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Dynamic changes of membrane structure in chemically and electrotonically transmitting synapses[1]

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Introduction

One of Walter Rudolf Hess' pioneering contributions to Neurobiology[2] was his well known method by which point-by-point electrical stimulation of deep brain structures could be achieved in the freely moving animal (Hess[15]). This procedure combined with a painstaking histological localization of the stimulation sites opened the way for the mapping of the waking brain. Behavioral effects such as sleeping, drinking, eating, orienting, locomotion, grooming, flight, defence and attack could be elicited in a manner unknown to previous investigators who had performed their experiments on animals kept in the deeply anesthetized state.

Twenty years ago when we began to explore the fine structure of synaptic membranes in the central nervous system, we were facing a similar situation. Thus, the commonly accepted technique of preparing the specimens was primarily aimed at an optimal preservation of cells and tissues, and little attention was paid to the functional state of the neurons immediately preceeding death. The best fixation of the tissue was achieved when the animals were kept under deep nembutal anesthesia, while their vascular system was perfused with carefully buffered Ringer followed by aldehyde solutions. Every laboratory at that time developed its own ritualized procedure in order to compete for the best results in tissue preservation.

It soon became apparent, however, that under these conditions it would never be possible to reveal structural correlates of functional states of the nervous system such as one would expect to observe when nerve cells and their excitable membranes are active at the time of death.

Considering the dramatic contrast between neurons firing off bursts of action potentials and neural networks which are electrically silent, it seemed to us a challenging task to examine synaptic membranes for the corresponding morphological features.

Initially the strategy consisted of two major steps: 1. to compare synapses which were kept in widely differing functional states: e.g. anesthetized vs unanesthetized or treated with an excitant drug, and 2. to examine the membrane structures electronmicroscopically by means of the freeze-etching technique of Moor and Mühlethaler[22] which allows one to survey relatively large internal membrane faces under nearly natural conditions. Conventional methods of thin section electronmicroscopy were also used and enhanced by contrasting the major synaptic features (vesicles, membrane densities) with the aid of iodinated compounds[4].

Chemically transmitting synapses

Investigations on the dynamic aspects of synaptic membranes were mainly carried out on the spinal cord of rats. While Streit and coworkers[29] compared the presynaptic membranes in anesthetized (sodium pentothal, i.p. 50 mg/kg) and unanesthetized animals, Tokunaga et al.[30] carried out analogous studies in barbiturized rats which were treated with 4-aminopyridine (1 mg/kg i.v.) and compared with untreated controls. 4-Aminopyridine (4-AP) is known to activate the calcium currents thereby enhancing transmitter release[17]. In both groups the structural changes that could be observed in the activated state were conspicuous.

The main feature of the 'active zone'[9] consisted in tiny membrane modulations which were first described by Pfenninger et al.[25,26] and interpreted as vesicular attachment sites. Their contacts between synaptic vesicles and presynaptic membrane are characterized at the cytoplasmic membrane face (P-face) as clustered pits within a slightly indented membrane district and in the external membrane face (E-face) as clustered protuberances many of which bear a craterlike

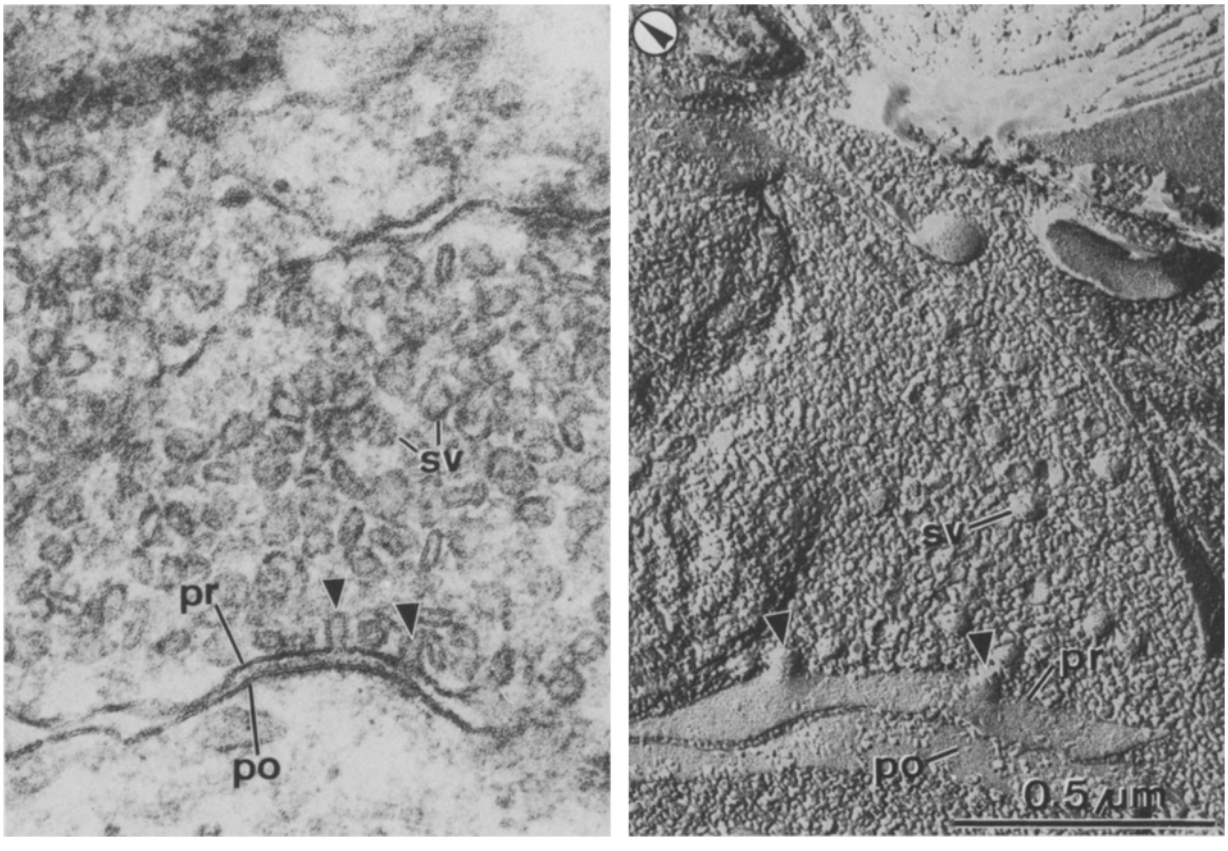

Figure 1. Profiles of the chemically transmitting synapse. Rat spinal cord, ventral horn. The main feature concerns the vesicle attachment sites (arrow heads) at the presynaptic membrane. Left: thin section electron micrograph. Right: replica of freeze-etched specimen. Primary magnification: ×40,000. pr, presynaptic membrane; sv, synaptic vesicles; po, postsynaptic membrane. Encircled arrow indicates the direction of shadow casting. (From Streit et al., Brain Res. *48* (1972) 18, figs 6 and 7, 1972; Elsevier, Amsterdam).

opening at the top (figs 1 and 2) It was concluded that these membrane modulations represent complementary views of the attachment sites between synaptic vesicles and presynaptic membrane, the underlying process being exo- or endocytosis related respectively to the release or re-uptake of transmitter molecules. Our findings were in agreement with those of Couteaux and Pécot-Dechavassine[10] who observed omega-shaped unions ('poches') at the presynaptic membrane of frog neuromuscular junctions by means of thin section electronmicroscopy.

Freeze-etching analysis of activated vs inactivated synapses enabled us to quantify the occurence of vesicle attachment sites under the influence of physiological activation and drugs. It turned out that the increase in the activated state (physiologically or 4-aminopyridine induced) was statistically highly significant. This observation seemed to provide strong support in favor of the 'vesicle hypothesis'[20] of transmitter release, an issue which is still controversial today[16,19,32].

Figure 1 illustrates the vesicle attachment sites as seen in classical thin section electronmicrographs and in replicas of freeze-etched preparations. The cross sectional profiles of a synapse reveal complementary views on the opening of synaptic vesicles into the synaptic cleft. It is not possible to decide as to whether exocytosis or endocytosis is the underlying functional state of the vesicle-to-membrane relationship. However, it is important to point out that such observations are not obtained under deep nembutal anesthesia[29]. An interpretation of these findings is given in figure 2. The temporary union between vesicle and plasmalemma is characterized by two main phases: 1. the attachment site at the presynaptic membrane is slightly curved (microcontraction?), 2. the interior of the vesicle is in direct connection with the intercellular space.

Under inactive conditions the synaptic vesicles are clustered in the vicinity of the presynaptic membrane, but they fail to contact the plasmalemma directly. This observation is facilitated by impregnating the synaptic vesicles by means of the zinc iodide-osmic acid stain. In contrast, in the unanesthetized synapse multiple attachment sites of vesicles are clearly seen[5].

Figure 3 shows the freeze-etching aspects of 4-AP treated synapses as compared with untreated controls. It shows in complementary fracture faces that the presynaptic membranes in 4-AP treated animals contain conspicuous membrane modulations while those of untreated animals are nearly spared. Tokunaga et al.[30] have examined the frequency of vesicle attachment sites at presynaptic membranes of the rat spinal cord and found that the counts obtained from activated synapses were markedly above control levels and the difference significant at the $p = < 0.001$ level.

In summary, then, chemically transmitting synapses have revealed striking structural changes when exposed to physiological or drug induced activation. The most obvious feature consists in a marked increase of vesicle attachment sites which can be visualized as omega forms of membrane union between vesicle and plasmalemma in thin sections, or in the form of specific membrane modulations in the replicas of freeze-etched presynaptic membranes. Whether the vesicle attachment sites represent the crucial substrate of impulse triggered exocytosis of transmitter packages is still not definitevely decided.

Electrotonically transmitting synapses

The two concepts of chemical and electrical transmission of synaptic signals have been antagonistic for some time. However, recent interdisciplinary and comparative studies have made it clear that both

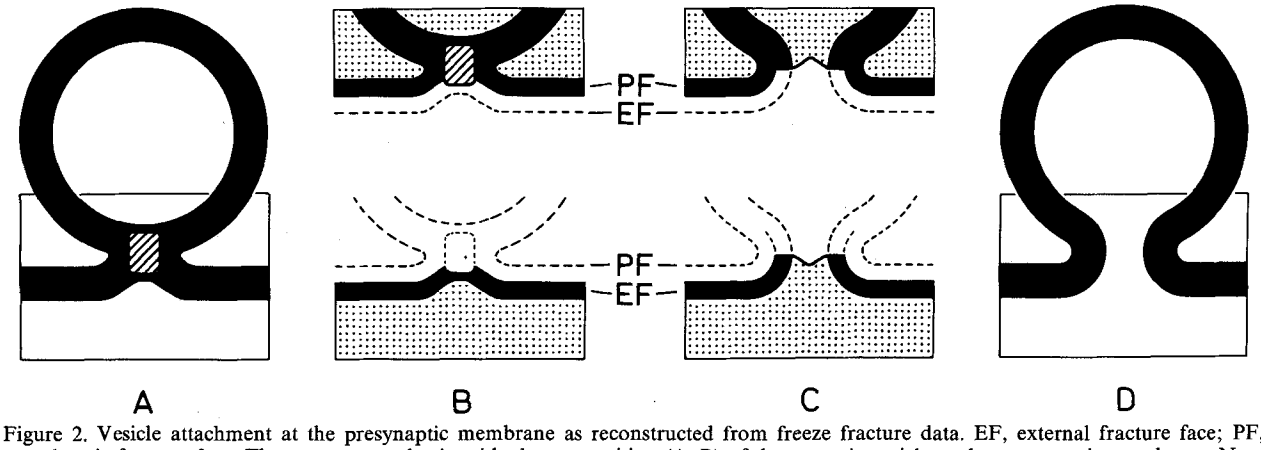

Figure 2. Vesicle attachment at the presynaptic membrane as reconstructed from freeze fracture data. EF, external fracture face; PF, cytoplasmic fracture face. The process may begin with close apposition (A, B) of the synaptic vesicle to the presynaptic membrane. Note that the latter is slightly curved (microcontraction??). Transmembrane particles may act as recognition sites. At the other end of the process (C, D) one finds the inside of the vesicle opening into the synaptic cleft (so-called omega-form of membrane union). (Modified from Pfenninger et al.[26]).

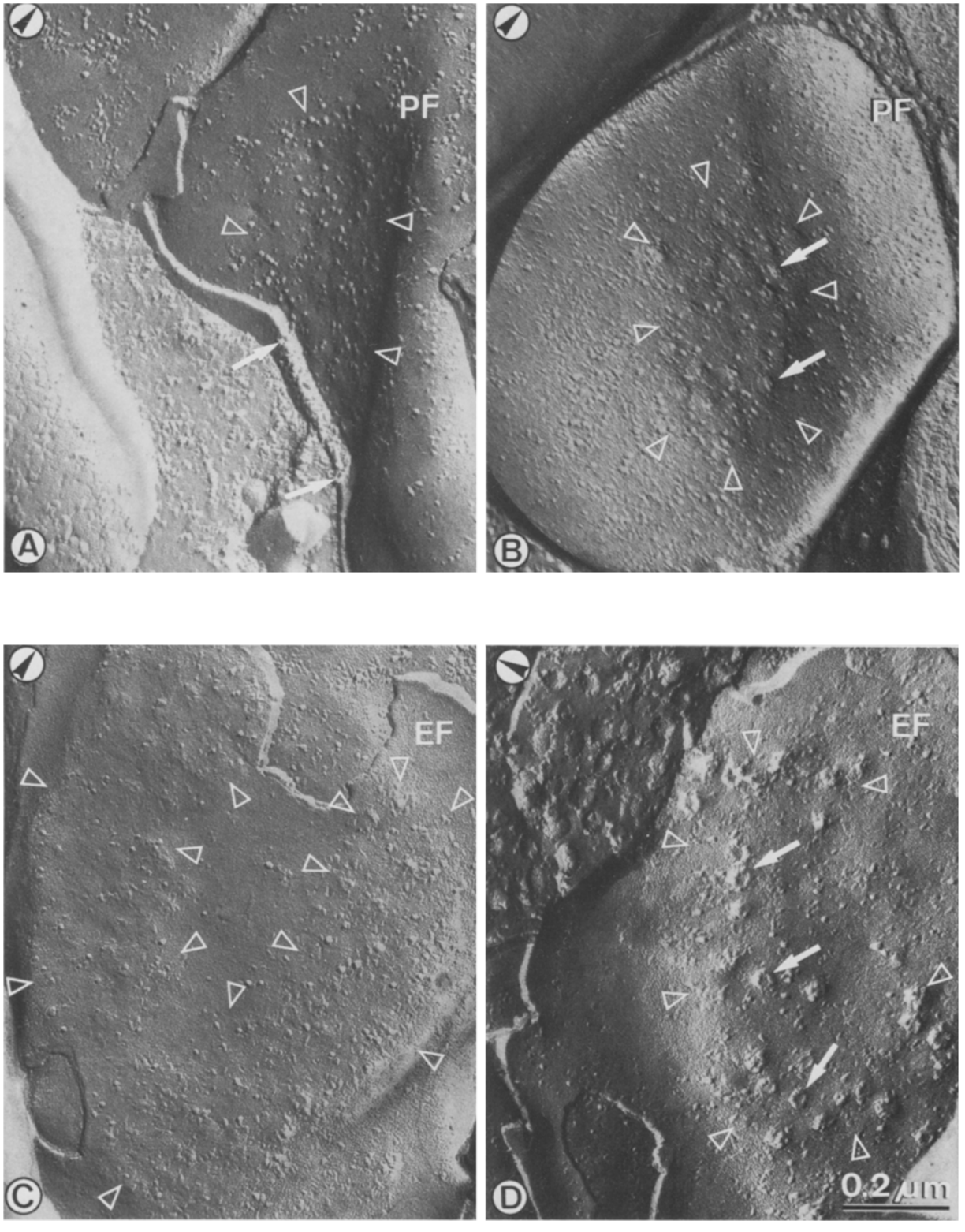

Figure 3. Comparison between activated and inactivated rat spinal cord synapses. Freeze fracture replicas of presynaptic membrane. *A* and *C* Pentobarbital sodium (50 mg/kg) was given i.p. 20 min before perfusion fixation. *B* and *D* The barbiturized rats were given 1 mg/kg 4-aminopyridine i.v. 15 min before perfusion fixation. EF, external membrane face, PF, cytoplasmic membrane face (see fig. 2). Encircled arrow indicates direction of shadow casting. White arrows point to the cross fractured synaptic site in A, and to vesicle attachment sites in B and D. White arrow heads define the 'active zones'. Note the increase of vesicle attachment sites especially in D in 4-aminopyridine treated animals. This drug is believed to enhance transmitter release by activating calcium inward currents across the presynaptic membrane. Primary magnification: ×40,000.

concepts are fully justified since both modes of synaptic transmission seem to occur both in invertebrates and in the vertebrate nervous system.

The crucial ultrastructural feature of electrical synapses as seen in thin section and freeze fracture electronmicroscopy were described respectively by Robertson[27] and by Zampighi and Robertson[31] at the goldfish Mauthner cell (club endings to lateral dendrite junctions). One can conclude from these studies that the morphology of these contacts corresponds with that of gap junctions, and that their freeze-etching aspects are mainly characterized by specific particle-to-membrane relationships.

Our initial experience with electrical synapses concerned the gap junctions in the electromotor control pathways of Gymnotid fish[6,28], whose transmission seem to be predominantly electrotonic at all levels between medullary pacemaker and spinal motoneurons.

Figure 4 shows the freeze-fracture view of a neuronal gap junction. It is important to note that transmembrane particles are arranged in a more or less regular hexagonal array and attached to the P-face (cytoplasmic leaflet of the membrane); their center-to-center distance is about 8–10 nm. The complementary E-face is studded with tiny pits representing vacated attachment sites of the P-face particles. This crystalline particle array is surrounded by an indented membrane district forming a halo in which particles are rarely seen. This halo corresponds to an *annulus adherens* (intermediate or desmosome like junction) whose function is believed to secure the precise fit between the 'connexons', i.e. the particles of apposed membranes which contain channels for cell-to-cell communications.

Figure 5 represents the cross sectional view of this arrangement.

Peracchia and Dulhunty[24] reported structural changes in low resistance junctions of crayfish nervous system under the influence of a chelator of calcium ions which caused the uncoupling of the junctions. These authors observed a tighter packing of particles and a decrease in the overall size of the junctions. A possible reduction in the size of particles was also mentioned. Hanna et al.[14] reported an increased lattice regularity of gap junctions in axolotl blastomeres which were uncoupled by exposing them to CO_2.

More recently we could confirm these observations in gap junctions of epithelial cells in early developmental stages of amphibia. The coupling and uncoupling occured under physiological conditions and not under the influence of artificial agents.

The experiments were performed by Prof. Chuang Hsiao-hui and his collaborators at the Shanghai Institute of Cell Biology. The first observations of this group date back more than 20 years[7]. These authors after removing the neural plate during neurulation grafted several amphibian embryos together serially, i.e. head-to-tail, in a chain-like fashion. In *Cynops orientalis,* at stage 26, with pronephros and 6–7 somites already formed, the normal embryo is still in a nonmotile stage, and no responses to external stimuli can be observed. However, during the subsequent development the embryonic epithelium is capable of generating and conducting spike-like impulses.

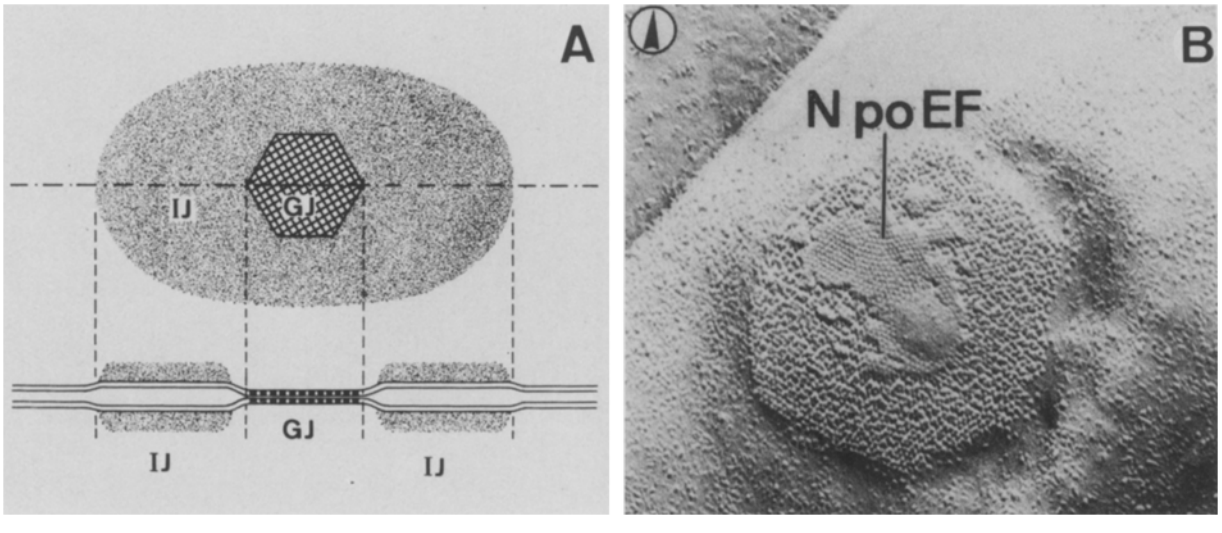

Figure 4. Portrait of an electrotonically transmitting synapse in the electromotor pathway of a gymnotid fish. Left: graphic reconstruction. Right: freeze fracture replica. Primary magnification: ×40,000. Encircled arrow indicates direction of shadow casting. The fairly regular hexagonal array of particles (=connexons) at the P face is characteristic of gap junctions in neural and non-neural tissues. Note that the halo surrounding the gap junction (GJ) contains relatively few particles. This membrane district corresponds to an intermediate junction (IJ) *(annulus adherens)* whose possible significance is mentioned in the text. NpoEF, E face of neuron membrane. (The specimen, *sternarchus albifrons,* was kindly provided by Dr M. V. L. Bennett, New York).

The first proof of conductivity was obtained by grafting a chain of 2–5 nerveless embryos to a normal 'leading' embryo[7]. When the skin of any one of the nerveless embryos was pricked with a glass needle, the normal embryo at the rostral end of the chain showed an intense muscular response, as if the stimulus had been applied to his own body surface, indicating that the excitation was conducted along the skin of the nerveless embryos to the central nervous system of the normal larva which responded with a reflex contraction of the body.

Subsequent systematic studies demonstrated that impulse conduction appeared only during a certain critical stage of development. It began at stage 26 and ended at stage 37, i.e. it lasted for only 180–200 h (at room temperature). Similar observations were made in *Xenopus laevis*.

The second proof came from electrophysiology[11,13]. It was shown that skin impulses can be elicited during the critical period of development by electrical stimulation and recorded intracellularly in the form of relatively slow spike-like potentials which are tetrodotoxin sensitive and propagated at a relatively slow speed (70–80 mm/sec).

These experiments on nerveless larvae of *Cynops orientalis* and *Xenopus laevis* were recently repeated by Chuang et al.[8]. Aldehyde fixed specimens were taken from the skin of these animals at 4 developmental stages: I (stage 21–22) before acquisition of conductivity, II (stage 26) during acquisition of conductivity, III (stage 32) when impulse propagation was fully established, and IV (stage 38) when propagation was no longer present.

The results obtained in our laboratory are summarized in figure 6. It turned out that epithelial cells were connected by gap junctions at all stages. However, marked differences with respect to junctional area and fine structural detail were noted. In group I and IV, i.e. before and after the critical developmental period, the gap junctions were extremely hard to find even in freeze-etch replicas, and even though a very large number of replicas was examined. These few gap junctions were relatively small (0.1–0.5 µm in diameter). In group II the gap junctions were notice-

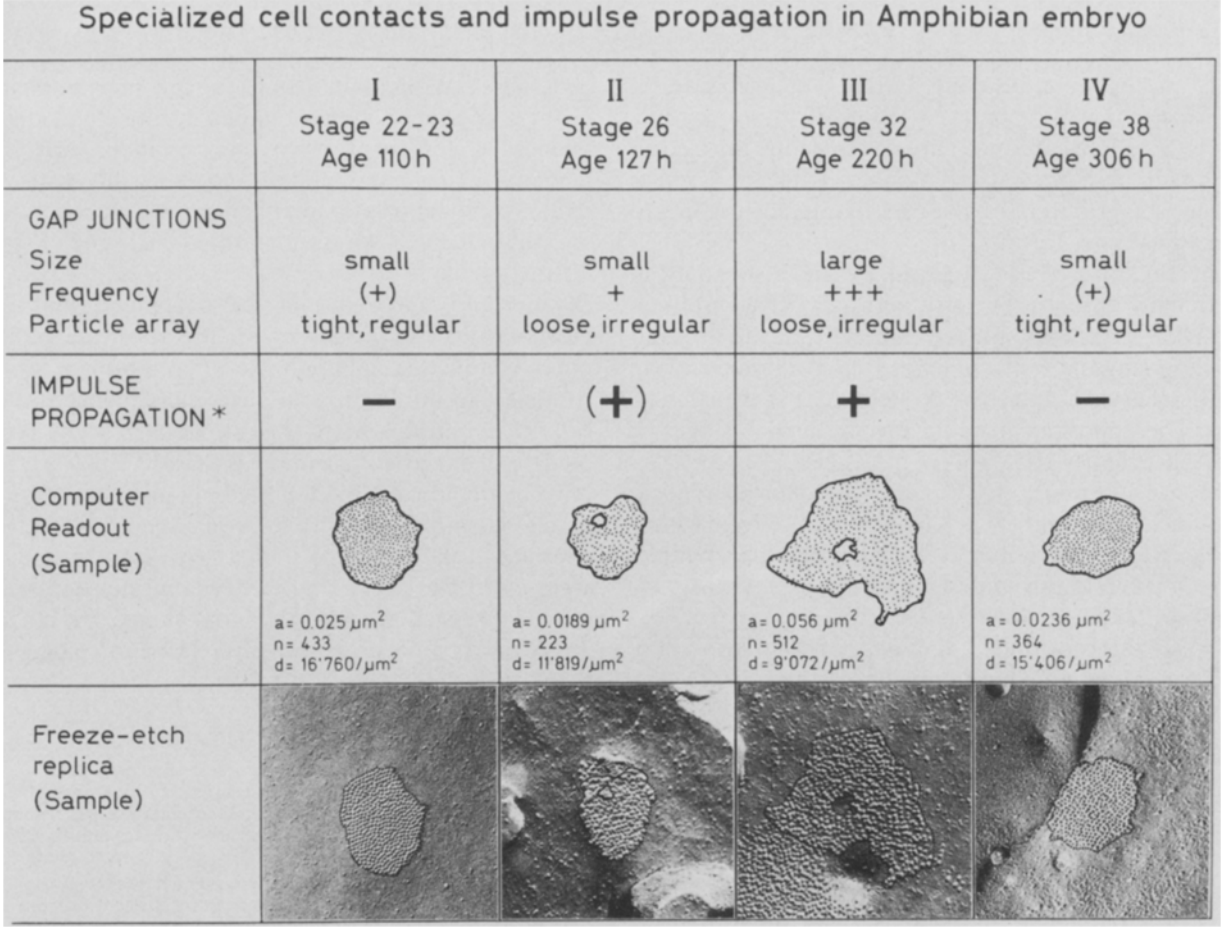

Figure 5. Correlation of physiological and morphological data on gap junctions connecting skin epithelia of *Cynops orientalis* (urodele) during 4 different stages (I–IV) of embryonic development. Explanation see text (Modified from Chuang et al.[8]). The electrophysiological studies were performed by Dai and Sun[11]). Primary magnification of freeze-etch replica: × 20,000.

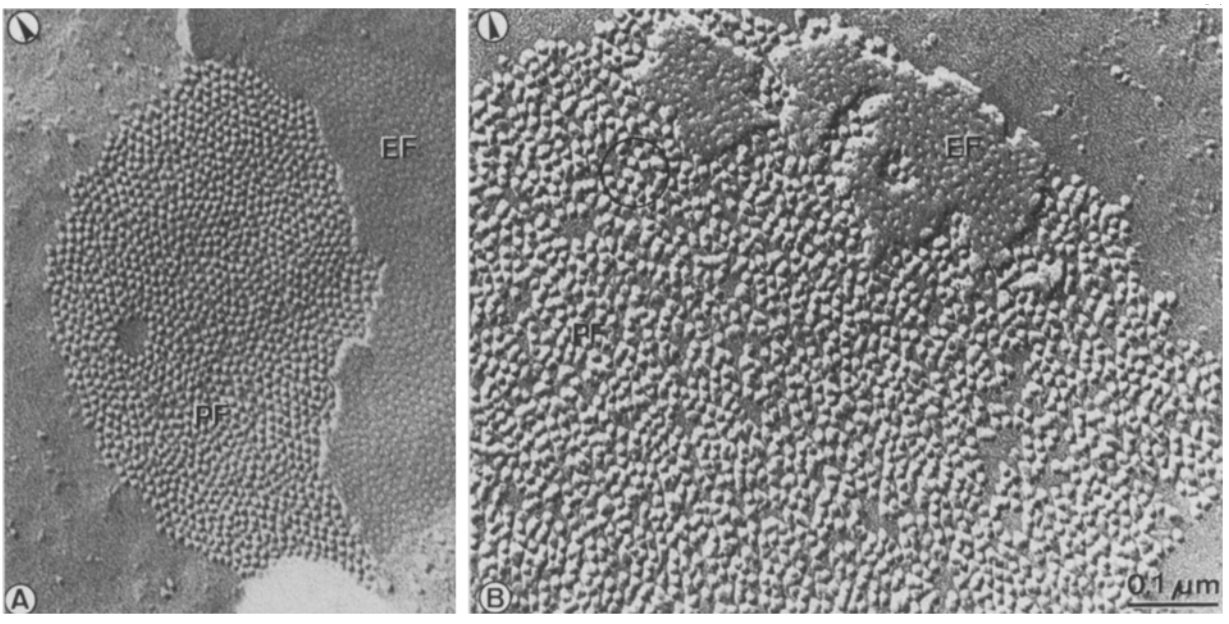

Figure 6. Comparison of fine structural details of electrotonically transmitting cell contacts. Skin epithelium, *Cynops orientalis* (urodele), freeze fracture replicas. *A* Uncoupled gap junction, stage 26 (I). *B* Coupled gap junction, stage 32 (III). Primary magnification ×25,000. Encircled arrows indicate direction of shadow casting. EF, external fracture face; PF, protoplasmic fracture face. Note the higher degree of order in the uncoupled state (A); the opposite is true in the coupled state (B) which contains larger and more loosely packed particles with tiny pits in the center (see encircled area). The two samples (A and B) are taken at identical magnification! These electronmicrographs were obtained in collaboration with Prof. Chuang-Hui and his colleagues in Shanghai, who provided the specimens and performed the experimental work (see text).

ably more numerous, but hardly larger in size. In contrast, the gap junctions in group II (when conductivity is fully established) are conspicuous in size (up to 1.4 µm in diameter) and number. Thus, the samples taken from group III suggest a striking increase in gap junctional area.

Gap junctions of group I and IV differ not only in frequency and size but also with respect to arrangement of connexons. Figure 6 shows that the nonconducting stages are characterized by a tighter packing and increased regularity ('crystallinity') of the connexons. The opposite is true for the conducting stages (II and particularly III).

The size of connexons was measured in 20 replicas and in a total number of 2000 particles. While striking differences were noted between individual samples (fig. 7) taken at the same magnification, the statistical analysis failed to show significant differences between coupled and uncoupled junctions. Obviously the sample was relatively small and the method at the very limit of resolution. Nevertheless, it seems significant that the enlarged particles of the coupled stage in figure 7A seem to contain a tiny pit in the center which is not seen in the particles of the uncoupled stage illustrated in figure 7B and which may represent the opening of a channel. Current hypotheses concerning the functioning of gap junctions[23] hold that contractions and dilatations of the connexon lattice are related respectively to the closing and opening of the ionic gates between adjacent cells. Our own observations with regard to the morphological changes of gap junctions obtained during coupled and uncoupled stages of embryonic epithelia, are not inconsistent with this notion and especially with the dynamic model of Makowski et al.[21] which was based on physiological, electronmicroscopical and X-ray-diffraction data.

In summary, then, it may be concluded that our morphological observations on the substrate of impulse conduction in epithelia of amphibian larvae confirm that ultrastructural differences in electrotonically transmitting synapses exist between active (coupled) and inactive (uncoupled) states. They are not only characterized by the striking increase of gap junctional area, but also by a change of the lattice constant of the junctions. Yet, a great deal of study is still required to prove that the dynamics of the lattice as an expression of conformational changes in macromolecular complexes is directly related to changes in junctional conductivity.

1 Acknowledgment. This work was supported by grants 3.505.79 and 3.580.79 of the Swiss National Foundation for Scientific Research and by the Dr Eric Slack-Gyr Foundation in Zürich. The technical assistance by C. Sandri, E. Schneider, U. Fischer, B. Meili and J. Künzli is gratefully acknowledged.

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Oxygen supply to tissues: The Krogh model and its assumptions

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The human organism can survive anoxia for only a few minutes. Its regular energy requirements are met predominantly by oxidations; this is not surprising since anaerobic glycolysis is about 20 times less efficient in energy supply than oxidation. The basic functions of ventilation, circulation, blood carriage of gases and gas exchange in the lungs and in the tissues serve to supply the tissues with oxygen from the ambient air. Enough oxygen must be transferred per unit of time to ensure a proper function of the terminal oxidases in the cells. Thus, during steady state conditions, the amount of oxygen taken up by the lungs and conveyed along the sequential steps of oxygen transport must equal the amount of oxygen used by the tissue (Kreuzer[29]).

All movement of substance is governed by the general law of transport which states that the flux or flow is proportional to a force, with a proportionality constant having the dimension of conductivity or the reciprocal of resistance. The transfer of oxygen from the air to the tissues may thus be represented in terms of resistances to the flux where the total resistance equals the sum of all individual resistances in series. These specific resistances include those involved in