Receptors and transporter for serotonin in Merkel cell-nerve endings in the rat sinus hair follicle. An immunohistochemical study*

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Summary. Serotonin (5-HT) has been a candidate for neurotransmitters in cutaneous type I mechanoreceptors (i.e., Merkel cell-nerve endings). Although recent electrophysiological studies have suggested the presence of the 5-HT₂ and 3 receptors in the Merkel cell-nerve endings, the histological localization of these receptors are obscure. We thus immunohistochemically examined the presence of 5-HT₁, 2, 3 receptors in Merkel cell-nerve endings in sinus hair follicles of the rat whisker pad. We also studied the immunohistochemical localization of the 5-HT transporter to confirm the site of 5-HT secretion. For this purpose, we used antibodies for the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT₃ receptors, and for the 5-HT transporter, as well as antibodies for cytokeratin 20 (as a marker of Merkel cells) and neurofilament H (a marker of type I sensory nerve terminals). The immuno-stained sections were analyzed under a laser-scanning microscope. It was found that the sensory nerve terminals in the Merkel cell-nerve endings showed strong positive immunoreactions of 5-HT_{1A} and 1B receptors but not 5-HT_{2A}, 2C, and 3 receptors. Furthermore, both the Merkel cells and related axon terminals showed strong immunoreactions of the 5-HT transporter. These findings support the idea that 5-HT molecules are released

from the Merkel cells during mechanical reception and indirectly regulate neural actions of sensory neurons via 5-HT₁ receptors. The localization of the 5-HT transporter found in this study also suggests a possibility that axon terminals in the Merkel cell-nerve endings also release 5-HT.

Introduction

Cutaneous type I mechanoreceptors (i.e., Merkel cell-nerve endings) are composed of an axon terminal of the type I sensory neuron, and a Merkel cell. These receptors are known to be slowly adapting mechanoreceptors (Iggo and Muir, 1969) and are distributed in the basal layer of stratified squamous epithelia of the skin and oral mucosa, as well as the external epithelial sheath of hair follicles of vertebrates (for reviews see: Tachibana, 1995; Halata *et al.*, 2003). The true function of the Merkel cell in the receptor is still enigmatic; some physiologists have suggested a mechanical transduction function for this cell on the basis of results of electrophysiological studies (for reviews see: Iggo and Muir, 1969; Iggo and Findlater, 1984; Ogawa, 1996; Halata *et al.*, 2003), while other researchers have opposed this idea (Gottschaldt and Vahle-Hinz, 1982; Diamond *et al.*, 1988; Miles and Diamond, 1995).

Various kinds of neuroactive substances—such as serotonin (5-HT), VIP, substance P, CGRP and ATP—have been demonstrated by immunohistochemical studies to be localized in Merkel cell granules (specific dense cored granules of about 100 nm in diameter) (for reviews see: Hartschuh *et al.*, 1986; Tachibana, 1995). If Merkel cells act as mechanical transducers, some of these substances should act as neurotransmitters that generate impulses in the axon terminal *via* ligand-gated ion channels during mechanical reception.

Recently, He and colleagues (2003) showed that antagonists for 5-HT₂ and 3 receptors reduced type I

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responses to mechanical stimuli in an *in vitro* rat skin preparation. It is known that the 5-HT₃ receptor is the only family of ionotropic receptors for 5-HT, while 5-HT₂ receptors, which include three subclasses, are known to be metabotropic receptors that couple to Gq/11 proteins (Raymond *et al.*, 2001). We previously showed that Merkel cells in the rat sinus hair follicles express positive immunoreactivities of α -subunit isoforms of Gq, Go, Gi, and Gz proteins, and the related axon terminals limitedly express immunoreactions of α_o and α_i isoforms of G proteins (Tachibana *et al.*, 2001). Taking these facts into consideration, the reduction of the type I responses in the Merkel cell-nerve endings caused by antagonists for 5-HT₂ receptors as shown by He *et al.* (2003) might be explained as an indirect response through impairment of the function of Merkel cells that have the Gq protein coupled to 5-HT₂ receptors. It is also considered that the

type I axon terminals in the Merkel cell-nerve endings may have 5-HT₁ receptors, since the 5-HT₁ family couple to Gi and Go proteins (Hoyer *et al.*, 1994; Hoyer and Martin, 1997; Raap *et al.*, 1999; Raymond *et al.*, 2001; Albert and Tiberi, 2001). Therefore, the primary purpose of the present study was to investigate the presence of 5-HT receptors in Merkel cell-nerve endings.

The actions of 5-HT in a synapse are limited by a re-uptake of the molecules by 5-HT transporter proteins located on the pre-synaptic membrane (Attwell and Mobbs, 1994; Brownstein and Hoffman, 1994). On the other hand, from their observations using cultured and intact Merkel cells from the rat skin of late embryonic stages, Hansson and Hoffman (2000) suggested that Merkel cells in the developing rat skin receive 5-HT from outgrowing sensory nerves or amniotic fluid *via* the 5-HT transporter on Merkel cells. Although mammalian Merkel

Table 1. Antibodies and conditions for serotonin receptors and transporter.

Antibody	Host	Source	Dilution	Control
Serotonin receptors				
5-HT1A (AB5406)	guinea pig	Chemicon* ¹	1 : 1,000	brain, PBS
(sc-1459)	goat	Santa Cruz* ²	1 : 400	brain, blocking
5-HT1B (AB5410)	guinea pig	Chemicon	1 : 1,000	brain, PBS
(sc-1460)	goat	Santa Cruz	1 : 100	brain
5-HT2A (sc-15073)	goat	Santa Cruz	1 : 100	brain
5-HT2C (sc-15081)	goat	Santa Cruz	1 : 100	brain
5-HT3 (sc-19152)	goat	Santa Cruz	1 : 100	brain
Serotonin transporter	guinea pig	Chemicon	1 : 5,000	brain, PBS

*1: Chemicon International, Inc., Temecula, CA, USA. *2: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Table 2. Sources and conditions of fluoro-labeled secondary antibodies and the anti-cytokeratin 20 antibody.

Antibody	Host	Label	Source	Dilution
Cytokeratin 20	mouse	–	Progen* ¹ (It-Ks20.8)	1 : 20
Neurofilament H	rabbit	–	Affiniti* ²	1 : 1000
Mouse IgG	goat	Alexa 488	Molecular Probes* ³	5 μ g/ml
Mouse IgG	donkey	FITC	Rockland* ⁴	20 μ g/ml
Goat IgG	rabbit	Alexa 594	Molecular Probes	5 μ g/ml
Guinea pig IgG	rabbit	Texas red	Rockland	20 μ g/ml

*1: Progen Biotechnik, Heidelberg, Germany. *2: Affiniti Research Products Ltd., UK.

*3: Molecular Probes, Inc., Eugene, OR., USA. *4: Rockland, Inc., Gilbertsville, PA., USA.

cells in adult stages express 5-HT-like immunoreactions (Garcia-Caballero *et al.*, 1989; English *et al.*, 1992), it is not known whether Merkel cells sustain the expression of 5-HT transporter mRNAs. The second purpose of this study is thus to immunohistochemically examine whether Merkel cells possess the 5-HT transporter in the adult rat.

Materials and Methods

Tissue preparation

Five male and four female Wistar rats of 2 to 6 months of age were used in this study. Seven of these were killed by carbon dioxide inhalation, and their whisker pads were immediately excised out. These pads were quickly rinsed in cold phosphate-buffered saline (PBS) and sliced manually with thin double-edged blades into thick sections, each of which contained one row of sinus hair follicles. These sections were embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo) and frozen in liquid nitrogen. The remaining two animals were deeply anesthetized with a peritoneal injection of an overdose of pentobarbital sodium and perfused with 4% paraformaldehyde dissolved in a 0.1 M phosphate buffer (pH 7.4). Whisker pads and brains were excised and immersed in the same fixative for 2 h at 4°C, washed in a 0.01 M phosphate buffer, mounted in O.C.T. compound, and then frozen in liquid nitrogen.

Fresh frozen sections of whisker pads, each 10–15 µm in thickness, were cut and placed on silan-coated slides and then fixed in 4% formaldehyde dissolved in a 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C. The sections were sequentially washed in distilled water and 0.01 M PBS, and then immersed in 0.2% triton X-100 dissolved in PBS for 30 min at room temperature prior to submission to the following fluorescence immunohistochemistry.

Frozen sections of pre-fixed specimens, 10–15 µm in thickness, were also cut and placed on silan-coated slides and permitted to air-dry for 30 min at room temperature. These sections were also subjected to fluorescence immunohistochemistry after sequential washing and saponification.

Antibodies and immunohistochemical procedures

Sources and dilutions of the primary and secondary antibodies used in this study are listed in Tables 1 and 2.

Sections prepared as described above were incubated in primary antibodies for 5-HT receptors or the 5-HT transporter overnight at 4°C. After being washed in PBS, reaction products were labeled with Alexa 594-conjugated

Fig. 1. A confocal laser scanning microscope image of a longitudinal section of the neck portion of the rat sinus hair follicle (HF) doubly stained with antibodies against CK20 (green) and neurofilament-H (red). Merkel cells stained with CK20 (green) and type I axon terminals stained with neurofilament H (red) are localized in the outermost layer of the follicular epithelium. BS: blood sinus.

anti-goat or anti-guinea pig IgG according to the host for generation of the primary antibody. The sections were washed in PBS and the presence of positive reactions in Merkel cell-nerve endings were briefly checked using a fluorescence microscope. The sections were then incubated in a 1:20 dilution of the mouse monoclonal anti-cytokeratin 20 antibody (CK20; Progen Biotechnik, Heidelberg, Germany) overnight at 4°C for the identification of Merkel cells. Reaction products of anti-CK20 in the post-fixed frozen sections were labeled with FITC-conjugated donkey anti-IgG, while those in the pre-fixed sections were labeled with Alexa 488-conjugated goat anti-mouse IgG. All specimens were finally washed in PBS and mounted in a water-permeable gel and observed under a laser-scanning microscope (Zeiss LSM; model 510-v3.0, Germany) by means of the multitrack scanning method.

Controls

For a negative control to the anti-5-HT_{1A} receptor (sc-1459; Santa Cruz Biotechnology, Inc., USA), 20 µl of

the antibody solution that contained 4 μg IgG was mixed with 100 μl of a 200 $\mu\text{g}/\text{ml}$ concentration of the corresponding antigen peptide solution purchased from the same company and refrigerated overnight. The antigen-antibody mixture was centrifuged and the supernatant was used at the same dilution as that in the experiment. Negative controls for other primary antibodies were made by using normal sera of host animals at the same dilutions as those in the experiments.

For positive controls, frozen sections of brains were subjected to fluorescence immunohistochemistry in the same manner as that in the experiments.

Some frozen sections of whisker pads were incubated in an equal mixture of 1:10 dilution of the anti-CK20 antibody and 1:500 dilution of neurofilament H (200 kDa neurofilament) to confirm the topographical distribution of Merkel cell-nerve endings. CK20 and neurofilaments were visualized with Alexa 488-conjugated anti-mouse IgG and Alexa 594-conjugated anti-rabbit IgG, respectively.

Results

5-HT receptors

Double immuno-labeling with the anti-CK20 and anti-neurofilament-H antibodies revealed the presence of numerous Merkel cell-nerve endings located in the external epithelial sheath of the neck portion of sinus hair follicles (Fig. 1). Two antibodies against the 5-HT_{1A} receptors from different sources (AB5406 and sc-1459) both

showed a strong affinity to the small structures facing the Merkel cells (Fig. 2, 3, Table 3), though sc-1459 was only reactive in pre-fixed frozen sections. These structures were topographically and morphologically identical to axon terminals stained with the anti-neurofilament H antibody (cf. Fig. 1). The pre-adsorbed sc-1459 antibody produced no positive immunoreactions on Merkel cell-nerve endings (Fig. 3). Positive controls using brain tissues revealed that many neuronal cells in the hippocampus and cortex were positively stained with both antibodies AB5406 and sc-1459 (Table 3; micrographs not shown).

As for 5-HT_{1B} receptors, two kinds of antibodies from different sources (AB5410 and sc-1460) showed different results as follows: In both pre- and post-fixed frozen sections used for the antibody sc-1460, no positive reaction was seen in the Merkel cell-nerve endings, in spite of clearly positive reactions of neurons in control brain sections (Table 3; micrographs not shown). In contrast, axon terminals of the Merkel cell-nerve endings were stained with AB5410 (Fig. 4, Table 3), and the negative control using the normal guinea pig serum yielded no positive reactions in sinus hair follicles (Table 3).

Antibodies against 5-HT_{2A} and 2C receptors used in the present study yielded no specific immunoreactions on the Merkel cell-nerve endings in both pre- and post-fixed sections, even at the highest concentrations introduced in the protocols for these antibodies. In positive controls, these antibodies positively stained neurons in the cortex and the choroid plexus of brains at 1:400 dilutions (Table 3; micrographs not shown).

The antibody against the 5-HT₃ receptor used in the

Table 3. Localization of immunohistochemical labels of serotonin receptors and transporter in Merkel cell-nerve endings in rat sinus hair follicles.

Antibodies	MC	AT	Negative control		Positive control (Brain)
			MC	AT	
5-HT _{1A} (AB5406)	+/-	++	-	-	reactive
(sc-1459)	+/-	++	+/-	-	reactive
5-HT _{1B} (AB5410)	+/-	++	-	-	reactive
(sc-1460)	-	-	-	-	reactive
5-HT _{2A} (sc-15073)	-	-	-	-	reactive
5-HT _{2C} (sc-15081)	-	-	-	-	reactive
5-HT ₃ (sc-19152)	-	-	-	-	reactive
Serotonin transporter	+	++	-	-	reactive

++: strong, +: mediate, +/-: very weak, -: negative

MC: Merkel cell, AT: type I axon terminal

Fig. 2. Confocal laser scanning microscope images showing a comparison of immunohistochemical reactivities of two kinds of anti-5-HT_{1A} receptors, AB5406 and sc-1459, for the Merkel cell-nerve endings in rat sinus hair follicles (HF). Specimens are doubly labeled with anti-CK20 combined with Alexa 488 (green) and antibodies against 5-HT_{1A} receptors combined with Alexa 594 (red). **a** and **c** are images for CK20-immunoreactivity, and **b** and **d** are the corresponding images merged with 5-HT_{1A} immunofluorescence in the same sections. Note that both antibodies show equally intense affinities to type I axon terminals. BS: blood sinus.

present study was not reactive to Merkel cell-nerve endings in either pre- or post-fixed sections (Fig. 5) though positive controls clearly showed that the antibody was reactive to nerves in brain stem nuclei at a 1:400 dilution (Table 3; micrographs not shown).

5-HT transporter

The antibody against the 5-HT transporter showed a high

affinity to both the axon terminals and Merkel cells though the reactivity in the Merkel cells was somewhat weaker than that in the axon terminals (Fig. 6, Table 3). In the positive control, many nerve fibers in the cortex and the hippocampus were labeled with the antibody (micrographs not shown), whereas the negative control the using the normal guinea pig serum yielded no reaction products on the Merkel cell-nerve endings (Table 3).

Fig. 3. Confocal laser scanning microscope images demonstrating the 5-HT_{1A} receptor (sc-1459) immunoreaction. **a** shows the oblique section of a rat sinus hair follicle displaying the CK20 immunoreaction (green) and **b** is the corresponding image merged with anti-CK20 and non-adsorbed anti-5-HT_{1A} (sc-1459) immunoreactions (red). **c** and **d** show images from a section adjacent to the section in **a** and **b**, which was stained with anti-CK20 and anti-5-HT_{1A} (sc-1459) pre-adsorbed with the antigen protein. Note that 5-HT_{1A} receptor-like immunoreactions on axon terminals (**b**, asterisks) facing Merkel cells (green) have disappeared from the section incubated in pre-adsorbed anti-5-HT_{1A} (sc-1459) (**d**).

Discussion

Mammalian 5-HT receptors are classified into seven families: six of them are G protein-coupled receptors, and only the 5-HT₃ receptor has ligand-gated ion channels (Hoyer *et al.*, 1994). Although many of these receptors are distributed in the central and peripheral nervous systems, some subfamilies are predominant in peripheral tissues (Hoyer *et al.*, 1994). Pierce and his colleagues (1996) showed in their polymerase chain reaction (PCR)

studies that mRNAs for 5-HT_{1B}, 1D, 2A, 2C, 3, and 7 receptors are predominant in the lumbar dorsal root ganglia of the rat, whereas superior cervical ganglia predominantly express mRNAs for 5-HT_{1A}, 1B, 1D, 2A, 3, 6, and 7 receptors. Carlton and Coggeshall (1997) further reported that axon terminals in some kinds of cutaneous nerve endings, including Meissner's corpuscles, were immunocytochemically labeled with an antibody against the 5-HT_{2A} receptor. He *et al.* (2003) recently showed that antagonists for 5-HT₂ and 5-HT₃ receptors reduced electrophysiological responses to mechanical stimuli in

Fig. 4. Confocal laser scanning microscope images showing the distribution of 5-HT1B receptor-like immunoreactions in a rat sinus hair follicle (HF). **c:** The specimen was doubly labeled using anti-CK20 combined with Alexa 488 (green, **a**) and anti-5-HT1B (AB5410) combined with Alexa 594 (red, **b**). Type I axon terminals and some axons in the blood sinus area (BS) are positively stained with the anti-5-HT1B antibody (**b, c**).

the Merkel cell-nerve endings of the rat touch domes. However, no definitive study on the immunohistochemical localization of these 5-HT receptors in Merkel cell-nerve endings has yet been reported.

In the present study, we tried to demonstrate the immunohistochemical localization of several subtypes of 5-HT receptors (i.e., 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C and 5-HT3 receptors) using commercially available antibodies. Our results clearly showed that the axon terminals of the Merkel cell-nerve endings in the rat sinus hair follicles were strongly labeled with antibodies against 5-HT1A and 1B receptors but not with antibodies against 5-HT2A, 2C and 3 receptors.

As for 5-HT1 class receptors, we used two different kinds of antibodies against 5-HT1A as well as 5-HT1B receptors to obtain reliable information. The results indicate that both the 5-HT1A and 5-HT1B receptors are present in the axon terminals of the Merkel cell-nerve endings although one of the two antibodies against 5-HT1B receptors was not reactive to the Merkel cell-nerve endings.

5-HT1 class receptors are known to couple to Gi/o/z protein families (Hoyer *et al.*, 1994; Hoyer and Martin, 1997; Raap *et al.*, 1999; Raymond *et al.*, 2001; Albert and Tiberi, 2001). In our previous paper, *αo* and *αi* subunit isoforms of G proteins were found to be localized on Merkel cells and the type I axon terminals in the rat sinus hair follicles (Tachibana *et al.*, 2001). These findings strongly support the present findings regarding the localization of 5-HT1A and 1B receptors in the Merkel cell-nerve endings. Functionally, 5-HT1 receptors mediate inhibitory signal pathways, including those for the inhibition of cAMP formation and the opening of K⁺ channels and closing of Ca⁺⁺ channels (Albert and Tiberi, 2001; Raymond *et al.*, 2001). It is therefore reasonable to assume that the family of 5-HT1 receptors are involved in the suppressive modulation of neural actions during mechanical reception in the Merkel cell-nerve endings.

Although He *et al.* (2003) suggested the involvement of 5-HT2 receptors in the mechanical reception in the Merkel cell-nerve endings, we could not find positive

Fig. 5. Confocal laser scanning microscope images showing the distribution of 5-HT3 receptor-like immunoreactions in a rat sinus hair follicle (HF). **c:** The specimen was doubly labeled using anti-CK20 (green, **a**) and anti-5-HT3 receptor (red, **b**). No positive reaction to the anti-5-HT3 receptor can be seen in the hair follicle (**b**, **c**).

immunoreactions for 5-HT_{2A} or 2C receptors in these structures in the present study. Merkel cells express a positive immunoreactivity for the α q subunit of the G protein (Tachibana *et al.*, 2001). Thus, a possibility that Merkel cells possess the 5-HT_{2B} receptor cannot be excluded at present although previous studies have shown that this receptor is predominant in non-neural cells. If Merkel cells possess 5-HT_{2B} receptors, the suppression of the mechanical reception in the Merkel cell-nerve endings by antagonists for 5-HT₂ receptors shown by He *et al.* (2003) may be induced by the impairment of Merkel cells. On the other hand, it is also possible that kentaserin and ritanserin, which were used by He *et al.* (2003) as specific antagonists for 5-HT₂ receptors, reacted with 5-HT_{1B} receptors on axon terminals because these drugs are also known to non-selectively bind to 5-HT_{1B/D} receptors (Slassi, 2002).

He *et al.* (2003) also reported that an antagonist for 5-HT₃ receptors, MDL72222, partially suppressed type I mechanical reception in the Merkel cell-nerve endings.

This is also contradictory to our finding of an absence of any positive immunoreaction of the 5-HT₃ receptor in the Merkel cell-nerve endings. However, the expression of mRNAs for the 5-HT₃ receptor limited to small-sized neurons in peripheral sensory ganglia (Tecott *et al.*, 1993) suggests the absence of this receptor in type I nerve terminals in the sinus hair follicles. Further immunohistochemical studies using antibodies from other sources and *in situ* hybridization will be required before this query can be settled.

5-HT transporter proteins are related to the uptake and reuse of 5-HT molecules (Blakely *et al.*, 1991; Hoffman *et al.*, 1991; Borowsky and Hoffman, 1995). In the present study, both Merkel cells and the related type I nerve terminals showed immunoreactions for the 5-HT transporter. Hansson and Hoffman (2000) previously showed that Merkel cells of the rat whisker pad transiently express mRNAs for the 5-HT transporter during late gestation and early postnatal development. Furthermore, there are reports that developing trigeminal neurons

Fig. 6. Confocal laser scanning microscope images showing the distribution of 5-HT transporter-like immunoreactions in Merkel cell-nerve endings in the sinus hair follicle (HF). **c:** The specimen was doubly labeled using anti-CK20 (green, **a**) and anti-5-HT transporter (red, **b**). Both Merkel cells and type I axon terminals are positively stained with the anti-5HT transporter antibody (**b, c**), though reactions in the Merkel cells appear to be rather weak. BS: blood sinus.

transiently contain significant levels of 5-HT (Jacobs and Azmitia, 1992) and express transient mRNAs for the 5-HT transporter (Hansson *et al.*, 1999). Thus, the present study is the first to demonstrate the presence of the 5-HT transporter in mature Merkel cells of the adult rat. Our results suggest that both the Merkel cells and type I axon terminals reciprocally release 5-HT molecules in the mature Merkel cell-nerve endings of adulthood. Actually, English *et al.* (1992) reported that both Merkel cells and their related axon terminals in the touch domes from the belly skin of adult rats showed a 5-HT-like immunoreactivity although Lazarov (1994) stated that 5-HT did not exist in type I nerve terminals. On the other hand, it is also possible that mature Merkel cells in adulthood receive 5-HT from the related sensory nerves in the same manner to those in the developing Merkel cell-nerve endings. It will be necessary to determine whether Merkel cells have an ability for serotonin synthesis before a conclusion can be reached.

In summary, we have demonstrated in the present study that the type I sensory axon terminals in the Merkel

cell-nerve endings in the sinus hair follicles of the adult rats express positive immunoreactions for 5-HT_{1A} and 1B receptors. We also showed that both the type I axon terminals and Merkel cells express immunoreactions for the 5-HT transporter. These results suggest that 5-HT is actually released from Merkel cells towards the type I axon terminals and that the 5-HT molecules in the synaptic cleft of Merkel cell-nerve endings are taken into both the axon terminals and the Merkel cells.

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