Quantitative evaluation of gap junctions during development of the brown adipose tissue

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Abstract Gap junctions of rat brown adipose tissue (BAT) were studied with the freeze-fracture technique during development. The frequency and the mean area of gap junctions increased after birth, reached a maximal development during the functional phase of the tissue (birth to 3–4 weeks) and decreased while brown adipose tissue's function regressed (from 3–4 weeks on). Gap junctional area per cell volume, an indirect estimate of intercellular coupling, followed closely the phases of BAT activity, suggesting an involvement of these junctions in the functioning of brown adipose tissue.—**Schneider-Picard, G., J-L. Carpentier, and L. Orci.** Quantitative evaluation of gap junctions during development of the brown adipose tissue. *J. Lipid Res.* 1980. **21:** 600–607.

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Brown adipose tissue (BAT) is an effector organ of regulatory heat production (non-shivering thermogenesis) in neonatal, hibernating, and cold-adapted mammals (1–3). BAT is composed of adipocytes supplied with an extensive network of capillaries and nerve endings (4–8). Adipocytes are electrically coupled (9, 10) via gap junctions between adjacent plasma membranes (11) and since heat production in BAT appears to be controlled by sympathetic innervation (3, 12–14), it has been suggested that gap junctions conduct the nerve stimulus throughout the tissue (10).

In the rat, the development of the BAT has been divided into three distinct phases (15): a formative phase (differentiation until birth), an active phase (birth to 3–4 weeks), and an involution phase (3–4 weeks to normal death) in animals not exposed to cold. To test the hypothesis of a role of gap junctions in the functioning of the BAT, we have evaluated quantitatively, by freeze-fracture electron microscopy, the development of gap junctions during the three phases of BAT function. By exposing the inside of membranes in face view (16), freeze-fracture allows the accurate measuring of the two-dimensional extension of gap junctions in the plane of the plasma membrane. The data obtained show that the development of gap junctions and the activity of BAT are correlated.

MATERIALS AND METHODS

Tissue preparation

Male Wistar albino rats kept at 24°C were killed by decapitation. Four animals were used at each of the following ages: 2 hr, 12 hr, 2 days, 17days, 34 days and about 90 days after birth. Four 21-day-old fetuses (1 day prepartum) were obtained by cesarean section. Interscapular BAT was quickly removed, cut into small pieces and fixed at room temperature in 4% glutaraldehyde, in 0.1 M phosphate buffer, for 30–60 min.

For freeze-fracturing, the samples were immersed in 30% glycerol, buffered with 0.1 M phosphate buffer, pH 7.4, for 30 min and frozen in Freon 22 cooled with liquid nitrogen. Fracturing and replicating was done in a Balzers freeze-fracture device (BAF 301, Balzers High Vacuum Corp., Liechtenstein) at −110°C according to Moor and Mühlethaler (17). Replicas were cleaned from tissue debris and fat by a sequential treatment with sodium hypochlorite, a chloroform–methanol mixture, pure dimethyl formamide, and distilled water. Replicas were recovered on copper grids and examined in a Philips EM 300 electron microscope.

For phase contrast microscopy, the glutaraldehyde-fixed tissue was post-fixed in 2% phosphate buffered OsO_4 for 2 hr, dehydrated in a graded series of ethanol, and embedded in Epon.

Quantitative evaluation

From each animal, a minimum of twelve membrane areas of brown adipose cells present in at least two different replicas were taken. Low magnification micrographs (about 6,000×) were taken in order to include an entire membrane face in one or two pictures. Higher magnification micrographs (about 10,500×) were taken of all gap junctions present on the membrane faces for size evaluation. The exact magnifica-

Abbreviations: BAT, brown adipose tissue.

tions were determined with a calibration grid (E. F. Fullam Inc., Schenectady, NY, 2.160 lines/mm). Evaluations were carried out on positive prints enlarged three times.

The areas of membrane faces and of gap junctions were determined with a planimeter (Aristo 1130L, Dennert and Pape, Hamburg, Germany). The precision of measurement of small areas (gap junctions) was 4% and of larger membrane regions (entire membrane faces), 1%.

As seen in Fig. 2 a–f, gap junctions in BAT appear under three main forms: single, well-delimited particle aggregates ("typical" gap junction, Fig. 2d); small islands of aggregated particles grouped in a particle-poor patch of membrane (= plaque, Fig. 2b, f); and single large islands with associated small aggregates similarly situated in a particle-poor area (Fig. 2c). To account for this variable morphology, two ways of expressing the development of gap junctions were used: *a*) the "individual gap junction" represents any single particle aggregate irrespective of the number of its constituting particles (the area of an aggregate containing as few as 10 particles could be measured accurately by planimetry); *b*) the "gap junctional plaque" represents the entity formed by individual islands (large or small) of aggregated particles grouped in a particle-poor area of the membrane. The area of a gap junctional plaque is obtained by summing up the areas of its constituting individual aggregates. In the case of a typical single gap junction, the values characterizing the individual gap junction or the gap junctional plaque are identical. Both terms and their underlying interpretation allow us to describe changes (i.e., aggregation or fragmentation) occurring in the constitution of gap junctions during postnatal development.

For the evaluation of gap junction frequency, a minimum of 200 junctions was counted for each age.

Most of the measurements were done on adipocyte P-faces. For some ages, when E-faces were present on the micrographs, gap junctions were evaluated and the data recorded separately.

The cell size, (measured as the cell diameter) was evaluated on semithin (1 μm) sections of Epon embedded tissue, photographed in a phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany) at a magnification of 400× for fetal tissues and 160× for neonatal and adult tissues. Cells were approximated by circles and diameter of the circles determined by comparison with calibrated circles. For each age, about 400 cells were measured. The average diameters, corrected according to Weibel (18), were used for calculation of cell volume.

Statistical analysis of the data was carried out with the χ^2 test.

RESULTS AND DISCUSSION

Qualitative observations

Characteristic ultrastructural features of the BAT adipocyte as seen with the freeze-fracture technique are shown in **Fig. 1.** The plasma membrane shows randomly distributed particles and numerous invaginations. Gap junctions, the only type of junctional specialization found in brown adipocytes, are recognized as close aggregates of intramembranous particles on the P-face and as aggregates of closely-spaced pits on the E-face (19, 20).

Considerable variations in gap junction shape and size were observed throughout the stages of development of BAT (**Fig. 2 a–f**). Besides "typical" gap junctions (Fig. 2d) present at all stages examined, other configurations were observed. Plaques (21) formed by individual particle aggregates of various sizes (Fig. 2a and b) were prominent in 21-day-old fetus and in 2-hour-old animals. Large particle aggregates accompanied by small satellites (Fig. 2c) were commonly observed in adipocytes from postnatal (12 hr to adult) animals. In 17-day, 34-day, and adult animals, gap junctional plaques with irregular contours (Fig. 2f) were found with increased frequency while large, irregularly-shaped particle aggregates with particle-free islands (Fig. 2e) characterized 17-day-old animals. The morphologic pattern described above is compatible with a pattern of formation, growth, and decay of gap junctions (22) in brown adipose cells as previously described in other differentiating and/or regenerating tissues (23–29).

Quantitative evaluation

The frequency of individual gap junctions and gap junctional plaques (cf. Materials and Methods) at the different ages studied is shown in **Table 1. Fig. 3** illustrates the frequencies of gap junctions in function of age: both individual gap junctions and gap junctional plaques increase in number between 21-day fetal life and birth; individual gap junctions then decrease between 2 hr and 12 hr after birth, while from 12 hr until 17 days, both parameters remain constant; in older animals (34 days and 90 days) individual gap junctions and, to a greater extent, gap junctional plaques decrease. The fact that the increase of individual gap junctions is larger than the increase of gap junctional plaques at 2 hr can be explained by the presence of plaques containing numerous small gap junctions. The subsequent decrease of the frequency of individual gap junctions can be accounted for by the confluence of small gap junctions into larger junctions, while in older animals the reverse takes

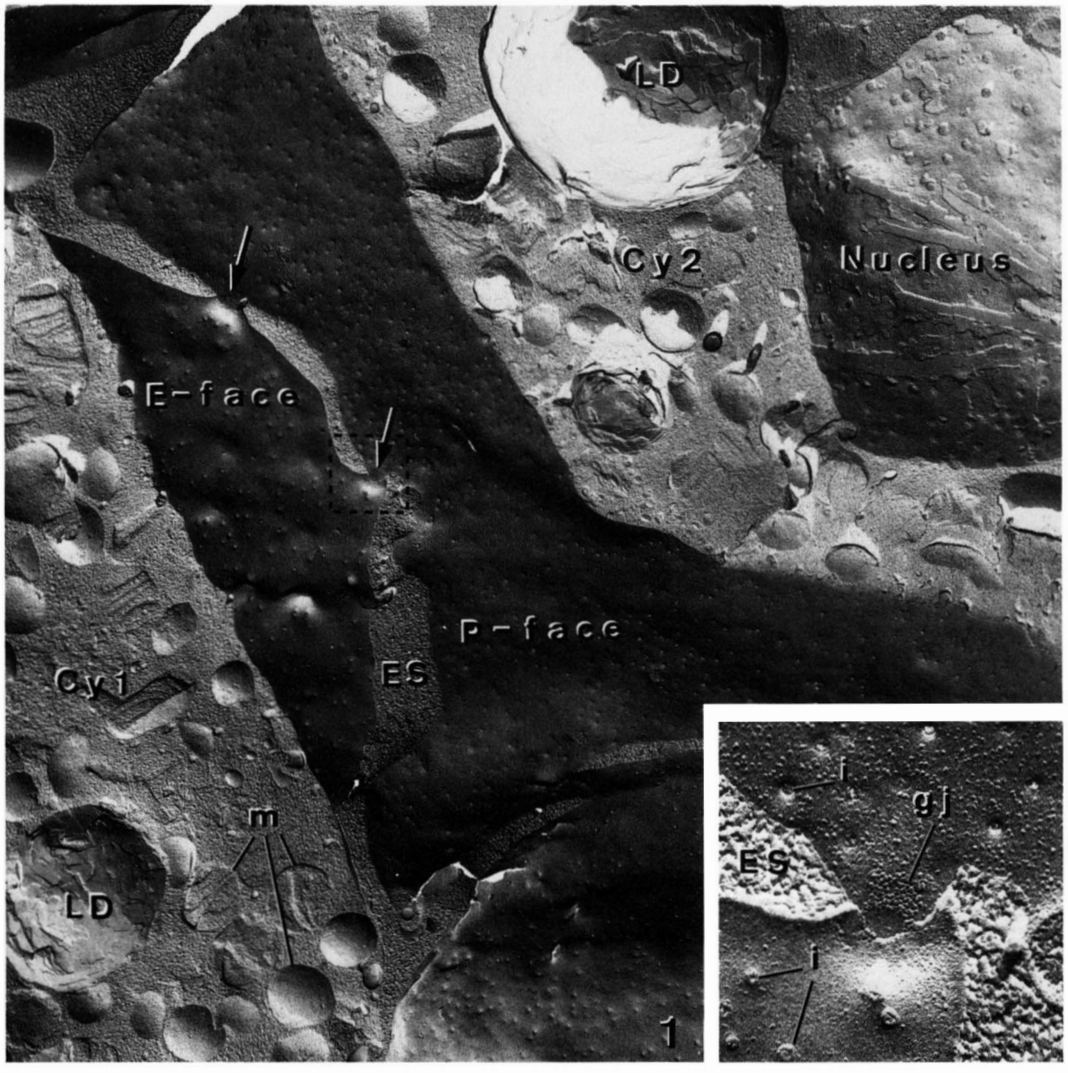

Fig. 1. Freeze-fracture replica of brown adipose tissue (BAT) showing the cytoplasm of two neighboring adipocytes (Cy1, Cy2) and parts of their respective plasma membrane. The cytoplasms contain mitochondrial profiles (m) and lamellar lipid droplets (LD). The exposed plasma membranes (E-face of cell Cy1, P-face of cell Cy2), display numerous punctiform invaginations. The extracellular space (ES) separating the two membranes is reduced at two focal regions (arrows) one of which (dotted square) is shown at higher magnification in the inset. The inset demonstrates a typical gap junctional aggregate (g.j.) on the P-face in a region of narrowed extracellular space (ES); (i) indicates plasma membrane invaginations. 2 hr after birth. ×11,000; Inset: ×58,000.

place, i.e., large gap junctions fragment into small ones. This hypothesis is confirmed by the fact that the ratio between the frequency of individual gap junctions and gap junctional plaques is higher at 2 hr and 90 days, as compared to 17 days (1.49 ± 0.11, 1.45 ± 0.11, and 1.16 ± 0.08 respectively, $P < 0.05$).

Fig. 4 shows the distributions of gap junction area in relationship to age. The smallest gap junctions (i.e., $<3 \times 10^{-2}$ μm^2) reach a maximum 2 hr after birth and then gradually decrease until 17 days. Within the same period of time, the larger junctions (i.e., $>18 \times 10^{-2}$ μm^2) progressively increase. In 34- and 90-day-old animals, the percentage of small gap junctions increases again while the proportion of large gap junctions decreases. The pattern of Figs. 3 and 4 is compatible with junction formation between fetal life and birth and with gap junction disaggregation between 17 and 34 days.

The mean area of individual gap junctions and gap junctional plaques calculated at the different ages is given in **Table 2.**[1] These values are plotted in func-

[1] The values have been calculated from histograms of gap junction area distribution shown in Fig. 4, assuming that these distributions resemble to an exponential function e^{-x/x_0}, where x_0 is the mean gap junction area. The best fitting x_0 and its error have been determined according to the maximum likelihood method (30).

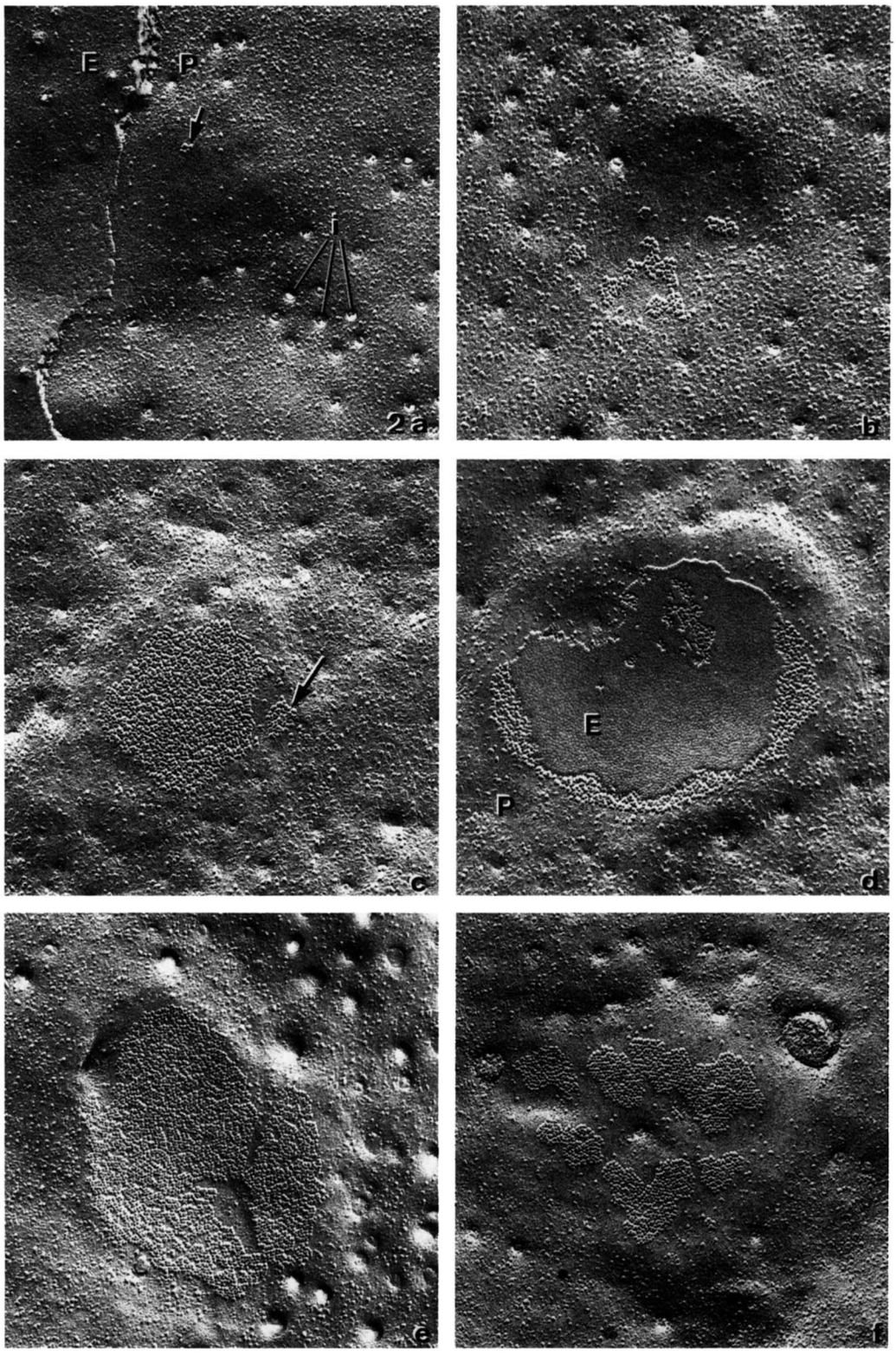

Fig. 2. Proposed sequence of gap junction development in brown adipose tissue. a) Plaque consisting of an ovoid, particle deprived area in the P-face of the plasma membrane; the plaque is situated in a P-E fracture face transition across a reduced extracellular space. The arrow points to a tiny aggregate of particles in the plaque; i, plasma membrane invaginations; 2 hr after birth; ×51,000. b) Plaque containing several individual gap junctions of various sizes; 2 hr after birth; ×59,000. c) Large-gap junctional aggregate in a plaque accompanied by a small satellite (arrow); 2 hr after birth; ×52,000. d) Large gap junction in a P-E fracture face transition; 2 hr after birth; ×54,000. e) Gap junction containing particle-free islands of various shapes and sizes; 17 days after birth; ×47,000. f) Gap junctional plaque (cf. definition in Materials and Methods) containing several individual gap junctions; 90 days after birth; ×52,000.

TABLE 1. Number of gap junctions per 100 μm^2 of brown adipocyte plasma membrane (±SEM)[a]

Age	Individual Gap Junctions		Gap Junctional Plaques	
	P-face	E-face	P-face	E-face
Fetus (21 days)	12.2 ± 0.7 (295)[b]	12.0 ± 1.6 (53)	8.9 ± 0.6 (215)	9.2 ± 1.4 (41)
2 Hours	17.7 ± 0.8 (451)		11.9 ± 0.7 (302)	
12 Hours	12.8 ± 0.9 (213)	10.2 ± 1.3 (59)	10.8 ± 0.8 (180)	8.8 ± 1.2 (51)
2 Days	14.4 ± 1.0 (203)	10.9 ± 1.5 (54)	11.9 ± 0.9 (167)	9.9 ± 1.4 (49)
17 Days	12.4 ± 0.6 (485)	12.1 ± 1.1 (129)	10.7 ± 0.5 (416)	11.0 ± 1.0 (117)
34 Days	11.6 ± 0.7 (311)		9.7 ± 0.6 (259)	
90 Days	9.7 ± 0.5 (377)	7.2 ± 0.8 (73)	6.8 ± 0.4 (264)	5.5 ± 0.7 (56)

[a] Since the proportion of the cell surface occupied by gap junctions represented only about 1% of the total cell surface area, the relative error of frequency was predictable by the Poisson distribution, N-½, where N is the number of gap junctions counted.

[b] Numbers in parentheses indicate sample size (number of gap junctions counted).

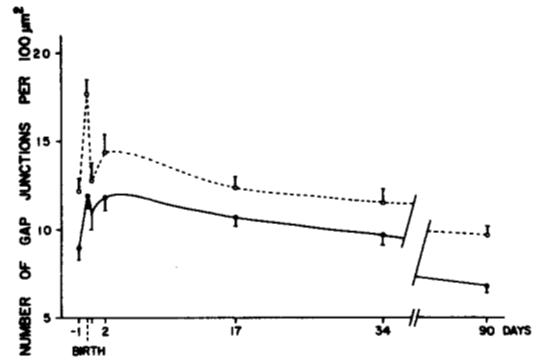

Fig. 3. Number of gap junctions per 100 μm^2 of plasma membrane (P-face) plotted as a function of age. ○ - - - ○, individual gap junctions; ● —— ●, gap junctional plaques. Means ± SEMs are represented.

tion of age in **Fig. 5:** individual gap junctions and gap junctional plaque areas show a three-fold increase between 2 hr and 2 days, and fall nearly towards fetal values in 34- and 90-day-old animals.

Since gap junctions permit the exchange of small molecules between adjacent cells (31–33), it was suggested that they are the structures responsible for

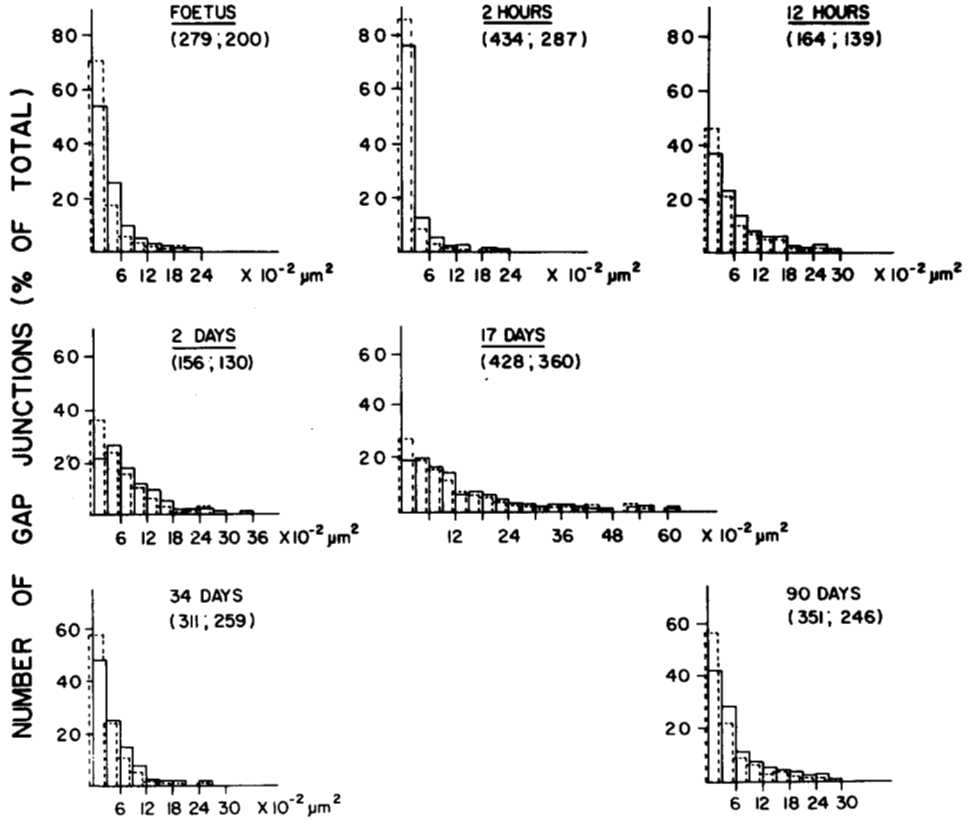

Fig. 4. Distribution of gap junction areas in plasma membrane P-faces at the different ages studied. Dotted columns, individual gap junctions; plain columns, gap junctional plaques. Numbers in parentheses represent the numbers of individual gap junctions and of gap junctional plaques studied.

TABLE 2. Mean gap junction area in brown adipocyte plasma membrane

Age	Individual Gap Junctions		Gap Junctional Plaques	
	P-face	E-face	P-face	E-face
	($\times 10^{-3}$ μm^2)			
Fetus (21 days)	28.7 ± 1.8	30.0 ± 4.5	40.1 ± 2.9	39.0 ± 6.6
	$P < 0.001$		$P < 0.001$	
2 Hours	19.8 ± 1.1		26.4 ± 1.6	
	$P < 0.001$		$P < 0.001$	
12 Hours	58.0 ± 4.6	76.8 ± 11.7	67.4 ± 5.8	82.1 ± 11.8
	N.S.		N.S.	
2 Days	63.6 ± 5.1	73.9 ± 10.4	77.7 ± 6.9	80.5 ± 12.5
	$P < 0.001$		$P < 0.001$	
17 Days	100.0 ± 4.9	99.4 ± 9.2	114.3 ± 6.0	111.4 ± 11.0
	$P < 0.001$		$P < 0.001$	
34 Days	34.5 ± 2.2		41.6 ± 2.8	
	$P < 0.05$		$P < 0.01$	
90 Days	42.4 ± 2.3	47.4 ± 5.8	57.3 ± 3.7	60.5 ± 8.5

The sample size is given in Fig. 4.

intercellular communication and, as such, that they play a role in the control of cell differentiation, growth, and function (31, 34–36). For this reason, we tried to correlate the variations of gap junctions observed in the different stages of BAT development with the functional activity of this tissue. A quantitative estimation of intercellular communication proposed by Sheridan (37) was used for this purpose. This author has shown that the ratio of gap junction area over the cell volume reflects the "capability for communication" of the cell studied. To calculate this ratio in our system, the diameter (and hence the volume) of adipocytes was measured in function of age (**Table 3**); the ratio of gap junctional area over the cell volume of the different ages is shown in **Fig. 6.** The pattern of the curve is similar to those describing mitochondrial development and respiration (15, 38, 39), and activities of cytosolic enzymes (40, 41) in the BAT. This suggests a correlation between the capability for communication and the degree of functional activity of the tissue. Although our data do not demonstrate the permeability of the gap junctions at the various ages studied, i.e., they do not show that a transfer of molecules occurs during BAT functional stages, they are at least consistent with the hypothesis

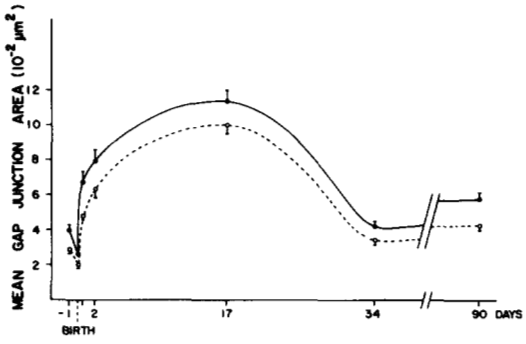

Fig. 5. Gap junction areas on plasma membrane P-faces plotted as a function of age. ○– – –○, individual gap junctions; ●——●, gap junctional plaques. Means ± SEMs are represented.

TABLE 3. Diameter of brown adipocytes corrected for sectioning artifact (18)

Age	Diameter (μm)
Fetus (21 days)	18.2 ± 0.2 (315)[a]
2 Hours	17.6 ± 0.2[b] (389)
12 Hours	22.2 ± 0.2 (357)
2 Days	24.6 ± 0.3 (534)
17 Days	28.6 ± 0.2 (369)
34 Days	35.4 ± 0.3 (396)
90 Days	36.2 ± 0.3 (384)

[a] Numbers in parentheses indicate sample size.
[b] The slight decrease of adipocyte diameter 2 hr post partum is explained by the depletion of triglyceride stores; the subsequent increase is accounted for by the augmentation of the number and volume of mitochondria and/or triglyceride droplets (15). Adipocyte size increase in adults is due to triglyceride accumulation (8).

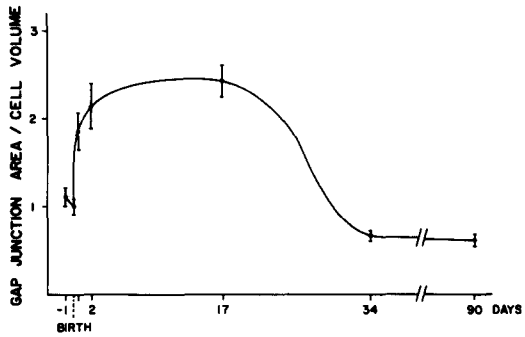

Fig. 6. Ratio of total gap junctional area (P-face) to adipocyte volume as a function of age. Means ± SEMs are normalized to the time point 2 hr after birth. $P < 0.001$ between 2 hr and 12 hr, between 17 days and 90 days, and between 21 fetal days and 90 days post natal.

that the transmission of an intracellular mediator of the stimulus for heat production, such as cAMP (10, 13, 42), might be modulated by permeable gap junctions.

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