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Fine Structural Analysis of the Synaptic Junction of Merkel Cell-Axon-Complexes

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The ultrastructure of synaptic contact areas in Merkel cell-axon-complexes from sinus hair follicles and touch domes of various mammals (nude mice, rats, cats, rabbits, opossums and monkeys) was investigated by electron microscopy of ultrathin sections from perfusion fixed tissue.

Synapses between Merkel cells and axons were a common feature in all analyzed species. Special staining with digallic acid and goniometric tilting facilitated the resolution of the membranous and paramembranous synaptic elements. The synaptic contact revealed the typical characteristics of a chemical synapse, except for presynaptic clear vesicles: a postsynaptic membrane thickening and dense projections at the presynaptic membrane (i.e., the Merkel cell membrane). The cleft material was resolved as a fuzzy coating of the outer leaflets of the synaptic membranes with occasional bridges across the synaptic cleft.

The presence of a synapse in the Merkel cell-axon-complexes emphasizes the receptor function of the Merkel cell besides other possible functions of this cell.

Merkel cell-axon-complexes (MCAC) have been investigated in various sites in differing species [1,2] and the association of these complexes with mechanoreceptors is generally accepted [3,4] although clear physiological data are still lacking. Electron microscopy has revealed that the predominant structural features of MCAC are rather uniform in different species and localizations [2]. Conflicting results are reported concerning the occurence of synaptic membrane specializations in MCAC in various mammals. Synapse-like structures were first described in MCAC of the cat [1] and later were also found in those of the other species [2]. However several authors failed to observe any synaptic specialization in various mammals [5–8], thus suggesting species differences.

The aim of the present study is to describe the substructure of the synaptic contact in MCAC of various mammals and to elucidate whether or not species differences exist. The sinus hair follicle was preferentially investigated due to the advantage it offers in its abundance and favorable stereological arrangement of the MCAC compared with other localizations [9]. Different postfixations and stainings were introduced because it is established that the synaptic image, visualized by electron microscopy, varies with different fixations and staining procedures [10–12].

In addition we carried out goniometric analysis of the Merkel cell-axon contact area in order to ensure that no specialized membrane apposition remained undetected and also to demonstrate the feature of the synaptic junction with different observation angles. Special attention was paid to the occurence of "clear vesicles" in the axon profiles and their distribution pattern, specially with regard to different section planes.

MATERIALS AND METHODS

Ten nude mice, 10 rats, 5 Tupaia Belangeri, 5 rabbits, 3 opossums (all adult animals of both sexes) and 5 cats (3 adult, 2 newborn) were fixed in ether anaesthesia by retrograde vascular perfusion through the abdominal aorta according to Forssman et al [13]. This was carried out in 2 consecutive fixation steps. The composition of the fixatives and the duration of perfusion is as follows: fixative I for 3 min: 1.5% formaldehyde, 1.5% glutaraldehyde in 0.09 M phosphate buffer at ph 7.3, 2.5% polyvinyl-pyrrolidone (PVP M.W. 40,000). Fixative II, similar to fixative I but with 3% formaldehyde, 3% glutaraldehyde plus 0.05% picric acid was introduced for another 3 min. Single hair follicles and touch domes from the glabrous snout were separated from the surrounding tissue and were rinsed in 0.1 M cacodylate buffer at ph 7.2. The tissue was postfixed in 1% OsO_4 buffered with 0.1 M cacodylate or with ferrocyanide-reduced OsO_4 [14]. A few probes were treated before postfixation with 1% digallic acid for ½ hr [15] or ethanolic phosphotungstic acid [12], while others were stained with bismuth iodide [16] without the OsO_4 postfixation. Tissues were dehydrated in ethanol and embedded in Epon. Longitudinal semithin sections through the upper part of the hair follicle, including the entire Merkel cell cylinder were cut and examined with the phase contrast microscope. In touch domes semithin sections were cut perpendicular to the skin surface through the center of the dome. The Merkel cell regions were selected and subsequently cut with a Reichert ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate [17] and examined with a Siemens Elmiscop Ia and a Zeiss EM 10 electron microscope. Goniometric analysis was also performed with the Zeiss EM 10.*

RESULTS

In sinus hair follicles, several hundred Merkel cells form a close-meshed cuff in the external epithelium of the upper region of the hair follicle. In all ultrathin longitudinal sections of the entire Merkel cell region, up to 70 Merkel cells are identified. In touch domes, only 5–10 Merkel cells are arranged in groups on the base of the dome and per ultrathin section usually not more than 5 Merkel cells are found. In both localizations 70–80% of the Merkel cells are in intimate contact with intraepithelial axon terminals.

In the touch domes the axon is adjacent to the basal lamina and underlying the Merkel cell, while in the sinus hair follicle, the Merkel cell is interposed between the basal lamina and the axon. Except for this topographical difference the principal ultrastructural features of the MCAC are similar in both localizations in all investigated animals and correspond with that described by other authors [1,2]. Intranuclear rodlets, described in Merkel cells of the rabbit [18] and human [19] were not detected in our material. Merkel cells contain the typical dense cored vesicles of varying number and electron opacity, which are concentrated in that part of the cytoplasm facing the axon (Fig 1*a–c*). The axon terminals exhibit besides numerous mitochondria and a few neurotubules a variable amount of clear, coated and dense vesicles. The vesicles are loosely distributed at different sites of the axon membrane, i.e., at sites facing the keratinocytes as well as the Merkel cells and they are of variable

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Abbreviations:
MCAC: Merkel cell-axon-complexes

* The terms pre- and postsynaptic membranes etc, are used in this study to describe the synaptic contact basing on comparative ultrastructural criteria in terms generally used in the nomenclature of neural ultrastructure and not to characterize physiological properties. Thus the synaptic membrane bearing the dense projections is denominated "presynaptic," the other exhibiting a membrane thickening "postsynaptic."

size (Fig 1–3). Serial sections in different sectional planes of the neural disc give evidence that the vesicles are concentrated in the form of a ring in the equatorial subneurolemmal axoplasm. In section planes perpendicular to the equator plane of the neural disc, except for perpendicular tangential sections (see below), vesicles are concentrated in the "edges" of the nerve transsection (Fig 1*a–c*). In tangential perpendicular sections numerous vesicles are often randomly distributed in the axoplasm (Fig 3). Horizontal sections, parallel to the equator plane of the nerve disc, reveal groups of vesicles along the entire circumference of the subneurolemmal axoplasm facing keratinocytes as well as Merkel cells (Fig 2).

In conventionally *OsO_4-postfixed tissue* "synapse-like" structures are regularly found between Merkel cells and axon terminals in all species. The synapse-like structures, as described in the literature [1,2] show the following characteristics: a

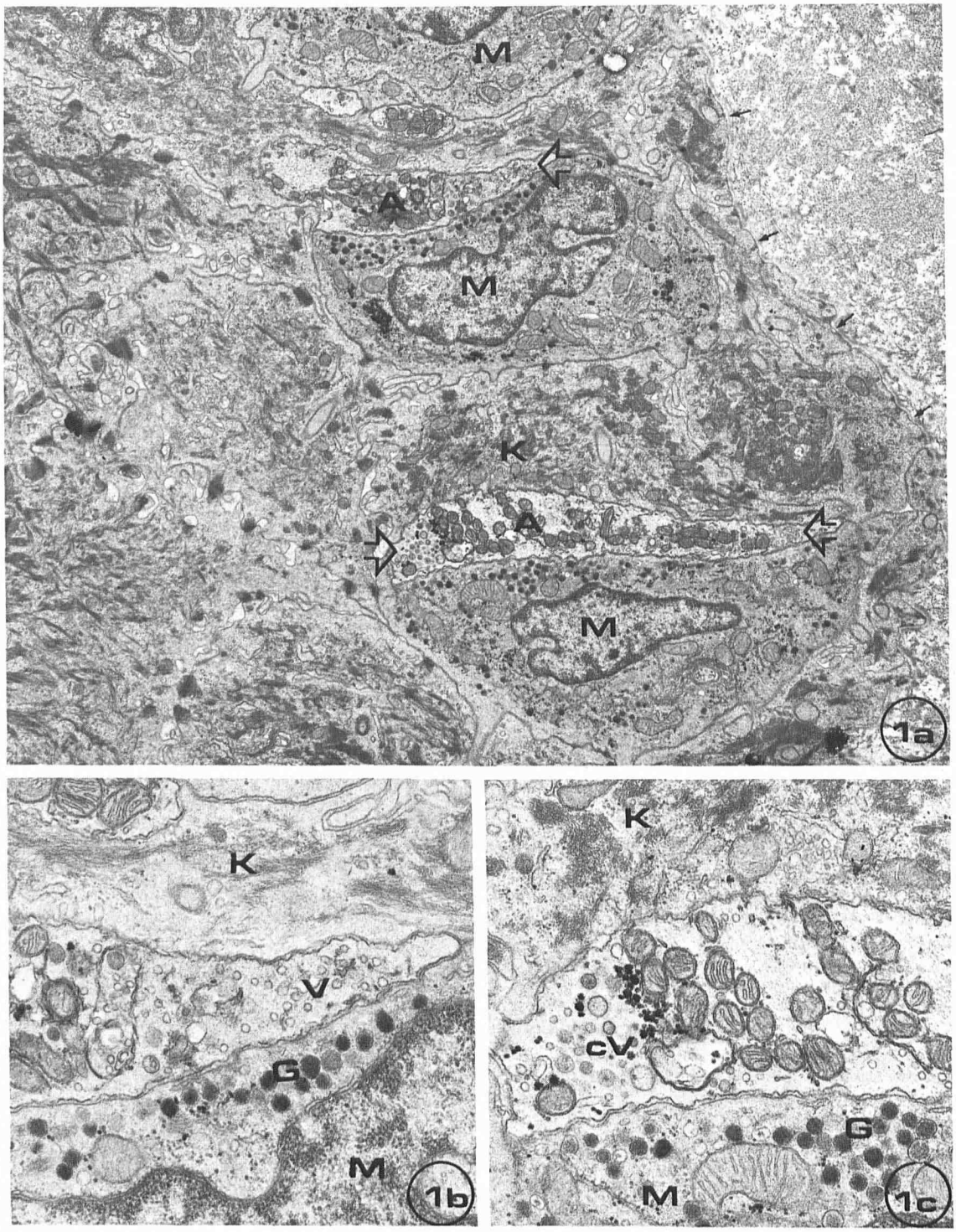

FIG 1. *a*, Low magnification showing 2 MCAC. Section plane perpendicular to the equator plane of the nerve disc. *Arrow heads* indicate numerous vesicles in the "edges" of the nerve transsection. *Small arrows* point to the basal lamina (× 21,000). *b* and *c*, Details of Fig 1a. Clear vesicles (*V*) and coated vesicles (*cV*) are distributed randomly in these parts of the axoplasm. No specific accumulation of vesicles at any site of the axon membrane. Synaptic structures are not detectable. Merkel (*M*) cell-axon (*A*)-complexes of sinus hair follicles of Opossums. Keratinocytes (*K*). Ferrocyanide-reduced osmium postfixation (× 61,000).

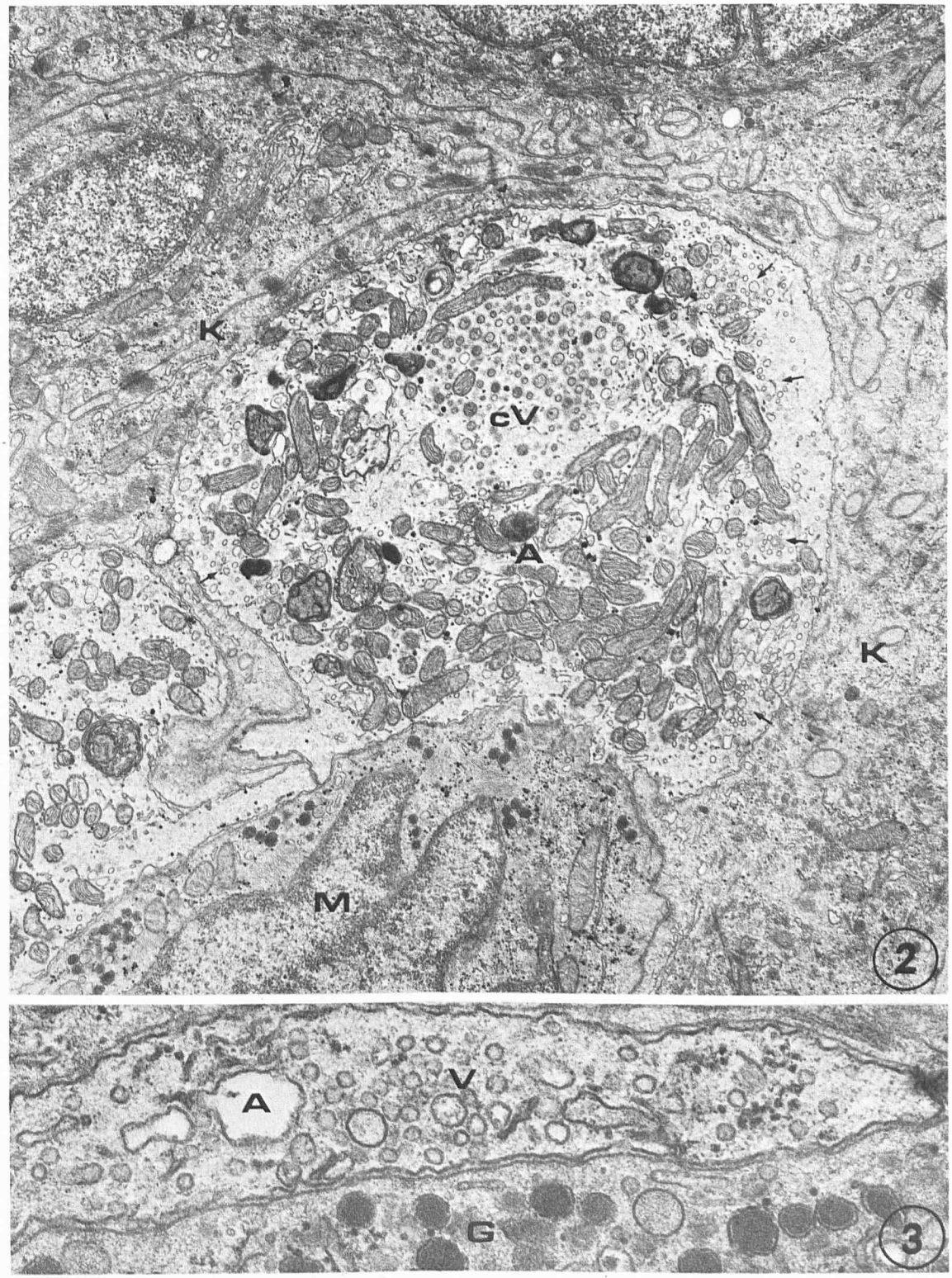

FIG 2. Horizontal section plane showing a Merkel (*M*) cell-axon(*A*)complex of the sinus hair follicle of Opossums. Groups of vesicles (*arrows*) are arranged along the entire circumference of the subneurolemmal axoplasm. No concentration of vesicles at the Merkel cell site. Coated and dense cored vesicles (*cV*) in the central part of the axon. *K* = keratinocytes. Ferrocyanide-reduced osmium postfixation (× 46,000).

FIG 3. Tangential perpendicular section of the nerv disc (opossum). Numerous vesicles in the axoplasm, no accumulation at the Merkel cell site. V = clear vesicles; A= axon; and G = granules. Ferrocyanide-reduced osmium postfixation (× 100,000).

postsynaptic membrane thickening and a granular, weakly electron dense cleft material (Fig 4). Some Merkel cell granules regularly are found near the densely stained presynaptic membrane (i.e., the Merkel cell membrane) which hardly exhibits distinct membrane specialisations. Occasionally less electron opaque Merkel cell granules are found which do not possess a continuous membrane, but are in intimate contact with the synaptic membrane (Fig 4). However we could not detect a fusion of a granule membrane with the presynaptic membrane, resulting in an Ω -figure. Such a close apposition of Merkel cell

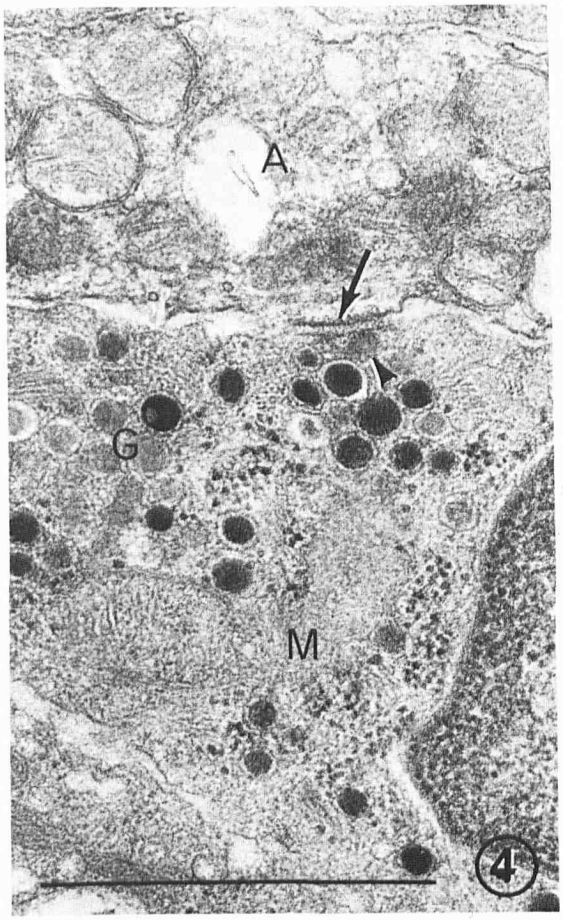

FIG 4. MCAC from the sinus hair follicle (nude mouse), showing a part of a Merkel cell (*M*) and an associated axon terminal (*A*). A synapse-like structure is indicated by an *arrow*. The Merkel cell granules (*G*) are of different electron opacity. A ghost-like granule is in intimate contact with the electron dense presynaptic membrane (*arrow head*). Bar = 1 μ. Postfixation with OsO_4 (× 52,000).

granules is never observed at other nonsynaptic areas of the Merkel cell membrane.

Occasionally dense projections at the presynaptic membrane are observed (Fig 5) which stain as densely as the postsynaptic membrane thickening. With goniometric tilting, the cleft material is shown to be variable with different tilting angles (Fig 6*a–c*). At the optimal view two longitudinally running bands are observed in the synaptic cleft (Fig 6*b*). One is separated from the postsynaptic membrane thickening by an electron translucent gap of approximately 40 Å and is continuously stained. The other band is discontinuous and opposed to the presynaptic dense projections also at 40 Å. The synaptic membranes are not resolvable as a trilayer, so it cannot definitely be decided whether these bands represent a material inside the synaptic cleft or the outer leaflets of the synaptic membranes plus a coating. Further it cannot be resolved whether the electron translucent gaps represent the middle layer of the unit membranes or a gap between the outer leaflets and the cleft material. The 2 bands appear to be interconnected by transversely running bars which never pass beyond the electron translucent gaps to the synaptic membranes. At other tilting angles the cleft material shows the common granular feature (Fig 6*c*) or even is not resolvable (Fig 6*b*).

Digallic acid treatment results in a better resolution of the synaptic membranes and in a reliable staining of the paramembranous densities. Thus the pre- and postsynaptic membranes are resolved as a trilayer (Fig 7*a–f*). The dense projections at the presynaptic membrane appear as clearly confined triangles and are less electron dense than the postsynaptic membrane thickening. At high magnification, the base fuses with the continuously stained inner leaflet of the presynaptic membrane (Fig 7*f*). The dense projections range between 180 Å and 600 Å in height. The highest ones are regularly found at sites where Merkel cell granules are in the closest apposition to the presynaptic membrane; here the Merkel cell granules appear embedded in 2 neighboring dense projections (Fig 7*g*).

The postsynaptic membrane thickening has a mean width of 230 Å and is composed of a highly electron dense flocculent material. At high resolution it is quite apparent that the membrane thickening represents a continuous coating of the inner leaflet of the postsynaptic membrane. The synaptic cleft has a width of 150 Å and is confined by the densely stained outer leaflets of both synaptic membranes (Fig 7*f*). The cleft material is resolved as a discontinuous, fuzzy coating of both outer leaflets of the synaptic membranes (Fig 7*f*). Occasionally the cleft material is arranged in bridges spanning across the synaptic cleft and resembles the transverse bars of OsO_4-postfixed tissue (Fig 7*f*). Tissue staining with *bismuth iodide* and *ethanolic phosphotungstic acid* for selective staining of the paramembranous synaptic material results in a poor tissue preservation and insufficient synaptic staining, perhaps due to an inadequate penetration of the stains in the tissue blocs.

DISCUSSION

This investigation of the MCAC reveals that the specialized membrane appositions between Merkel cells and axon terminals exhibit the typical paramembranous components of a chemical synapse, resembling synapse type II of Gray [20] or type B of Jones [11], presynaptic dense projections, a cleft material, arranged in 2 longitudinal bands joined together by transversely-running bars and further a postsynaptic membrane thickening.

These findings are in contrast to the observations of other authors [5–8] who neither observed dense projections nor analyzed the substructure of the cleft material. These authors

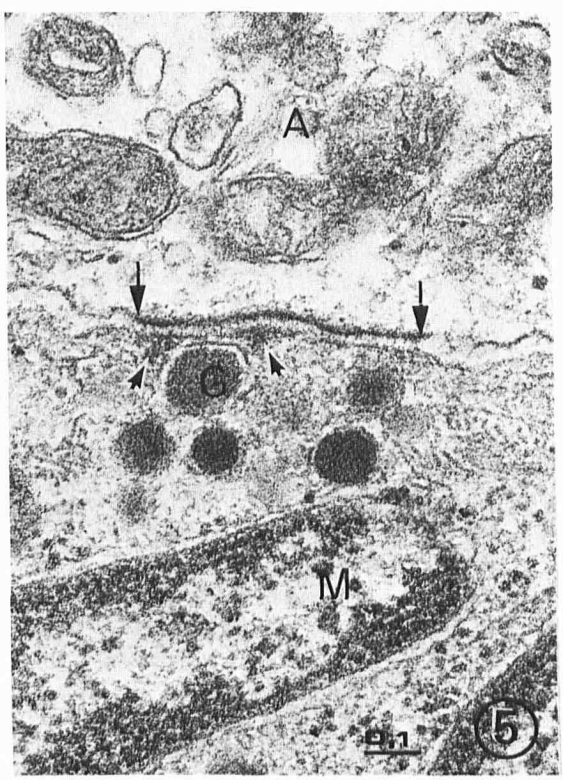

FIG 5. MCAC from a touch dome of the glabrous snout (opossum). The synaptic contact between Merkel cell (*M*) and axon (*A*) is delineated by 2 *arrows*. One Merkel cell granule (*G*) is intruded between 2 dense projections (*arrow heads*) and is in close contact to the presynaptic membrane. Postfixation with OsO_4 Bar = 1 μ. (× 65,000).

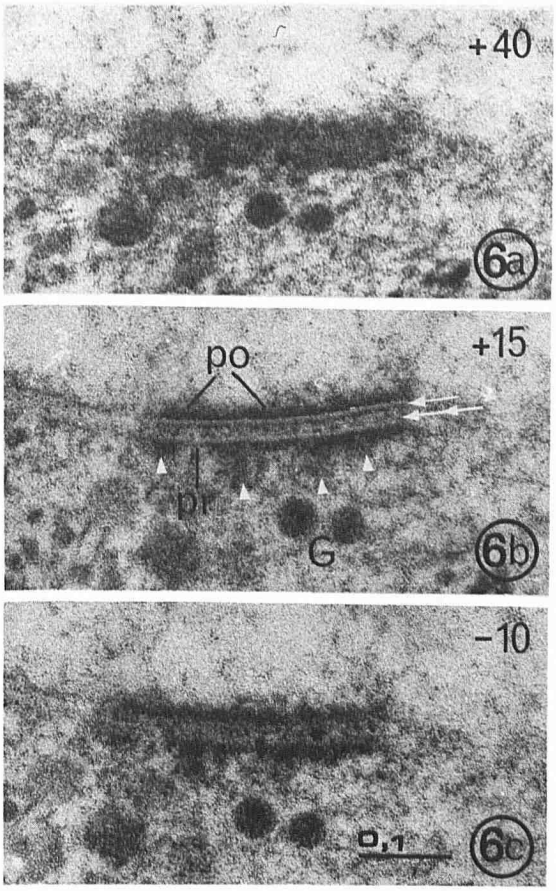

FIG 6. *a–c:* Goniometric tilting series of micrographs from a MCAC (sinus hair follicle, nude mouse). The section is tilted with the axis parallel to the synaptic membranes. The tilting angle is indicated in the upper right corner of each micrograph. Figure 6*b* best exhibits the synaptic substructure with the postsynaptic membrane thickening (*po*) and dense projections (*arrow heads*) at the presynaptic membrane (*pr*). Inside the synaptic cleft an electron dense continuous band (*arrow*) and a discontinuous one (*double arrow*) are seen. In Fig 6*c* the cleft material (*asterisk*) appears granular. There is no resolution of the synaptic substructure in Fig 6*a*. Postfixation with OsO_4 Bar = 1 μ. (× 120,000).

described only a thickening of the postsynaptic membrane, a granular weak electron-dense cleft material and an accumulation of the Merkel cell granules at the presynaptic membrane and denominated this specialized region as "synapse-like structure" [1,2]. In contrast to the literature [5–8] and in agreement with the findings in the Gandry's corpuscle of the Pekin duck [21], synapses are demonstrated in the present paper to be a common feature in MCAC of all investigated mammals.† It seems likely that the conflicting results are not due to species variations but to different methodological procedures of tissue preparation for electron microscopy, i.e., fixation by perfusion or immersion, choice of fixative and postfixative material and staining procedure. E.g., OsO_4 postfixation results in an unsufficient resolution of the synaptic membranes and in a rather weak and unreliable staining of the dense projections as already mentioned by Bloom and Aghajanian [12].

After digallic acid treatment, a reproducible staining of both the dense projections and the postsynaptic membrane thickening is found and further the substructure of the synaptic membranes is resolvable. Therefore the digallic acid procedure offers a useful tool in the simultaneous visualization of membranous and paramembranous synaptic constituents. This is an important advantage as compared with the synapse stains ethanolic phosphotungstic acid and bismuth iodide which selectively stain the paramembranous densities but do not allow a resolution of the synaptic membranes.

Further, with goniometric tilting, variability of the synaptic substructure, especially with regard to the appearance of the cleft material and of the dense projections is demonstrated, which is probably due to the different tilting angles and does not reflect the existence of different synapse types or functional states. In agreement with the literature [1,22] small clear vesicles, referred to as "synaptic vesicles" are never found near the presynaptic membrane. "Synaptic vesicles" are generally regarded as a main characteristic of synaptic ultrastructure. However conflicting data and hypotheses are reported [10] concerning their definite role and provenience. There is evidence that synaptic clear vesicles represent transmitter storing organelles, originating from the Golgi complex [23,24], or empty transmitter vesicles, as well as excess membrane material retrieved from the cell membrane [25]. They are even considered as a fixation artifact, originating from a disrupted presynaptic endoplasmic reticulum [10]. In any case the total lack of presynaptic clear vesicles in the MCAC synapse, never described in any other synaptic contact, as to our knowledge, is a hint that clear synaptic vesicles are no indispensable for synaptic ultrastructure and function. The close association of Merkel cell granules with the presynaptic membrane and their intrusion between the dense projections suggest that the Merkel cell granules themselves function as synaptic vesicles. The chemical composition of these granules has been for a long time a matter of speculation. All attempts to demonstrate an involvement of the Merkel cell in monoamine metabolism have been negative [26, 27]. Recently Merkel cells were shown immunoreactive to the neurotransmitter methionine-enkephalin. The strongest immunoreaction was observed in those parts of the Merkel cells with the highest granule density [28]. Thus the Merkel cell granules probably are the storing sites of the enkephalin-immunoreaction product.

It is likely that the granules discharge their content by stepwise release over a longer period rather than by exocytosis of the whole granule content. This assumption is based on the facts that in contrast to Chen and Gerson [29], we never observed a membrane fusion of a granule membrane with the presynaptic membrane, which results in a Ω -figure and also that the Merkel cell granules adjacent to the presynaptic membrane are mostly less electron opaque, due probably to a partial loss of their content. From the morphological data it seems likely that synaptic transmission occurs unidirectionally from the Merkel cell to the axon terminal. This would be consistent with an afferent relationship between the Merkel cell and the axon terminal.

In contrast to the findings of Munger [5] we never found clear vesicles to be selectively accumulated at a specialized region of the axon membrane, which is typical for synaptic vesicles. In newborn cats, which have fully developed synapses‡ the axoplasm contained conspicuously numerous clear vesicles, but also never preferentially accumulated at any site of the axon membrane. This is in agreement with recent findings in newborn rats [22]. Serial sections in different sectional planes of the neural disc revealed that the vesicles are concentrated in the form of a ring in the subneurolemmal axoplasm. From there results a characteristic distributional pattern in different section planes, as described in this study. Thus, from our results and in agreement with most other authors concerning this problem [1,2,22], there is no evidence that the axonal vesicles function as synaptic vesicles. They rather may be involved in pinocytotic processes, membrane turnover or fulfill a role in trophic pro-

† Since submission of this manuscript another study has appeared reporting synaptic contacts in MCAC of amphibians (Fox H, Whitear M: Observations on Merkel cells in amphibians. Biol Cellulaire 32:223–232, 1978).

‡ Synaptogenesis in the MCAC is described in greater detail elsewhere.

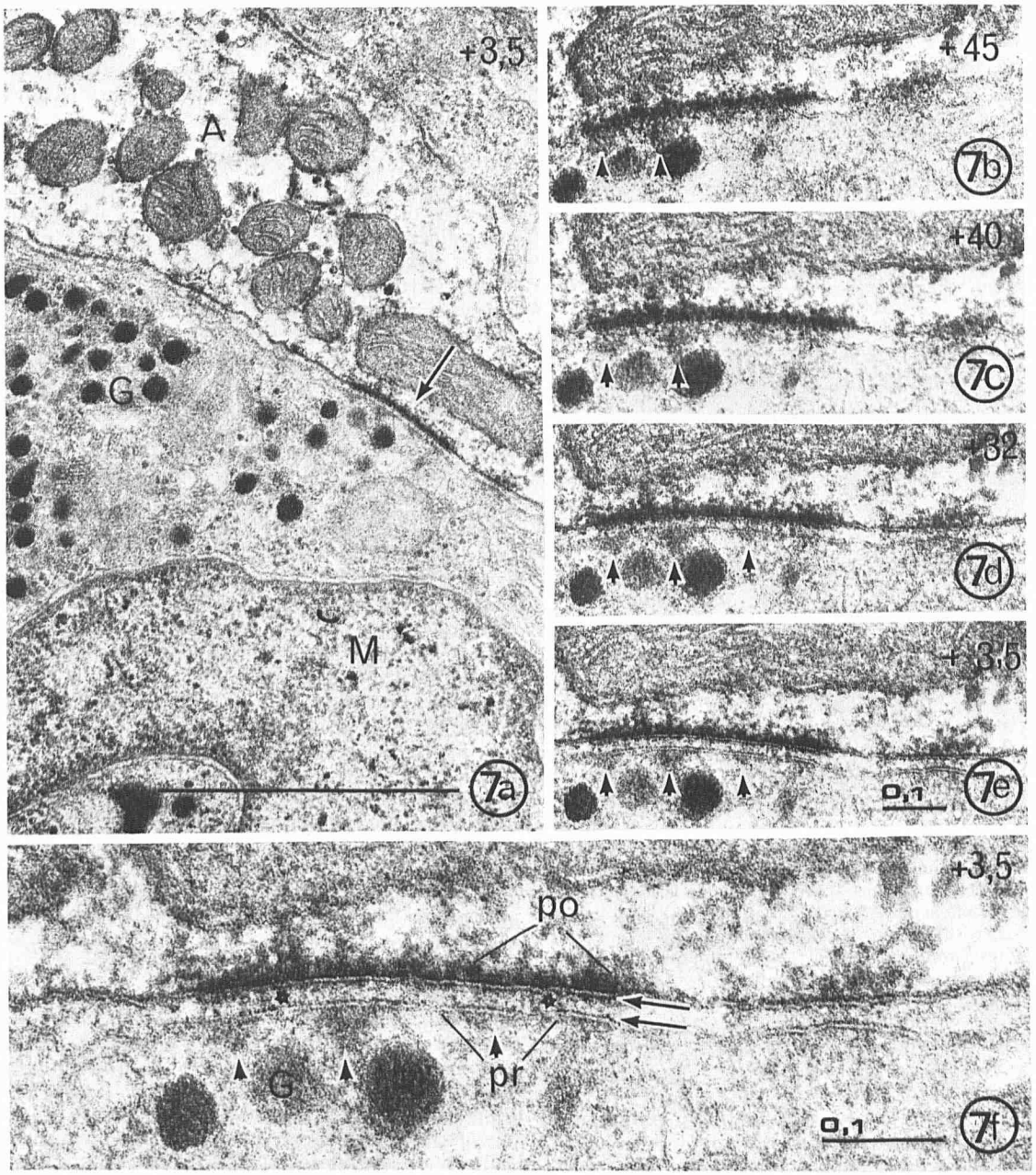

FIG 7. *a–f*, MCAC from the sinus hair follicle (adult cat) following digallic acid staining. Postfixation with OsO_4. Figure 7*a* exhibits the heavily stained membranes of the Merkel cell (*M*) and the axon (*A*). The *arrow* indicates the densely stained postsynaptic membrane thickening of the synaptic contact (× 43,000). Figure 7*b–e* analyzes in a series of goniometric tilting micrographs the synapse from Fig 7*a*. The section is tilted with the axis parallel to the synaptic membranes. The tilting angle is indicated in the upper right corner of each picture. In Fig 7*e* the synapse is in an optimal tilting position. *Arrow heads* point to the presynaptic dense projections. In Fig 7*b* and *c*, the synaptic cleft is not resolvable and the dense projections (*arrow heads*) appear rounded due to a projectional effect and can be mistaken as Merkel cell granules fusing with the presynaptic membrane (× 85,000). Fig 7*f*: High-power resolution of the synapse from Fig 7*a* and *e*. The synaptic membranes are resolved as a trilayer. The postsynaptic membrane thickening (*po*) is resolved as a flocculent coating of the inner leaflet of the postsynaptic membrane. *Arrow heads* point to the prominent dense projections at the presynaptic membrane (*pr*). The synaptic cleft is confined by the densely stained outer leaflets of the synaptic membranes (*arrows*). Both leaflets have a fuzzy coating which is occasionally arranged in bridges (*asterisks*) Bar = 1 μ. (× 170,000).

cesses, as proposed by K. English [22]. Likewise dense projections at the axon membrane were never observed. We therefore do not support the hypothesis that Merkel cells and axons also might have an efferent relationship via antidromic excitation and reciprocal synapses [5], which is based on our ultrastructural evidence.

As to the function of the MCAC, it is established by several correlated physiological and morphological investigation that the complexes might represent mechanoreceptors [3,4]. Based on comparative ultrastructural criteria it has been theorized that both the axon terminal as well as the Merkel cell might represent the primary touch receptor. Our demonstration of a highly developed synapse type in this paper together with the recent finding of methionine-enkephalin immunoreactivity of Merkel cells as described elsewhere [28] give further evidence that the Merkel cell is a receptor cell and transducer of physical to quantal chemical activity. However considering the widespread pharmacological effects of enkephalins in the nervous system [30], the definite perception modality of the Merkel cell has to be clarified by future investigations. A receptor or modulator role in a neuronal system related to pain may also be considered. Since Merkel cells are found without any nerve

association, as ascertained by serial sectioning, and persist ultrastructurally and immunohistochemically unaffected following denervation [9,28,31], further until now unknown functions of this cell might be taken in consideration.

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