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Essential components for a glutamatergic synapse between Merkel cell and nerve terminal in rats

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Abstract

The exact role of Merkel cells and their possible involvement in mechanosensation is unclear. The aim of this study was to determine, in the adult rat sinus hair follicle, the expression pattern of a number of vesicular proteins involved in neurotransmitter release to provide a clearer understanding of Merkel cell signalling mechanisms. We identified prominent expression and co-localization of the glutamatergic vesicle loading proteins VGLUT1 and VGLUT2 at the site of the sinus hair follicle known to be densely populated with Merkel cells. We also found expression of the vesicle recycling proteins synaptogyrin and syntaxin-6 in the same region of the hair follicle. Our data suggest that glutamate signalling is involved in Merkel cell mechanosensation and that vesicular trafficking is commonplace in the Merkel cell-neurite complex.

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Merkel cells exist within the basal layer of skin and oral mucosa epidermis in mammals. Although they were first described in 1875 [9], their function is still a matter of controversy. Recent evidence supports the theory that they act as mechanoreceptors, transducing sensory touch information to associated primary afferent nerve endings (reviewed in Ref. [5]). Subsequent investigations aimed at identifying candidates for neurotransmitters involved in signalling between the Merkel cell-neurite complex confirmed that a number of neuropeptides [8] in addition to serotonin [6] and ATP [1] were expressed by Merkel cells, or in regions populated by high numbers of Merkel cells. More recently, the excitatory amino acid glutamate has emerged as a potential neurotransmitter in mechanosensation [8].

We have previously shown expression of various glutamate signalling components during the differentiation of keratinocytes, including both ionotropic and metabotropic receptor types as well as glutamate transporter-1 (GLT-1) and excitatory amino acid carrier-1 (EAAC-1) [4]. Furthermore, expression of the NMDA receptor subunits

(NR1, NR2A/B and GLT-1) was identified in the outer root sheath and the rete ridge collar of the rat sinus hair follicle, a region of tissue known to be densely populated by Merkel cells [14]. Electrophysiological experiments monitoring the sinus hair slowly adapting type I (St I) responses evoked by mechanical stimulation demonstrated that the broad spectrum glutamate receptor antagonist kynurenic acid depressed St I responses in a dose-dependent manner [3], as did the non-competitive antagonist MK-801 [12]. However, their effects were not characteristic of conventional NMDA receptor blockade, suggesting that the NMDA receptor configuration may not be identical to those found in the central nervous system, or that the drugs evoke their response by alternative means.

In this study, we determined the expression of glutamatergic vesicle loading proteins VGLUT1 and VGLUT2 [10, 16], in addition to more promiscuous proteins involved in vesicle trafficking and docking, synaptogyrin [18] and syntaxin-6 [17], in the Merkel cell-neurite complex. Our results suggest that glutamatergic vesicle recycling operates in the vicinity of Merkel cells and that other vesicular proteins are expressed possibly by both the Merkel cell and the nerve ending.

Whisker pads were dissected from adult Wistar rats and

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sinus hair follicles extracted using a dissection microscope. Isolated follicles were processed for electron microscopy (see below) or frozen in chilled isopentane and mounted in Cryo-M-bed embedding compound (Bright Instrument Co., Huntingdon, UK) on brass chucks. Sections (7 μm) were cut using a Bright OTF500 cryostat (Bright Instrument Co.), collected on polysine slides (BDH, Poole, UK) and kept at $-20\text{ }^{\circ}\text{C}$ until use.

All incubations during the immunolocalizations were performed at room temperature and three phosphate buffered saline (PBS) washes, each for 5 min, were carried out between incubations. Sections were thawed at room temperature prior to fixation in 4% paraformaldehyde for 5 min. To block non-specific antibody binding, sections were incubated in 10% goat serum (Sigma, Poole, UK) for 30 min and then in primary antibody. These included mouse anti-

Cytokeratin (CK) 20 (1:20 dilution; Progen, Heidelberg, Germany), rabbit anti-VGLUT1 (1:1000 dilution; Chemicon, CA, USA), guinea pig anti-VGLUT2 (1:500 dilution; Synaptic Systems, Goettingen, Germany), mouse anti-Synaptogyrin (1:200 dilution) and mouse anti-Syntaxin-6 (1:1000 dilution; both Transduction Laboratories, Oxford, UK) for 1 h. Negative controls were treated with the same concentrations of either rabbit or mouse non-immune IgGs (Sigma). Unbound primary antibody was removed by washing and the samples were incubated for 1 h in the appropriate secondary antibody (VGLUT1, goat anti-rabbit FITC (Sigma); VGLUT2, goat anti-guinea pig Cy-3 (Chemicon); CK20, Synaptogyrin and Syntaxin-6, all goat anti-mouse FITC (Sigma)). The sections were washed and mounted in Vectorshield containing DAPI (Vector Laboratories, Peterborough, UK). Staining was visualized using a

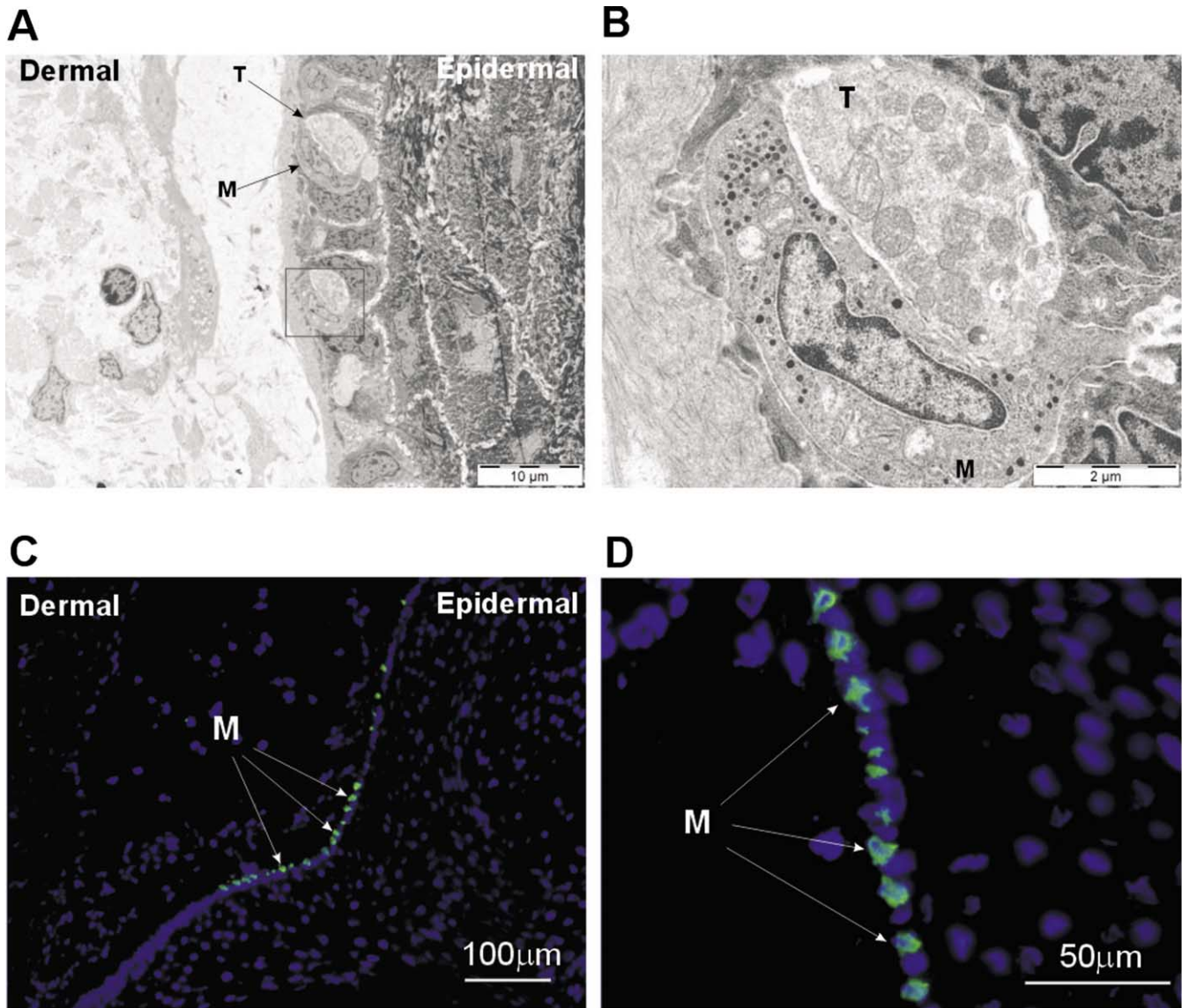


Fig. 1. Localization of Merkel cells in the adult rat sinus hair follicle. Transmission electron microscopy imaging of the adult rat sinus hair follicle identified Merkel cells (M) and their associated nerve termini (T) located in the outer root sheath (A,B). Positive immunoreactivity for the Merkel cell-specific marker CK20 was also identified in this region of the hair follicle by immunofluorescence (green, C,D; blue fluorescence, DAPI-positive nuclei).

Leica DMLA microscope (Leica, Milton Keynes, UK) under UV illumination.

The presence of Merkel cells in the outer root sheath of the rat sinus hair follicle was confirmed by transmission electron microscopy (TEM). Briefly, vibrissa were removed as previously stated and fixed in 4% paraformaldehyde/2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.0) for 90 min at room temperature. The specimens were then washed three times with PBS and subjected to secondary fixation in 1% osmium tetroxide for 1 h on ice and washed twice in PBS. The specimens were then dehydrated through graded ethanols, dried over a molecular sieve and washed twice in epoxypropane for 5 min. Embedding was performed by adding 60% epoxypropane/40% epon araldite for 30 min and left to desiccate with silica gel overnight. Fresh epon araldite was added the next day and was allowed to polymerize for 48 h at 60 °C before sections were cut and stained with saturated uranyl acetate in 50% ethanol with Reynolds lead citrate and viewed using a transmission electron microscope (JLJEM 1200 EX; Tokyo, Japan).

Using TEM, we identified Merkel cells in the rat sinus hair follicle as spherical shaped cells with multi-lobed nuclei and dense core bodies located within the cytoplasm (Fig. 1A). Each Merkel cell makes a synaptic interaction with a mitochondria-containing disc-shaped nerve terminal to form a Merkel-neurite complex (Fig. 1B) [11,13].

CK20 is now a widely used marker for Merkel cells, both in the identification of normal cells in heterogeneous tissues and for the detection of Merkel cell carcinoma [15]. CK20 expression in the rat sinus hair follicle was restricted to the same area in the outer root sheath which we previously identified as the location of Merkel cells by electron microscopy (Fig. 1C,D).

Expression of the vesicular glutamate transporter molecules VGLUT1 and VGLUT2 was localized to the same region as CK20-positive Merkel cells (Fig. 2A). VGLUT1 and VGLUT2 expression was in general co-localized although it was apparent that the proteins were not co-expressed in certain areas. In contrast to the expression of CK20, which was identified throughout the cytoplasm of Merkel cells (Fig. 1B), it appeared that VGLUT1 and VGLUT2 were localized to highly specific regions of selected cells. In view of the finding that glutamate signalling may be involved in the process of mechanosensation between the Merkel cell and the nerve terminal, it is tempting to speculate that VGLUT1 and VGLUT2 expression is localized to the region in the Merkel cell directly opposite the nerve terminal. Considering the apparent importance of glutamate signalling in mechanosensation [8], it could be hypothesized that the function of VGLUT1 and VGLUT2 in Merkel cells is to package glutamate into vesicles prior to docking and release. However, it is necessary to further characterize the exact location of VGLUT1 and VGLUT2 in Merkel cells to clarify their role.

Expression of the vesicular proteins synaptogyrin and syntaxin-6 was also restricted to the region in the sinus hair follicle where Merkel cells are present. Synaptogyrin expression patterning was very similar to that of VGLUT1 and VGLUT2, appearing at discrete cellular sites, whilst syntaxin-6 immunostaining was more cytoplasmic (Fig. 2B). Although both synaptogyrin and syntaxin-6 are considered to be vesicular proteins, syntaxin-6 is primarily involved in internal membrane trafficking, whereas synaptogyrin promotes the fusion of the vesicle with the plasma

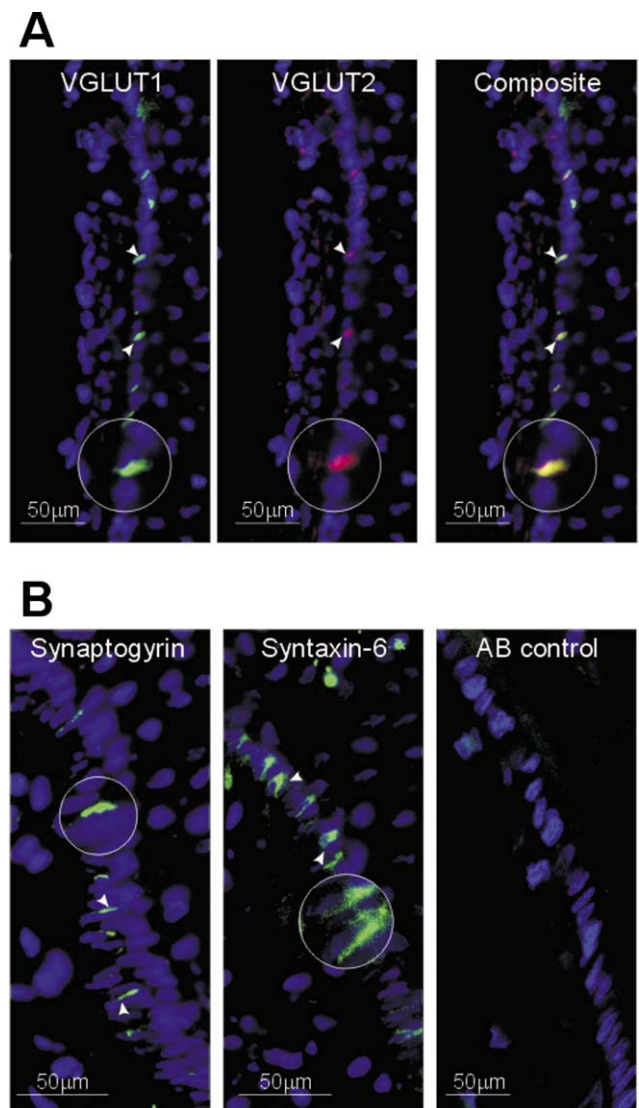


Fig. 2. Immunolocalization of synaptic vesicle proteins in the adult rat sinus hair follicle. Expression of the glutamatergic vesicle packaging proteins VGLUT1 and VGLUT2 was localized to the same vicinity of Merkel cells and associated nerve endings (arrowheads, A). VGLUT1 (green) and VGLUT2 (red) were predominantly co-localized (yellow) and expressed in confined areas only (circle, 2 × original magnification). Synaptogyrin and syntaxin-6 were also localized to the vicinity of Merkel cell complexes, with synaptogyrin expressed in confined regions and syntaxin-6 distributed throughout the cytoplasm of undefined cells (arrowheads, B, circle, 2 × original magnification). Antibody controls displayed low levels of background staining only.

membrane and subsequent exocytosis of the transmitter. Therefore, the differences in synaptogyrin and syntaxin-6 expression in the Merkel cell-neurite complex could be due to syntaxin-6 being involved in the trafficking of the Merkel cell dense-core granules and synaptogyrin having a role in the fusion of vesicles at the plasma membrane of either the Merkel cell or the nerve terminal. Unfortunately, due to the lack of appropriate commercially available antibodies, it was not possible to perform co-localization experiments with CK20 to confirm that these proteins are expressed by Merkel cells. Further investigations aimed at accurately determining the location of these vesicular proteins would provide a clearer understanding of their function.

Previous evidence suggests that glutamate is involved in Merkel cell-neurite signalling. However, it is unclear whether Merkel cells release glutamate which binds to receptors on the nerve ending to transduce mechanosignalling, or conversely, that glutamate release from the nerve terminals activates receptors expressed by Merkel cells, possibly acting as a retrograde feedback mechanism. Although immunohistological evidence of NMDA receptor expression by what appeared to be Merkel cells in the sinus hair follicle would favour the latter hypothesis, it is also important to consider evidence that cutaneous axons express NMDA receptors [7] and also release glutamate [2]. These findings suggest that glutamate signalling at this junction is more complicated than first expected, and warrants further investigation. The same questions can be addressed with regards to expression of synaptogyrin and syntaxin-6, as it remains unclear exactly which cell, or part(s) of the cell, is expressing these proteins, although the more cytoplasmic distribution of syntaxin-6 may indicate that it is expressed by Merkel cells, possibly for the purpose of trafficking the previously identified dense-cored granules. As direct electrophysiology of Merkel cells in situ is not possible due to inaccessibility, investigating what signalling components are expressed by immunohistochemistry remains one of the best techniques by which to determine Merkel cell function. Although this work needs expanding, we hope we have posed some further interesting questions on mechanosensory transduction from Merkel cell to nerve ending.

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