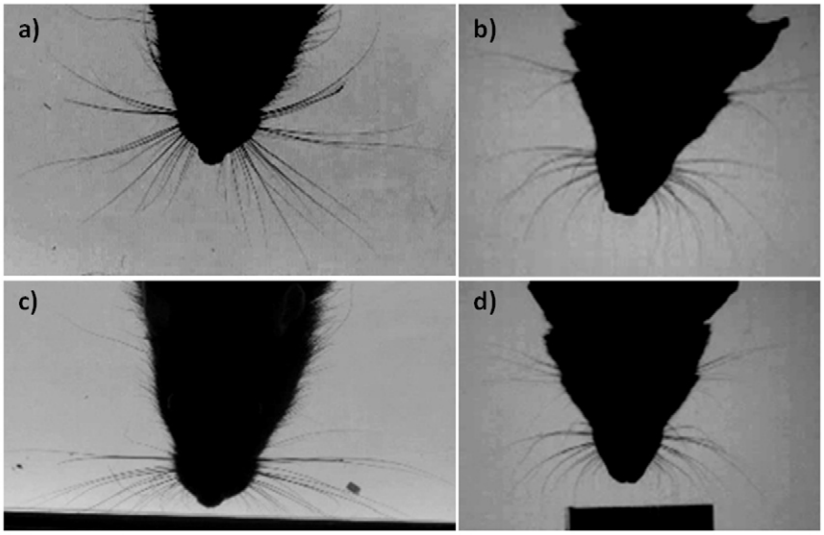

Fig. 9. Effect of surface contact on whisker spread in rat (left) and opossum (right). These digital video frames were selected to show the whiskers at maximum protraction during movement across a smooth floor (A,B) or after contact with a vertical surface (C,D). In rats, there is a visible reduction in whisker spread following contact with surfaces, suggesting that the whiskers are brought closer together in order to increase the number of surface contacts. In opossum, there was no evidence of a similar reduction in whisker spread, suggesting that these animals lack the capability to control their whiskers in this fashion. Note that head movement can cause changes in apparent spread viewed from an overhead camera; in our previous study (Grant et al., 2009), changes in whisker spread in rat were shown to be significant after controlling for changes in head tilt. This figure also illustrates the difference in vibrissal morphology between the rat and opossum, with the rat lacking the prominent genal vibrissae seen in the opossum but possessing a rounder, blunter snout that can provide better coverage, by the whiskers, of the region in front of the animal (see Discussion).

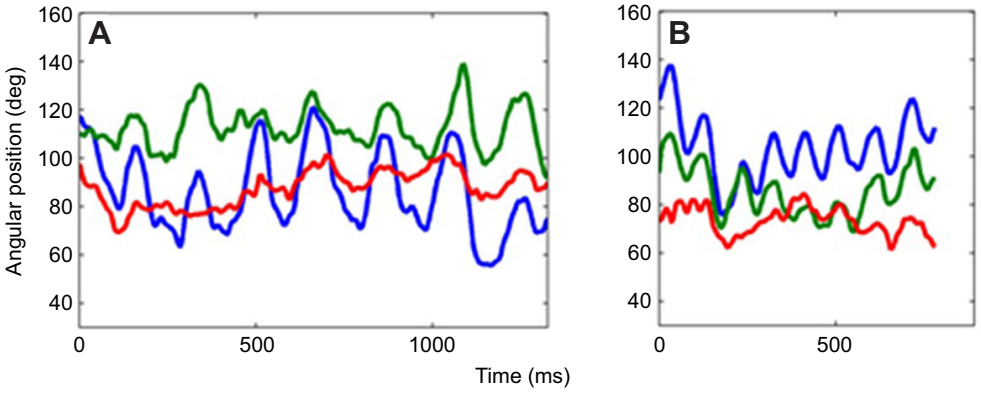

Fig. 10. Movement of the opossum genal whiskers. The results of two manual tracking examples are shown (A,B). Two macrovibrissae (blue, left whisker field; green, right whisker field) and one genal whisker (red) during two episodes of regular whisking. The genal whisker moves synchronously with the macrovibrissae, but at a lower amplitude, during some but not all whisk cycles.

(Haidarliu et al., 2010; Rinker, 1954). The deep retracting muscles of the m. nasolabialis profundus can be slightly different in placement or characteristics between rodent species (Wineski, 1985; Haidarliu et al., 2010); however, in general, these muscles pull the deep layers of the whisker pad forward, so that the whiskers are retracted back. In rat, whisker retractions are found to be faster than protraction movements, and both types of movement may be actively controlled (Berg and Kleinfeld, 2003). For instance, we previously found that, following a contact, whisker retraction velocities slowed down in the subsequent whisk cycle (Grant et al., 2009). In opossum, the equivalent deep retraction muscles are relatively reduced and attach rostrally to collagen fibres. This difference in musculature possibly also explains the lack of retraction velocity control when the opossum explores objects.

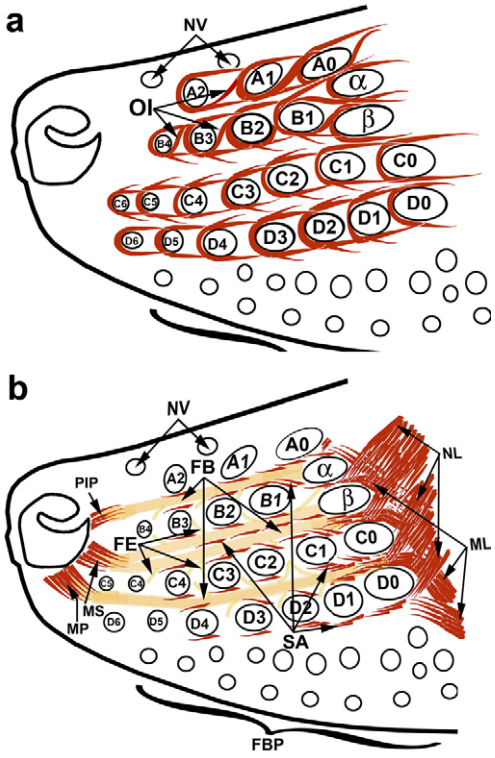

Fig. 11. Schematic representation of the mystacial pad muscles in the opossum *M. domestica*. (A) Intrinsic muscles. FBP, furry buccal pad; NV, nasal vibrissae; OI and RI, oblique and regular intrinsic muscles, respectively; marked black circles represent mystacial vibrissae. (B) Extrinsic muscles. FB, fibrous bundles passing between vibrissal rows; FE, fibrous extensions (branches) connecting FB with vibrissal follicles; ML, m. maxillolabialis; MP, MS and PIP, partes maxillares profunda and superficialis, and pars interna profunda of the m. nasolabialis profundus, respectively; NL, m. nasolabialis; SA, sites of attachment of ML and NL muscle fibre ends to the corium of the mystacial pad; other figure labels as in A.

The extrinsic protracting muscles also vary between rodent species (Rinker, 1954; Klingener, 1964), especially in placement. In some rodents they have been described as having characteristics similar to the superficial extrinsic muscles (Rinker, 1954; Klingener, 1964), being flat and running in the same plain. However, two previous studies (Dörfl, 1982; Haidarliu et al., 2010) have found them to run in a different plane. Dörfl (Dörfl, 1982) found the muscles to insert between the five rows of follicles in mouse, whereas Haidarliu and colleagues (Haidarliu et al., 2010) found them to be present in rat, between the five whisker rows and also dorsal and ventral to the whisker rows. In the opossum we see that they run between rows A–B, B–C, C–D and also lie ventral to row D. Haidarliu and colleagues (Haidarliu et al., 2010) used the term ‘focusing’ to describe the extrinsic protracting muscles, hypothesising that they help bring about the reduction in rostrocaudal spread of the whisker field that has been described during foveal whisking (Berg and Kleinfeld, 2003) and surface investigation (Grant et al., 2009; Grant et al., 2012). That the opossum does not appear to reduce whisker spread during object exploration could be due to a number of differences in these muscles. First, the difference in attachment positions of the muscles between rodents and opossums might cause changes in spread reduction behaviour (a comparison between the attachment sites can be seen in Fig. 12). Second, the muscle bundles in the opossum appear to be weaker, or attached partially to other places, which could change the direction of the muscle fibres. Overall, this could reduce the capability to reshape the mystacial pad in a manner that could be used to bring the whiskers closer together.

Genal whiskers

Many orders of mammals have genal whiskers, including marsupials (e.g. the grey short-tailed and Virginia opossums and the Tasmanian devil), edenta (e.g. hairy armadillos), insectivora (e.g. tenrecs), chiroptera (e.g. bats), primates (e.g. lemurs, aye ayes), carnivora (e.g. jackals) and rodents (e.g. dormice) (Pocock, 1914). However, their position on the face and the number of whisker can very immensely. In *M. domestica*, we usually see six genal whiskers positioned mid-way between the edge of the mouth and the bottom of the ear, well below the eye. During macrovibrissal whisking we have seen evidence of smaller amplitude movements in the genal whiskers, largely synchronised with those of the macrovibrissae. We believe this is the first report of sinoidal whisker motions in

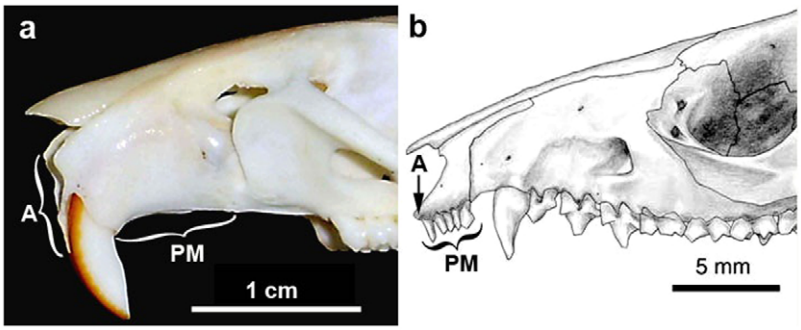

Fig. 12. Rostral skull sections of the rat (A) and opossum (B). PM is premaxilla and A is the attachment place of partes mediae superior et inferior. B is based on fig. 1A in Wible (Wible, 2003). The difference in the positioning of the attachment sites of PMS and PMI in opossum, compared with rat, could explain the absence of the spread reduction behaviour.

non-mystacial whiskers. In the current study we have also shown that these whiskers have sling muscles, similar to those involved in protracting the mystacial vibrissae, and suggesting that genal whisker movements may be actively generated rather than simply being a consequence of the movement of the extrinsic musculature of the nearby mystacial pad. Genal intrinsic muscles attach to the skin rather to other whisker follicles. During evolution of the mystacial whiskers it seems plausible that sling-like muscles, which originally anchored the whiskers to the skin, may have adapted to attach themselves to neighbouring follicles along the principal axis of whisker motion. Simulation results from Simony and colleagues (Simony et al., 2010) show that contraction of an intrinsic muscle in the rat causes movement of both attached whiskers. The evolutionary step of chaining the intrinsic muscles would therefore have enhanced their effectiveness in protracting the mystacial whiskers whilst also providing a stronger coupling of the movement of adjacent vibrissae.

Broader implications for understanding mammalian evolution

The presence of whisking in both rodents and marsupials, shown in our earlier study (Mitchinson et al., 2011), implies that a common ancestor to all modern therians (marsupial and placental mammals) may have also employed active vibrissal sensing. The current study provides compelling new evidence that whisking in rodents and marsupials uses similar underlying mechanisms and is therefore unlikely to have arisen by convergent evolution but is instead an inherited trait; in other words, that early mammals were also whisking animals.

If active vibrissal sensing was present at an early stage in the evolution of modern mammals then it will have had greater impact on the broader course of mammalian evolution than has hitherto been realised. Indeed, if early mammals actively moved their vibrissae, then this has important implications for many other aspects of mammalian evolution. For instance, the evolution of whisking musculature will have strongly shaped the broader evolution of the muscles of the face (see Huber, 1930). Further, the need to control the movement of the vibrissae will have brought about changes in sensorimotor circuits in the brain. Interestingly, the opossum cortex lacks a distinct motor area, and the only area of the body in which movement can be elicited by direct cortical microstimulation appears to the vibrissal region around the snout (Frost et al., 2000). If this represents an ancestral condition it suggests that the vibrissae musculature may have been one of the first, if not the first, areas of the motor system to have come under cortical control. Cortical influence on movement generally arises where more sophisticated patterns of control are needed than can be realised by brainstem or spinal circuits. Our results suggest the intriguing possibility that active vibrissal sensing may therefore have been a key driver for the evolution of cortical motor control in early mammals.

Opossums are generalists, having excellent eye sight and hearing, whereas rats are more specialised towards tactile sensing, having a comparatively poor visual sense. In progressing to become more of a specialist in vibrissal sensing, rodents such as the rat appear to have evolved a modified configuration of the snout and whiskers (compare e.g. Fig. 9A and 9B) in which, in addition to an increased number of whiskers and new degrees of freedom of whisker movement (spread control), the snout has become visibly shorter and blunter. Simulation and robotic studies (e.g. Prescott et al., 2009; Pearson et al., 2011; Towal et al., 2011) have shown that the morphology of the snout and vibrissal array shapes a sensory surface formed by the whisker tips and so helps determine the nature of the information that can be obtained by the animal. Moving from a more conical snout, as seen in the opossum, to the rounder shape of the rat, and adding additional macrovibrissae and microvibrissae, alters the shape of this surface and, in particular, substantially enhances the capacity of the animal to explore, through vibrissal touch, the area in front of the head and around the snout tip.

Conclusion

The grey short-tailed opossum, *M. domestica*, engages in active vibrissal sensing behaviours that have also been described in mice and rats. We found that the mystacial muscle system of the opossum is very similar to that of rodent whisker specialists, containing the four key muscle groups: the intrinsic, the superficial extrinsic, the deep retracting and the extrinsic protracting muscles. Similarities in whisking behaviour, and mystacial pad morphology and musculature, between these distantly related species lends confidence to the view that a common ancestor of modern therian mammals possessed an active vibrissal sensing system.

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AUTHOR CONTRIBUTIONS

R.A.G. contributed to study design and execution (sample preparation and behavioural studies), interpretation of the findings, and manuscript drafting and revising. S.H. contributed to study design, interpretation of findings and drafting the manuscript. N.J.K. contributed to sample preparation. T.J.P. conceived the

study and contributed to study design, interpretation of the findings, and drafting and revising of the manuscript.

COMPETING INTERESTS

No competing interests declared.

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