

ELECTRICAL COUPLING BETWEEN FAT CELLS IN NEWT FAT BODY AND MOUSE BROWN FAT

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ABSTRACT

White fat from the newt, *Triturus pyrrhogaster*, fat body, and brown fat from the interscapular fat pad of newborn mice have been tested for the presence of low-resistance intercellular junctions. 42 pairs of amphibian fat cells and 15 pairs of mammalian brown fat cells were found to be "electrically coupled." In most of these cases intracellular deposition of a dye, Niagara Sky Blue: 6B, was used to supplement and confirm direct observations of impalements. Coupling was often difficult to find in both preparations, but the mechanical disturbance of the tissue during the preparative procedures may have uncoupled many cells. The fact that, in both types of fat, coupling was observed between cells separated by one or more other cells suggests that coupling may be more widespread in vivo. Electron microscopy (provided by Dr. J. -P. Revel and Mrs. K. Wolken) of the brown fat revealed frequent intercellular junctions resembling "gap junctions" but possibly lacking the substructure usually visible with colloidal lanthanum infiltration. The results are discussed in relation to current ideas about the exchange of regulatory molecules via low-resistance junctions and about the control of brown fat by hormones and nerves.

INTRODUCTION

In many tissues, small ions, and perhaps larger molecules, can move directly between the cytoplasm of adjacent cells (see references 1, 9, 21 for reviews). There is considerable evidence that this movement occurs at points of cell contact where the extracellular space is essentially excluded and the apposed membranes have greatly increased permeability. Such specialized junctions were discovered first between certain excitable cells (1, 7, 8) where they function at least as "electrical synapses," but they also occur with widespread distribution between nonexcitable cells and here their function is not understood. An obvious possibility is that these junctions may permit cells to exchange substances important in regulating processes such

as cell movement, growth, and differentiation (3, 21-23, 31).

The earlier electrophysiological studies suggesting the presence of low-resistance junctions in nonexcitable cell systems were restricted to epithelia (15, 19, 20, 24, 25, 26, 30). However, recent studies on embryonic (3, 13, 14, 31, 38, 39, 41, 46) and tissue culture cells (4, 9, 28, 31) have detected similar junctions between certain nonepithelial cells as well, indicating an even broader distribution than first supposed. The present study now extends the list of coupled cells to include connective tissue cells of two types, amphibian white fat cells and mammalian brown fat cells. A preliminary report of some of these results has been published (34).

METHODS

Preparation

The newt, *Triturus pyrrhogaster*, was used for the initial electrophysiological studies. The animals were obtained from Japan and kept at 18°C in deionized water supplemented with Ca⁺⁺. They were fed pieces of beef liver twice weekly, a schedule sufficient to maintain an abundant store of fat. For each experiment, an animal was killed by decapitation and pithing, and then both fat bodies, which lie retroperitoneally near the gonads, were excised and placed in amphibian Ringer's solution (36) at room temperature (about 25°C). Attempts to impale the fat cells in the intact fat body were unsuccessful because of the tough connective tissue layer separating the mesothelium and the fat cells. Usable material was obtained from the surface of the fat body by peeling rectangular pieces which included the mesothelium, connective tissue, and a single layer of fat cells. These pieces were pinned, with the fat cells up, to a layer of soft plastic (Sylgard, Dow Corning Corp., Midland, Mich.) covering the bottom of a culture dish. Amphibian Ringer's at room temperature bathed the preparation. This arrangement exposed the fat cells for microelectrode penetrations observed directly with a compound microscope (see Fig. 1 A).

The second preparation used in this study consisted of brown fat from 1-5 day postnatal mice (Swiss-Webster). Thin pieces of brown fat tissue were taken from interscapular fat pads and immobilized, by the same method used for the newt fat, in a culture dish filled with Locke's solution (36) at room temperature. The whole fat pad could not be used because of poor visibility and the tough connective tissue capsule which, similar to the amphibian preparation, proved impenetrable by the microelectrodes used. Even with the thin pieces of brown fat, microelectrode impalements were difficult and unpredictable, and some preparations proved easier to impale than others. It is likely that this great variability depended on the amount and distribution of remaining connective tissue.

Electrophysiology

The methods for investigating electrical coupling between two fat cells were similar to those described elsewhere (38, 39, 40) and therefore will not be given in detail here. Each cell was impaled with a single microelectrode which could be used to pass current or to record potential changes. Valid impalements of fat cell cytoplasm were signalled by appearance of stable, negative resting potentials (10) and, in dye-marking experiments, by dye filling the cytoplasm. Penetrations of the fat droplet in the fat body usually

led to loss of lipid droplets from the cell accompanying loss of resting potential. The cells were considered "coupled" when a current pulse supplied to one cell produced an electrotonic potential change inside the second cell. An extracellular control was used to exclude the possibility (rather unlikely in these cases) that the coupling was due to high interstitial resistance or external barrier rather than to low-resistance intercellular junctions (see references 3, 31, 38, 39, for more thorough discussion of similar situations).

The position of the microelectrodes was ascertained visually and in most cases also by intracellular deposition of the dye, Niagara Sky Blue: 6B (31, 38). In histological sections, this dye appears blue with transmitted light, but red with dark-field illumination adjusted appropriately (40). The red fluorescence is the more sensitive detector of the dye marks. It can be discerned readily in cases where the mark is nearly invisible with transmitted light. This property of the dye proved useful in identifying marked fat cells in which the cytoplasm characteristically formed only a thin rim around the fat droplet(s). By looking for the red fluorescence it was possible to detect even the small amount of dye contained in very attenuated cytoplasm. Unfortunately, the red fluorescence in the fat cells could not be photographed with adequate contrast on black and white film. This problem did not arise with tumor cells studied previously (cf. reference 40, Figs. 3, 4, and 5) and may reflect differences in the optical properties of the fixed tissues.

In a number of experiments on the newt fat body, microelectrodes filled with 0.5 M K₂SO₄ were used to impale two adjacent cells. If coupling was present, the two electrodes were withdrawn and the cells were re-impaled with dye electrodes. After establishing that the cells were still coupled, they were marked with the dye. These experiments effectively excluded the possibility that coupling was an unphysiological side effect of dye injection.

Light Microscopy

The tissues were fixed overnight at about 0°C in modified Sanborn fixative (31, 37, 38-40) containing 6.5% glutaraldehyde and 2% acrolein, and maintained at pH 4.0 with 50% 0.1 M acetate buffer. The alcohol used in dehydration was acidified in later experiments and by this means the stains were retained more effectively (Kravitz, personal communication). As in previous studies (31), the tissue was embedded in Epon and serial 5- μ sections were cut with a steel knife. The sections were mounted on albuminized slides, and cover slips were applied with Permount (Fisher Scientific Company, Pittsburgh, Pa.). The thickness of the section, slide, and cover slip, as well as the nature of the mounting medium,

are all apparently critical for obtaining optimal detection of the red fluorescence of the dye.

Electron Microscopy

Dr. J. -P. Revel and K. Wolken carried out the electron microscopy on the fat. The relevant methods are mentioned in the text below and explanation for Fig. 4.

RESULTS

Amphibian White Fat

ELECTROPHYSIOLOGY: A typical case of coupling between two amphibian white fat cells is illustrated in Fig. 1. Similar coupling was found in 42 other cases; in most of these, intracellular dye marks were used to confirm the electrode positions although direct observation was often sufficient. It was usually necessary to impale many adjacent cells in a given preparation before finding a coupled pair. This may not, however, accurately reflect the extent of coupling in the intact fat body. It is likely that many junctions were disrupted during the process of peeling away the surface layer of fat cells and adhering connective tissue. Furthermore, the coupling found in the amphibian fat was noticeably labile: frequently two cells, coupled when impaled with K_2SO_4 electrodes, were uncoupled when impaled subsequently with dye electrodes; coupling occasionally diminished or disappeared during current passage even though there was no sizeable change in resting potential. Nevertheless, in a few cases, as many as four adjacent cells were coupled, and in two cases, two cells separated by an intervening cell were coupled. Thus, in the intact fat body, intercellular coupling may be more extensive than could be demonstrated in the split preparation.

Certain initial results clearly demonstrated the importance of using marking techniques to localize electrode position in such complex tissues as the fat body and fat pad. In preliminary experiments, coupling was found whenever the intact fat body was penetrated, and at first it seemed likely that fat cells were being impaled. In fact, even when the cells were marked with dye, the stains in the block appeared to be in fat cells. However, sections through the stains showed that they were usually in mesothelial cells, though sometimes possibly in fibroblasts in the connective tissue capsule. The tough outer covering of the fat body could not be

removed from the fat cells, and therefore it became necessary to use the split preparation described above.

ELECTRON MICROSCOPY: A detailed examination of the fine structure of the cells of the newt fat body proved impossible because appropriate preservation of the tissue was not obtained. Fixation with either potassium permanganate or glutaraldehyde followed by osmium tetroxide was adequate, however, to show that the cells were not separated at all points by a basement lamina. There were occasional blunt processes which penetrated the basement lamina and extended across the extracellular space between adjacent cells. It may be that cell contact occurred at such points. However, it was not possible to determine whether actual contact did take place, let alone the type of contact involved, since the quality of preservation was inadequate.

Mammalian Brown Fat

ELECTROPHYSIOLOGY: In order to determine if electrical coupling between fat cells was peculiar to the amphibian preparation, experiments were carried out on strips of interscapular fat taken from mice 1-5 days old. Brown fat, rather than white fat, was chosen so that parallel ultrastructural studies could be made (see below).

A few electrophysiological experiments were made on mammalian white fat from the mesentery and from epididymal fat pad. Coupling was found in a few cases, and dye marks were located in one case. However, the abundant collagen in these tissues greatly interfered with penetrations, and the experiments were not carried further.

It was possible to demonstrate 15 cases of coupling between brown fat cells; all of these cells were marked with dye and located in sections. Sometimes the coupled cells were direct neighbors, but often there was one or more interposed cell. Figs. 2 and 3 illustrate an example of the latter case. As in the study of white fat cells, negative results were suspect since it was not possible to rule out mechanical disturbance of normal coupling by the method used to prepare the tissue. Furthermore, the cells were often difficult to impale (perhaps because of connective tissue remnants), and the force required to pierce this barrier may have disrupted coupling. Very extensive coupling was demonstrated in one case: one electrode was kept

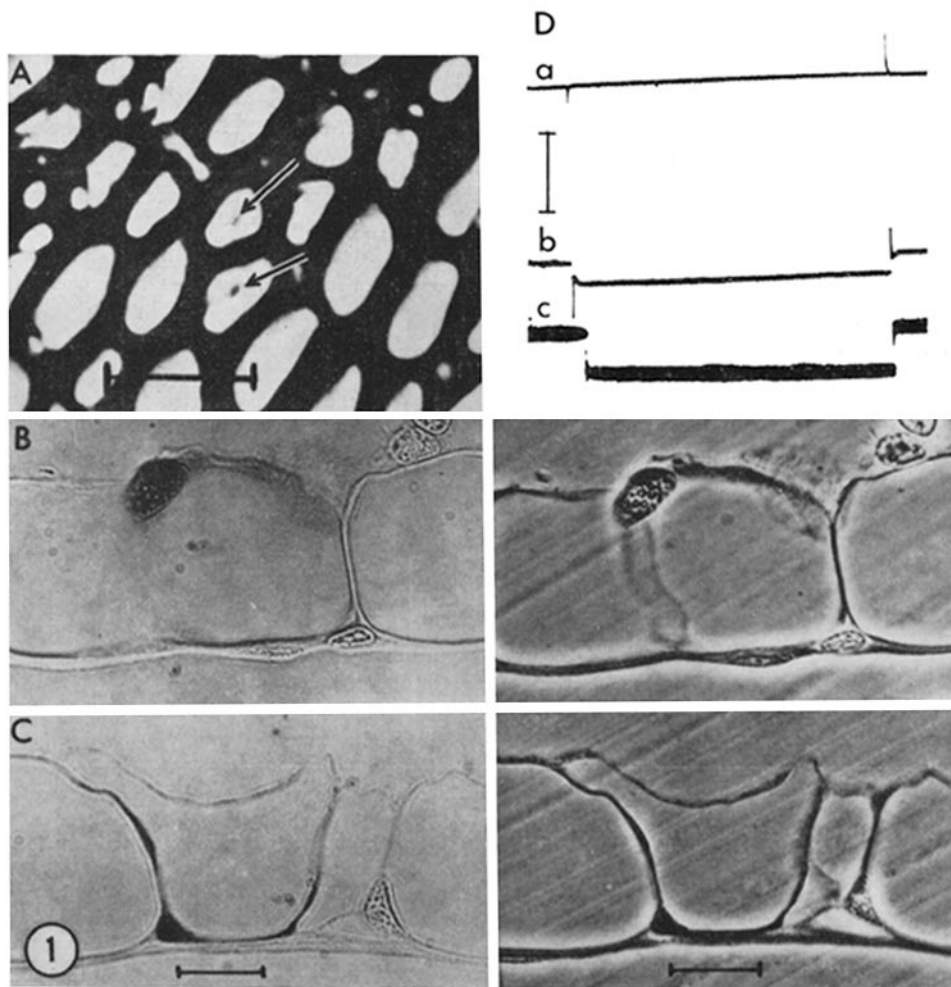


FIGURE 1 One experiment on amphibian white fat is illustrated. (A) Photomicrograph taken during an experiment. The arrows point to dimples produced by the microelectrodes impaling two adjacent fat cells. Visibility during the experiment was better than that indicated by the picture; (B) and (C) bright-field photomicrographs of representative sections through each cell shown in A. The nuclei are dark owing to the dye injected during the impalements. Some dye in the cytoplasm is apparent but in most places the cytoplasmic layer is too thin for reasonable contrast. Corresponding phase-contrast photomicrographs appear on the right. Calibration, 100μ in A ($\times 200$); 25μ in B and C ($\times 460$). (D) Electrical records demonstrating coupling between the cells shown in A-C. Trace *c* shows the current (1.5×10^{-8} A, 0.99 sec) passed into one cell which produced a potential change, *b*, across the membrane of the second cell. Trace *a* shows the extracellular control, and the distance between *a* and *b* indicates the resting potential. Calibration, 10 mv.

inside a cell while 5–10 others in the immediate vicinity were impaled at random; all of these cells were coupled to the first cell (these cells were not included in the 15 pairs noted above). Extensive coupling was also suggested by the demonstration of coupling between pairs of cells via intervening cells as in Fig. 2.

ELECTRON MICROSCOPY: Brown fat cells from neonatal and 5-day old mice were better suited for ultrastructural study than were white fat cells. As was the case for the amphibian fat, the space between brown fat cells was bridged at intervals by blunt pseudopodia extending from one cell toward its neighbor (see Fig. 4). In other regions

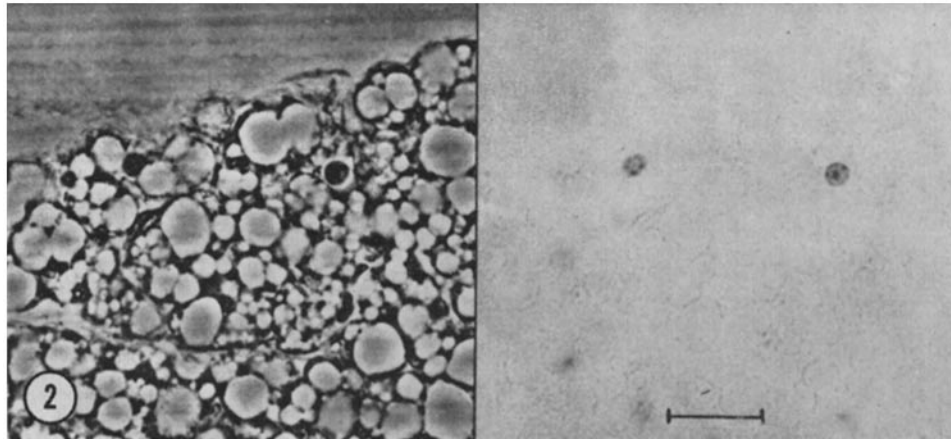


FIGURE 2 This illustrates an experiment on brown fat from a 5 day old mouse. A phase-contrast (left) and bright-field (right) photomicrograph of a section through two brown fat cells which were marked by dye deposition. Only the nuclei are prominent with bright-field illumination. The electrical records corresponding to this pair of cells appear in Fig. 3. Calibration, $25 \mu \times 500$.

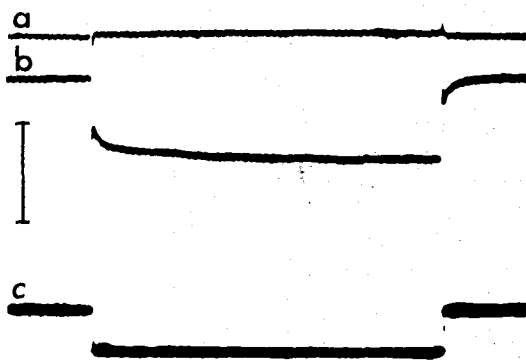


FIGURE 3 Coupling between the brown fat cells shown in Fig. 2 is illustrated. The convention is the same as in Fig. 1: *a*, extracellular control; *b*, potential change in one cell of pair; and *c*, current ($3.6 \times 10^{-8} \text{A}$, 0.88 sec) injected into other cell of pair. Calibration, 5 mv.

apposition occurred along broad fronts rather than at the tips of pseudopodia. Quite frequently, in a single section one cell was seen to make specialized contact with more than one neighbor. As we have reported previously (34), conformations reminiscent of gap junctions (32) were visible at both types of cell-to-cell approximations. Lanthanum staining techniques, which have often revealed a particulate substructure specific to the supposed low-resistance junctions (29, 32), allowed us to delineate the extracellular space between brown fat cells. However, although we obtained one

picture which closely resembled a cross-section of a gap junction penetrated by lanthanum, we found none which clearly revealed the typical polygonal array of closely packed subunits seen in tangential sections of gap junctions. Replicas produced by freeze-cleave methods also failed to indicate any of the structures found at gap junctions in liver (see references 11, 18).

DISCUSSION

Electrical coupling, similar to that present between various excitable cells and between numerous non-excitable cells, has now been observed between amphibian white fat cells and between mammalian brown fat cells. Although fibroblasts from a variety of sources are coupled in cell culture (4, 9, 28, 31), fat cells are the first connective tissue cells shown to be coupled in excised tissue.

In many of the epithelia exhibiting coupling, it has been possible to argue convincingly that each adjacent pair of cells is coupled. Similarly, coupling between fibroblasts in culture appears quite extensive. In the present study, however, coupling could not be detected with every pair of impalements. Although this may indicate that coupling in the intact tissues is not as widespread as in epithelia or in cell culture, the methods used to prepare the tissues for impalement were quite drastic and likely to have interrupted coupling in many cases. Furthermore, coupling between cells separated by an intervening cell occurred twice with the white

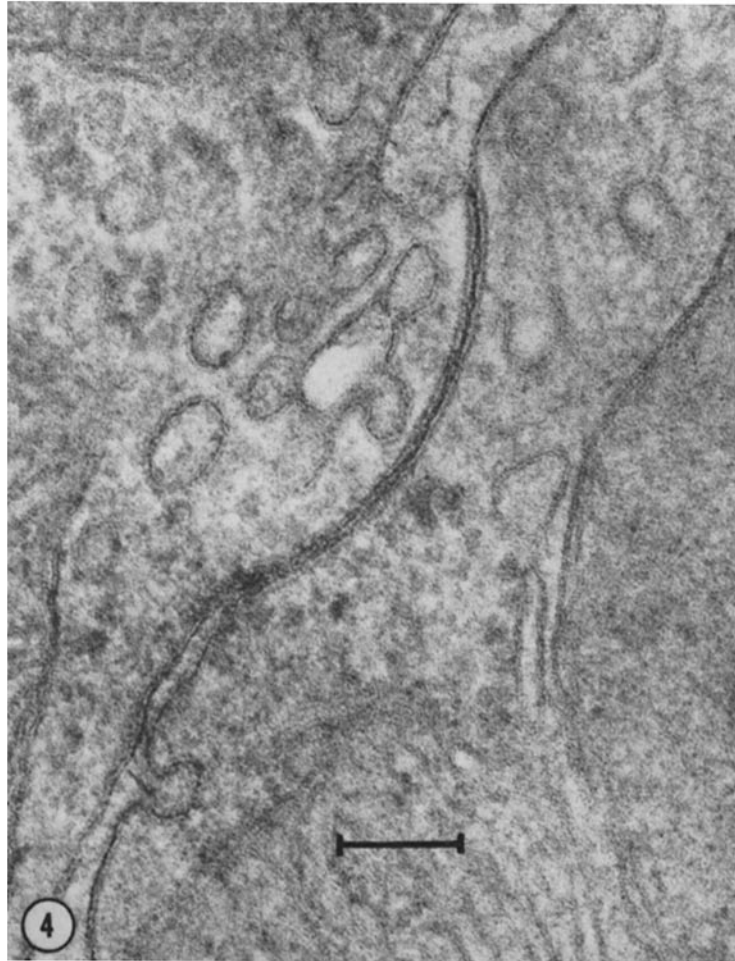


FIGURE 4 A cell junction between two cells of the brown fat of 5-day old mice. Such cellular contacts are typical, and closely resemble the gap junctions of liver, heart, or smooth muscle. The total width of the junction shown here is about 160 Å and an individual membrane of about 70 Å, suggesting a 90 Å gap in the junctional area. See text for further discussion. Calibration, $0.1 \mu. \times 116,000$. (Courtesy of K. Wolken, J.-P. Revel.)

fat and frequently with the brown fat, and one experiment with the brown fat demonstrated that a single cell can be coupled to numerous neighbors. Also, under the electron microscope gap-like junctions between brown fat cells were seen frequently. Thus coupling may well be extensive in the undisturbed fat tissues.

The structures responsible for coupling between brown fat cells are most likely the areas of close membrane apposition illustrated in Fig. 4. In cross-section these areas resemble the gap junctions (5, 29, 32, 33) between other coupled cells, but the characteristic polygonal substructure was not

found. It is possible that the intrinsic difficulties in preparing the brown fat for electron microscopy somehow interfered with the lanthanum procedure or directly affected the substructure itself. However, the alternative possibility, i.e. the brown fat junctions are fundamentally different, must remain open until more extensive electron microscopy has been carried out.

The uncertainties concerning the distribution of the coupling and the particular structure(s) responsible in the fat tissues make an extensive consideration of function unwarranted. However, some relevant comments can be offered. In other

tissues it has been suggested that the low-resistance junctions permit molecules larger than small inorganic ions to pass directly between adjacent cell interiors. Support for this idea has come from demonstration of the movement of tracer molecules between coupled cells. Especially convincing is the evidence for transfer of Procion Yellow (29, 40 *a*) as well as microperoxidase (31 *a*) between certain coupled cells. Fluorescein, the tracer used in the greatest variety of preparations (though not without criticism, see references 9 and 29), has been shown to pass between insect salivary gland cells (17, 24), between certain nerve cells (2, 28 *a*), and between cells in culture (9, 40 *a*). Although cells from *Xenopus laevis* embryos in early stages may not permit transfer of fluorescein (41), this dye has been seen to move between coupled cells dissociated from later stage *X. laevis* embryos and re-aggregated in vitro (unpublished observations). Fat cell junctions may behave similarly since, in the few cases tested in preliminary experiments, fluorescein passed readily between coupled but not between uncoupled amphibian fat cells. These incomplete results suggest a potentially fruitful direction for further studies.

Numerous functions have been suggested for the possible direct exchange of small molecules between neighboring cells. In the case of fat cells such exchange might insure a uniform metabolic response to circulating hormones and/or to nervous input. On the one hand, the amphibian fat body apparently provides a nutritional supply for the gonads (6, 27), and hormones would presumably be important links between the two tissues. Brown fat, on the other hand, is particularly responsive to a number of hormonal, physical, and nervous factors (e.g., 10, 12, 16, 43, 44, 45). One example is the thermogenic response of brown fat to catecholamines released from sympathetic nerves or carried in the blood (12, 42; but see also reference 43). Junctions permitting direct cellular interchange of certain low-molecular weight substances might be involved in distributing the final effect of catecholamines over the whole tissue. Thus, an homogeneous thermogenic response by the fat tissue might be obtained even if every cell were not innervated or affected to the same extent by circulating catecholamines. One substance which might pass from cell to cell is 3'-5' cyclic adenosine monophosphate (cAMP) which apparently mediates at least part of the response (12). It is interesting that the molecular weight of cAMP (330) is

very similar to that of fluorescein (332) which has been shown to pass between electrically coupled cells (see above). The possibility that cAMP does indeed move from cell to cell has far-ranging implications, since it has been found as a mediator of hormone action in many other systems (35).

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Note Added in Proof: Recently replicas of freeze-fractured brown fat from 2-day old mice have revealed the intramembranous subunits reported for gap junctions in other systems (J. P. Revel, Ann Yee, and A. J. Hudspeth, personal communication). These results strengthen the argument for the gap junction as the site of low resistance between the fat cells.

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