How many hair follicles are innervated by one afferent axon?

A confocal microscopic analysis of palisade endings in the auricular skin of thy1-YFP transgenic mouse

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Abstract: Hairs are known as a sensory apparatus for touch. Their follicles are innervated predominantly by palisade endings composed of longitudinal and circumferential lanceolate endings. However, little is known as to how their original primary neurons make up a part of the ending. In this study, innervation of the palisade endings was investigated in the auricular skin of thy1-YFP transgenic mouse. Major observations were 1) Only a small portion of PGP9.5-immunopositive axons showed YFP-positivity, 2) All of thy1-YFP-positive sensory axons were thick and myelinated, 3) Individual thy1-YFP-positive trunk axons innervated 4–54 hair follicles, 4) Most palisade endings had a gap of lanceolate ending arrangement, 5) PGP9.5-immunopositive 10–32 longitudinal lanceolate endings were closely arranged. Only a part of them were thy1-YFP-positive axons that originated from 1–3 afferents, and 6) Single nerve bundles of the dermal nerve network included both bidirectional afferents. Palisade endings innervated by multiple sensory neurons might be highly sensitive to hair movement.

Keywords: hair follicle, lanceolate ending, touch sense, single afferent, three dimensional analysis, thy1-YFP transgenic mouse

Introduction

Hairy skin is an essential sensory apparatus in mammals.1–6 Body surface of most fur-bearing mammals is densely covered by both guard and vellus hairs. In addition, a certain number of vibrissa hairs are provided to their facial hairy skin and the wrist.7,8 Vibrissa follicles are well known as an important tactile sensory organ. Over the years, a great deal of effort has been spent in anatomical and physiological investigations on their innervation.7–14 Several types of mechanoreceptors, such as the Merkel endings, the lanceolate endings, the Ruffini-like endings and the lamellated endings, were identified in the vibrissa follicles. Dense and well arranged distribution of all endings can be observed in each follicle.14

On the other hand, the follicles of common guard and vellus hairs are small in size compared to the vibrissa follicles, however, they are well-innervated by palisade endings.1–6 Each follicle has a palisade ending which is distributed around the outer root sheath at the level of just below the sebaceous gland.1–6,13 It is composed of a certain number of longitudinal lanceolate endings and a few circumferential lanceolate endings.2,9 The former is thought to be supplied by myelinated nerve fibers and the latter by a mixture of myelinated and unmyelinated nerve fibers.2 The circumferential lanceolate endings are sparsely distributed in vellus hairs.12 In these days, they call cluster of only longitudinal lanceolate endings as a palisade ending. It is known that the innervation of guard and vellus hairs is derived from afferents of the nerve plexus in deep dermis.12 And the longitudinal lanceolate endings are physiologi-
cally known as rapidly adapting (RA) receptors and the circumferential lancetlike endings as slowly adapting (SA) receptors. However, little is known about their precise innervation in the complicatedly distributed skin nerve plexus and about original primary sensory neurons.

The aim of this study was to clarify how single sensory afferents individually make their receptive fields. We focused our attention to the palisade endings in the murine auricular skin. The auricle is composed of two skin layers and cartilage in between them. Since the auricular skin contains thinner dermis, less hair follicle and less adipose tissue compared to any other body parts, the skin tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation. In previous studies, number of hair follicles, hair types, tissue is suitable for analysis of innervation.

No matter how specific neuronal markers well discriminate nerve characteristics, dense nerve distribution in the skin tissue was the biggest barrier to analyze individual afferent throughout from trunk afferent to the axon terminals. Then, we found availability of thy-1 gene-expressing YFP transgenic mouse for tracing particular axons. The mouse model has been demonstrated that only a part of specific types of peripheral nerves including DRG neurons and palisade endings were clearly labelled. Distribution of lamellated corpuscles and their afferents in the murine auricular skin were evaluated by means of acetylcholinesterase enzyme histochemistry and silver impregnation staining.

Several studies precisely described the ultrastructures of the palisade endings and turnover of the endings. However, little is known about their receptive fields and the precise three-dimensional architectures of the palisade endings.

We hope that these morphological observations will contribute to understand physiological response profiles of the hairy skin as a tactile sensory organ.

Materials and methods

Six normal mice (C57BL6, 4–7 weeks old, both sexes) and two thy-1 gene targeted YFP transgenic mice (Background; C57BL6, 4 and 7 weeks old respectively, female, fixed specimens were kindly provided by Prof. Raya Eilam (Weizmann Institute of Science, Rehovot, Israel)) were used. All procedures used in the present studies were approved by the Meiji University of Integrative Medicine Animal Care and Use Committee (No. 22–26).

Under deep anaesthesia (sodium pentobarbital, 40 mg/kg, i.p.), animals were transcardially perfused by fixative (4% paraformaldehyde in 0.1 M phosphate buffer (PB) at room temperature (RT), 30–40 ml) following 10 ml 0.1 M PB perfusion at RT. After fixation, auricles were removed and both sides of the auricular skin were slipped off from the cartilage with tweezers under a stereomicroscope. Excessive adipose tissues were removed and still thick and winding parts or most of densely hairy parts at the base of the auricles were removed. The flat and thin skin tissues were dealt as pieces of whole-mount tissues with 150–200 μm-thick (Fig. 1). They were processed after incubation in 0.1 M PB containing 0.3% triton-X100 (T-PBS) at 4°C for 1–2 weeks. A long immersion in T-PBS, if necessary with under reduced pressure (reduced-pressure incubator, Taitec), was necessary at several steps of the following procedures to get good staining results.

Immunohistochemistry. After one-day-long immersion in normal goat serum (1:10 in T-PBS) at 4°C, the tissues were stained for 1–2 weeks at 4°C with appropriate combinations of primary antibodies as mixture, anti-PGP9.5 as a pan-neuronal marker, anti-NF200 for Aβ-thick axons and anti-MBP for myelin sheath. The sections were placed on a shaker once a day for 1–2 hours at RT during the incubation period. After washing off excessive primary antibodies by rinsing in T-PBS, the sections were incubated within appropriate combinations of fluorescent-conjugated secondary antibodies. All primary and secondary antibodies were commercial products derived from rabbit, rat or goat (see Table 1). After rinsing in 70% glycerol in T-PBS for several hours at RT following washing out of excessive secondary antibodies, the sections were mounted between two coverslips using a hydrophilic mounting medium containing DAPI for nuclear staining (Vectorshield H-1200, Vector).

Observation. The skin tissues were observed from two sides using a spacer on stage of microscopes. High resolution photomicrographs were taken by means of a software (NIS-Element D, Nikon). One of its modules, Extended Depth of Focus (EDF) option, was available to allow images from different Z-axes captured by a digital camera (DXM1200, Nikon) equipped on a standard fluorescent microscope (Nikon E-800, objective lens; CFI Plan Apo 4X, 10X, VC 20X, 40X, VC 60X oil, 100X oil, numerical aperture respectively; 0.2, 0.45, 0.75, 0.95, 1.4, 1.4) to be combined into a single all-in-focus image. Photomontages to take overview of whole
auricular tissues were synthesized from those focused images.

**Three-dimensional analysis.** For image capture and 3D-analyses, the detailed procedure was described previously (Ebara et al., 2002, 2008). Briefly, for three-dimensional analysis, confocal stack images of appropriate sites on the skin tissues were captured by confocal laser microscope systems (BioRad LaserSharp-2000 equipped on a Nikon E-800, and Nikon C1 equipped on a Nikon Eclipse 90i, objective lens were the same as the above, z-step between adjacent optical images; 1.0–0.2 µm). All images were reconstructed by using a volume rendering software (VGStudio Max 2.0, VolumeGraphics, Heiderberg, Germany). Three-dimensional volume images were consistently made along the focused axon to avoid scrambling. And the axons were traced using photo montages of the images. Several reconstructed structure were observed from every possible angle by using the reconstruction software. Removals of distractions around focused labelled axons were often made in order to discriminate continuities of the ramifications. Commonly-used photo-retouched and presentation softwares (Adobe PhotoShop CS and Microsoft PowerPoint 2007) were appropriately used to add pseudo-colours or to make photo montages.

Arbitrarily-selected 8 large palisade endings labelled by anti-PGP9.5 in the normal mouse auricular skin were three-dimensionally analyzed and the number of all lanceolate endings that
constituted each palisade ending were counted. In the biggest one of those, every axon terminal of all longitudinal lanceolate endings was traced to the deep dermal nerve plexus. All ten thy1-YFP-positive afferents (trunk axons), that were recognized in a thick nerve bundle at the basal middle part of the cut edge of a posterior auricular skin, were three-dimensionally traced throughout to the nerve endings. The terminal structure of the endings, the number of innervated hair follicles, the distributional area of trunk axons and their branching pattern, derived from specified single trunk afferent, were analyzed and mapped on the focused photomontage of the auricular skin.

**Results**

Innervation in the auricular skin was well observed in our whole-mount preparations with two-side observations (Fig. 1).

**Murine auricular hairy skin.** Hairs distributed in both sides of auricular skin were mostly thick vellus hairs that mostly had short hair shaft in outside of the skin surface in addition to scattered guard hairs. Vellus hairs were individually distributed evenly across each other without showing any compact cluster of hair follicles and any eccentric distribution density of follicles throughout the auricular surface (Fig. 2a). No differences were recognized between inferior and posterior skin of the auricles in the hair distribution patterns.

**Innervation: General observation.** All axons from thick fibers in the nerve bundles to thin varicose fibers in the epidermis were well labelled by anti-PGP9.5 antibody and they showed a dense distribution in the auricular skin (Figs. 1d, 2a). A part of the axons were MBP-positive, thus they were myelinated (Fig. 4). Myelinated thick bundles came into at the basal cut end of the auricles and extended radially to the marginal area of the auricles showing several branching. Small nerve bundles firstly developed a rough network in the subcutaneous tissue, and then established a dense network, i.e., the dermal plexus, in which each of several hair follicles was surrounded by a network of nerve bundles (Fig. 2a). Finally, finer bundles originated from the plexus formed a finer network and then some single axons approached to the follicle to make a palisade ending (Fig. 2). All hair follicles without an exception had a palisade ending in the present studies. Merkel endings and lamellated corpuscles were scattered in the auricular skin (data not shown).

**Single palisade ending.** Each palisade ending was located beneath a sebaceous gland (Figs. 2, 3). Several myelinated fibers converged to the follicle from different directions. Beyond their myelin sheath, each fiber branched a few times and finally terminated as lanceolate endings in a fork-like shape. Individual lanceolate ending was a thickened and long axon terminal sandwiched mostly by two thin cell sheaths of the terminal Schwann cell (data not shown). A total of 10–32 lanceolate endings surrounded the hair shaft (n = 8) in a palisade arrangement. Occasionally a few circumferential lanceolate endings overlapped (Figs. 2, 3). Most palisade endings had only longitudinal lanceolate endings without circumferential lanceolate endings. On the other hand, a very few endings had only circumferential ones.

Most palisade endings had a gap in a circle of longitudinal lanceolate endings. The gap was 1/8–1/2 of the circle and usually faced to the epidermis, that is, superficial side of the hair root which canted over to the skin surface (Fig. 3). Thus, longitudinal lanceolate endings were generally distributed in the deeper side of the hair root at the resting position. These endings tended to form a population having those gaps in the similar direction on the dorsal auricular skin (Fig. 3).

**Thy1-YFP-positive palisade endings.** Only a small portion of PGP9.5-immunopositive axons showed thy1-YFP-positivity in the skin (Fig. 4b). All of thy1-YFP-positive axons were MBP-positive, thus myelinated and NF200-immunopositive, thus Aβ-fibers. Only a part of MBP-immunopositive axons were thy1-YFP-positive.

Ten thy1-YFP-positive trunk axons were observed in a branch of the posterior auricular nerve at the basal middle edge of an auricular skin. All of them were fully traced to the endings individually by confocal three-dimensional analysis (Figs. 5, 6). Individual thy1-YFP-positive trunk axons innervated 4–54 hair follicles in each territory which showed different sizes of distribution area (Fig. 6). Only one of them showed a large isolated territory of terminals at the basal portion of the auricle. The others were distributed with overlaps with neighbouring territories in the marginal zone. All territories in the marginal zone included several follicles that were innervated by more than one afferents (Figs. 6, 7). One to 3 trunk axons were involved to form one palisade ending terminating as longitudinal lanceolate endings around hair follicles (Fig. 7f). Each terminal
Fig. 2. PGP9.5-immunopositive dense nerve distribution in an auricular whole-mount tissue. a; Thick, middle and fine arrows; trunk, small and fine axon bundles. asterisks; all palisade endings in this rectangular area. b1; a view from the skin surface. A hair shaft comes out from the mouth of the follicle (dashed line) to the skin surface. b2; the same view as b1 but the surface layer was removed on the 3D-image. A palisade ending (circle) with a gap at a direction to the skin surface (arrowhead) could be seen. b3; a side view of b1. c; A view from subcutaneous side. Most palisade endings showed a gap respectively (arrowhead). All gaps were arranged to the same orientation. d; A palisade ending with entirely arranged lanceolate endings. A hair shaft should insert inside the circle (asterisk). The far side of this picture towards skin surface.
axon provided 1–5 lanceolate endings. They were distributed as a large-tooth combe. Lanceolate endings originated from different axon terminals infrequently showed crossover distribution (Figs. 5, 7f).

Tracing of individual afferents made it clear that each of the small myelinated nerve bundles that comprise the network of the dermal nerve plexus included nerve fibers in both centrifugal and centripetal directions (Fig. 7d–g).

Discussion

One afferent is always connected to only one type of nerve ending. In this study, we investigated detailed morphology of the palisade endings in the auricular skin by three-dimensional analysis and also with tracing methods using thy-1 gene-expressing YFP transgenic mouse. Only a part of myelinated innervation was thy1-YFP-positive in the transgenic mouse, and unmyelinated fibers were
thy1-YFP-negative. A recent study demonstrated that 21–46% of innervation was thy1-YFP-positive. Such sparsely labelled neurons were useful to be traced as single axons in complicated skin innervation of the receptive field.

Although a certain number of myelinated afferents that originated from the dermal nerve plexus normally terminate as Merkel endings and lamellated corpuscles, while majority terminate as palisade endings, only palisade endings were thy1-YFP-positive in the auricular skin. Incidentally, Merkel endings at the level of the rete ridge collar in the mystacial vibrissa follicles of the same mice used in this study occasionally could be observed as...
as a small part of the lanceolate endings at the level of the ring sinus and several palisade endings surrounding ordinal guard hairs distributed in the skin between vibrissae (data not shown). Those facts and the small proportion of thy1-YFP suggested that thy1-YFP positivity was specific to the lanceolate endings at least in the murine auricular skin. Furthermore, complete tracing of individual labelled axons in this study strongly suggested that one afferent is always connected to only one type of nerve ending.

**Original afferents of a palisade ending.** In the murine skin, different layers of dense nerve plexus, subepidermal plexus/superficial dermal plexus, deep cutaneous plexus/dermal plexus and subcutaneous plexus, are distributed in the dermis and subcutaneous tissues, respectively.\(^5\) Axons originating from one of them come close to the final destination and terminate as different type of nerve endings. As for palisade endings, both longitudinal and circumferential lanceolate endings are generally said to be originated mainly from dermal plexus.\(^2\),\(^12\) In this study, clear discrimination could not be well pursued since subepidermal plexus was very close to the dermal plexus in such a thin tissue of the mouse auricle. Subepidermal plexus was mainly composed of unmyelinated fibers, so that we may just say that palisade endings were originated from dermal plexus. Our observations are consistent with previous studies.

Longitudinal lanceolate endings have been well demonstrated to originate from myelinated afferents and terminate as fork-like axon terminals. Each axon terminal is sandwiched by two pieces of thin cell
Fig. 6. Mouse hair follicle innervation
Fig. 7.
sheath of terminal Schwann cells. These cells also made networks in connecting with each other by gap junction in lanceolate endings in the rat vibrissae. Axon and Schwann cell complex may work for mechanoacceptation and mechanotransduction in most mechanoreceptors. Circumferential lanceolate endings in all types of hair follicles except for lanceolate endings in vibrissa hairs have been considered to originate from unmyelinated afferents. However, our observation indicated that they also originate from myelinated fibers in auricular skin since they were positive to both thy1-YFP and MBP. In addition, PGP9.5-immunopositive innervations showed unmyelinated nerve distribution surrounding near palisade endings in both guard and vellus hairs and vibrissae. Most of unmyelinated nerves showed varicose appearance. PGP9.5-positive but Thy1-YFP-negative lanceolate endings might possibly originate from myelinated afferents that were incidentally negative thy1-YFP expression. Further tracing study will be required to clear it.

Numbers of palisade endings and lanceolate endings to a trunk afferent. Ten to 32 PGP9.5-immunopositive thick lancelet-like axon terminals and their myelinated afferents were counted in individual palisade endings. These observations are largely in agreement with earlier studies that showed 4–30 myelinated afferents in individual palisade endings. The difference in the number may depend on the size of follicle in different animals. On the other hand, our tracing analysis of individual thy1-YFP-positive afferents to their endings showed for the first time that individual palisade endings might be innervated by at least 2–4 different trunk afferents. The reason for this is that most palisade endings were innervated by 1–3 thy1-YFP-positive trunk afferents in addition to singly innervated by PGP9.5-immunopositive lanceolate endings.

Numbers and characteristics of neurons that innervate a palisade ending. The hair type found in murine skin of the back is distinguished into four (guard, awl, auchene and zigzag) on the basis of the length of hair shaft, the number of medulla cells and the presence of kinks in the shaft. Zigzag hair follicles are innervated by both C- and Aδ-low-threshold mechanoreceptors (LTMRs). Guard, auchene and zigzag hairs by Aβ, Aδ- and C-LTMRs and guard hairs by Aβ-LTMRs. The longitudinal lanceolate endings of the zigzag and awl/auchene hair follicle suggest innervations by different neurons. In this study, even a palisade ending of the guard hair was also always innervated by 1–3 thy1-YFP-positive different trunk axons. In addition, each of thy1-YFP-positive axons showed the first ramification close to the terminal area. Further investigations are needed to show if each thy1-YFP-positive axon originates from an individual neuron. If that is the case, each palisade ending would be innervated by at least 2–4 primary sensory neurons.

This study is the first clear demonstration of complete trajectories of individual Aβ-fibers that constitute a single palisade ending in non-vibrissal hairs. Although such multiple innervation was shown in mouse dorsal skin by Li et al. (2011), Aβ-fibers of different cell origin were not distinguishable from...
one another by their labeling technique. On the other hand, this study appears to lack information concerning interdigitation among C, Aδ- and Aβ-axon terminals as demonstrated by Li et al. (2011).32

**Morphology and function of palisade endings.** Such interdigitating manner of lanceolate endings may suggest not only innervations by multiple neurons on a follicle but also a possibility of responses to hair shaft movements in wider directions than a restricted distribution. The responsiveness may be facilitated by that single afferent innervating considerable number of hair follicles at the same time. A single thyl-YFP-positive axon terminated in various directions on the multiple palisade endings.

On the other hand, most of individual palisade endings in the murine auricular skin showed a gap in longitudinal lanceolate ending arrangements. This is consistent with previous studies in the murine body hairy skin.13 Palisade endings have been regarded to evoke nerve firings when the hairs were bristled up.20,21 According to our observations, lanceolate endings might ordinarily form a gap in the direction to which the hair goes forward during the hair protraction. Those gaps of lanceolate endings were found at the similar orientation in neighbouring hair follicles. In addition to the possibility of innervations by multiple sensory neurons on every palisade ending, these results may suggest that palisade endings would represent an ideal structure to be exquisitely sensitive to hair bristling/protration although their directional discrimination may be poor.

A previous study showed that 150–579 corpuscles were distributed in the murine auricular skin and that at least 20 and at most 40–60 corpuscles might be innervated by a single myelinated afferent.20 Another study on the cat finger tip showed that more than 150 Merkel-nerve complexes of a touch dome converged to a single myelinated afferent.25 In this study, individual thyl-YFP-positive axons innervated 4–54 palisade endings. All of those observations confirmed previous neurophysiological studies that suggested each of single trunk afferents should have a restricted receptive field in which the afferent innervated only one kind of multiple nerve endings.33 However, in any cases, it is still uncertain whether each of trunk afferents in the tissue originated from a separate primary sensory neuron unless a single neuron is successfully traced from the cell body to the nerve ending.

Most palisade endings in the murine auricular skin showed complicated interdigitating distribution of lanceolate endings. And individual palisade endings may be innervated by multiple neurons. Most of small myelinated nerve bundles in the dermal plexus included both bidirectional afferents. Functions depend on those morphological characteristics of palisade endings are still uncertain. Further histological investigation supported by electrophysiology will required to well understand touch sense.

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