

# Glutamate Receptor-Like Immunoreactivity in Rat Vibrissal Merkel Cells

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## Summary

Recent functional data has raised the possibility that an excitatory amino acid, such as glutamate, may act as neurotransmitter in vibrissal type I slowly adapting (St I) mechanoreceptors. It is supposed that Merkel cells respond to mechanical stimulation by releasing a neurotransmitter which acts on the nerve terminals in contact with the Merkel cells. Therefore, we sought to establish whether or not the appropriate signalling molecules for glutamate neurotransmission are to be found in the vibrissa. Using frozen sections from vibrissa isolated from adult Wistar rats, we stained for NMDA and AMPA glutamate receptors and the glutamate transporter GLT-1. Distinct patterns of expression of NMDA-type receptors were found in the vibrissal sections. There was intense immunoreactivity to the NR1 antibody in the layer of cells in the outer root sheath, adjacent to the glassy membrane. However, NR2A/B staining was restricted to a subpopulation of cells adjacent to the glassy membrane, but only above the level of the ring wulst and the innermost cells of the rete ridge region – suggesting that the NR2A/B stained cells are Merkel cells. GLT-1 immunoreactivity was largely restricted to the epithelial cells lining the thick sinus body capsule enclosing the blood sinuses. There was no remarkable staining with the AMPA-type GluR1–3 antibodies. The data suggest the presence of glutamate signaling in the outer root sheath cells, including Merkel cells. However, it remains to be seen if this is related to mechanosensory function in Merkel cells or whether it is part of the already described glutamate signaling in keratinocytes.

## Introduction

The anatomical association between Merkel cells and nerve terminals in the skin and appendages across many species is suggestive of a synaptic relationship. Although functional and other data (Yamashita and Ogawa 1991; Chan et al. 1996; Senok and Baumann 1997) continue to support the notion that the Merkel cell is a mechanosensory transducer which responds to mechanical stimulation by releasing a transmitter, the identity of the transmitter has remained elusive (see reviews in this volume; Ogawa (1996). Recent functional data has raised the possibility that glutamate may be a chemical transmitter at vibrissal Merkel cell touch receptors (Fagan and Cahusac 2001; Senok et al. 2001). It was hypothesized that if glutamate served a transmitter function between Merkel cells and nerve terminals, the appropriate glutamate signalling molecules should be present wherever Merkel cell-neurite complexes are found.

Vibrissae were used because they have a high concentration of Merkel cells and nerve terminals in a compact, well-defined location and the fact that the functional work (Fagan and Cahusac 2001; Senok et al. 2001) used isolated vibrissae.

Glutamate is the major excitatory neurotransmitter in the brain. It exerts its effects through ionotropic (fast synaptic transmission) and metabotropic receptors (modulation of pre- and post-synaptic activity). The ionotropic receptors are subdivided into NMDA and non-NMDA (comprising AMPA and Kainate receptors), based on their sensitivity to ligands. The NMDA receptor is made up of a multimeric assembly of four or five subunits, comprising NR1 and one or more NR2 (A–D) subunits. Expression of both types of subunit is required to form functional channels. The non-NMDA receptors are also made up of subunits – GluR1–4 for AMPA, and GluR5–7 and KA1–2 for Kainate. The transporters EAAC1, GLT-1 and GLAST transport glutamate away from the synaptic cleft. Therefore, we immunostained the vibrissae for the following signalling molecules: NR1, NR2 A/B, GluR1–3 and GLT-1.

## Materials and Methods

### Antibodies

All antibodies were obtained from Chemicon (Harrow, UK), unless otherwise stated. Rabbit polyclonal anti-NR1, NR2A/B, GluR1, and GluR2/3 (UBI, New York, USA) were used. Anti-GLT-1 polyclonal antibody was provided by Dr. Jeffrey Rothstein (Johns Hopkins, USA).

### Immunolocalisation of Glutamate Receptors and Transporters in Rat Vibrissae

Vibrissae were dissected from adult Wistar rats as previously described (Senok et al. 1996). Isolated vibrissa were dipped in 10% polyvinyl alcohol (PVA; Sigma,

Poole, UK), immediately frozen in chilled isopentane ( $-70^{\circ}\text{C}$ ) and mounted in 10% PVA on brass chucks. Sections (5–7  $\mu\text{m}$  thickness) were cut using a Bright cryostat (Bright Instrument Co., Huntington, UK), collected on polysine slides (BDH) and stored at  $-35^{\circ}\text{C}$  until use.

The sections were fixed in 4% paraformaldehyde for 5 min and endogenous peroxidase activity depleted with 3% hydrogen peroxide (Sigma) for 30 min. A further pre-incubation was performed with 10% normal goat serum (Vector Laboratories) for 30 min to block non-specific antibody binding. Sections were incubated for 30 min with primary polyclonal antibody (individual antibodies were titrated on each tissue to determine optimal concentration and a range of 0.5–1  $\mu\text{g}/\text{ml}$  was used) followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories; 1:200 dilution) for 15 min and avidin-biotinylated-peroxidase reagent (ABC Elite, Vector Laboratories, 1:50 dilution) for 20 min. Peroxidase activity was disclosed with 0.5 mg/ml 3, 3'-diaminobenzidine (Sigma) and 0.3% hydrogen peroxide as substrate. All dilutions were made up in phosphate-buffered saline (PBS), pH 7.4 (GLT-1 antibodies were diluted in PBS containing 0.1% Triton X-100) and incubations were performed at room temperature with three PBS washes between each incubation. Negative controls received the same concentration of normal rabbit IgGs (Vector Laboratories) in place of primary antibody. Sections were counterstained with haematoxylin prior to mounting in glycerol/PBS.

## Results

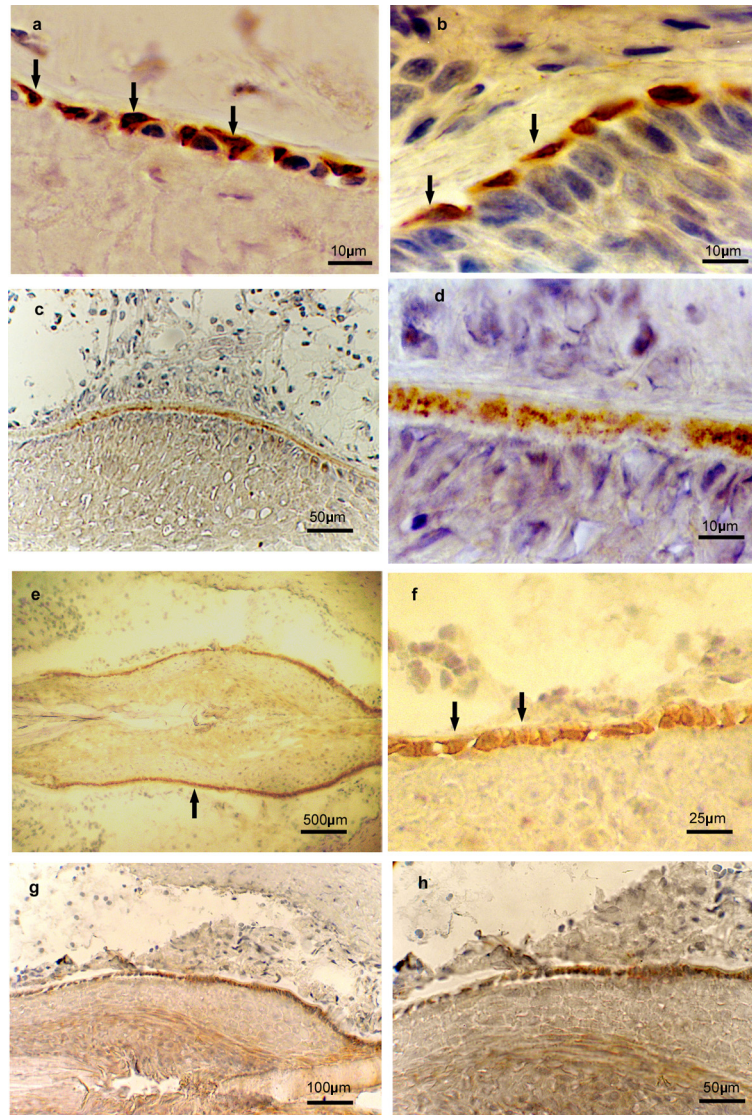
### NMDA Receptor-Like Immunoreactivity

**NR1.** There was uniform immunostaining of the monolayer of cells of the external root sheath both above and below the ring wulst (Fig. 1e,f).

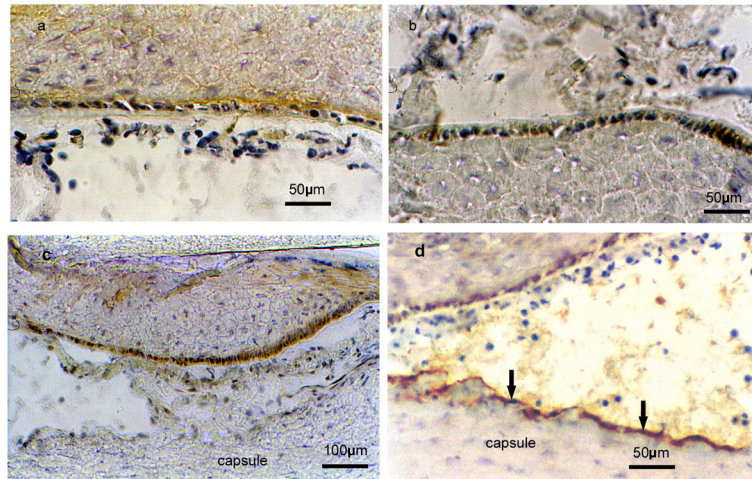
**NR2 A/B.** There was staining of a subset of the outer root sheath cells above the ring wulst (Fig. 1a). A layer of cells in the rete ridge (Fig. 1b) was also clearly immunopositive for NR2 A/B, suggesting that these are Merkel cells. A circumscribed band of non-cellular profiles that appear to be embedded in the glassy membrane was seen about the middle third of the area distal to the ring wulst, in the area to the cavernous sinus (Fig. 1c,d).

**GluR1, GluR2 and GluR3** did not show any clear staining in the hair follicle (Fig. 2a–c).

**The anti-GLT1** transporter antibody clearly stained a layer of apparent endothelial cells lining the capsule of the blood sinus (Fig. 2d).



**Fig. 1.** NR2 A/B immunoreactivity (*brown* reaction product) of a subset of external root sheath cells above the ring wulst (**a**, *arrows*) and the group of cells in the rete ridge collar known to be Merkel cells (**b**, *arrows*). **c**, **d** Band of intensely stained profiles in contact with the glassy membrane at the level of the cavernous sinus. Unlike the NR2 A/B staining, NR1 staining was intense and uniform in the external root sheath cells above and below the ring wulst (**e**, *arrow*; and **f**, higher power showing the stained cells). Negative control slides show no staining of external root sheath cells (**g**, **h**)



**Fig. 2.** AMPA-type GluR1, 2 and 3 showed no clear immunoreactivity in the external root sheath cells (**a**, **b**, **c**). GLT-1 is seen in the endothelial lining of the sinus capsule (**d**, *arrows*; cf. **c** and Fig. 1g). Elements of the mesenchymal tissue in the blood sinus also appear to be positive for GLT-1

## Discussion

The positive staining for NR1, NR2 A/B and GLT-1 in the outer root sheath and the rete ridge collar of the sinus hair follicle suggests that the signalling molecules for glutamate transmission are present in the appropriate location of the vibrissa. The differential distribution of the NMDA receptor subunits in the external root sheath cells might reflect functional differences between the cells.

If glutamate is the transmitter, the ionotropic receptors ought to be on the nerve terminals rather than the Merkel cells (the metabotropic receptors would be expected on the Merkel cells as reported by Tachibana and Nawa (this Vol.)). While it is clear that the NR1 staining is in cells, we are unable to categorically say whether the NR2 A/B is on the Merkel cells or the expanded nerve terminals. Further work is required to clarify this. In any event, the processes involving (non-sensory) glutamate signalling described in the skin (Genever et al. 1999) are likely to operate in the sinus hair follicle as well. Whether the presence of glutamate signalling molecules associated with Merkel cell-neurite complexes is a part of that system remains to be determined.

We have shown that MK-801, a blocker of the ionotropic NMDA channel blocks the responsiveness of the St I receptors in isolated vibrissae (Senok et al. 2001), but we have since been unable to show a clear effect of other classical NMDA and non-NMDA antagonists on the function of the receptor. This suggests that either the pharmacological profile of the NMDA receptor in these mechanoreceptors is different from that in the CNS, or that the MK-801 is acting via a different mechanism.

In conclusion, the appropriate signalling molecules for glutamate transmission appear to be present in the Merkel cell-nerve terminal complexes, but it remains to be shown whether glutamate (or another excitatory amino acid) is involved in Merkel cell mechanosensory function.

## Acknowledgements

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