

## Muscle cells, nerves, fibroblasts and vessels in the detrusor of the rat urinary bladder

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### Abstract

All the cells of rat detrusor muscle fall into one of five ultrastructural types: muscle cells, fibroblasts, axons and glia, and vascular cells (endothelial cells and pericytes). The tissue is ~79% cellular and 21% non-cellular. Muscle cells occupy 72%, nerves ~4% (1/3 axons, 2/3 glia), and fibroblast >3% of space. Muscle cells (up to 6  $\mu\text{m}$  across and ~600  $\mu\text{m}$  long, packed to almost 100,000 per  $\text{mm}^2$ ) have surface-to-volume ratio of 2.4  $\mu\text{m}^2/\mu\text{m}^3$  ~93% of cell volume is contractile apparatus, 3.1% mitochondria and 2.5% nucleus. Cell profiles are irregular but sectional area decreases regularly towards either end of the cell. Muscle cells are gathered into bundles (the mechanical units of detrusor), variable in length and size, but of constant width. The musculature is highly compact (without fascia or capsule) with smooth outer surfaces and extensive association and adhesion between its cells. Among many types of intercellular contact and junction, digitations are very common, each muscle cell issuing minute finger-like processes that abut on adjacent cells. Sealed apposition are wide areas of specialized contact, possibly forming a chamber between two muscle cells, distinct from the extracellular space at large (stromal space). The innervation is very dense, virtually all intramuscular axons being varicose (including afferent ones). There are identifiable neuro-muscular junctions on each muscle cell, often several junctions on a single cell. There are also unattached terminals. Fibroblasts (involved in the production of collagen), ~1% of the total number of cells, do not make specialized contacts.

**Key words:** bladder, smooth muscle, cell junctions, fibroblasts, innervation

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## Introduction

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The endogenous motility of the urinary bladder and the characteristic mechanical properties of the organ are founded on the structural features of the muscle coat (a smooth muscle labeled detrusor muscle) and the lamina propria (a substantial connective tissue coat, part of the mucosa). Early investigations by electron microscopy have documented the structure of this musculature, including similarities and dissimilarities with other visceral smooth muscles, in humans and other mammals (reviewed by Ref. 1). However, many recent studies have rather focused on cells that support signaling within the wall and on hypothetical elements generating spontaneous rhythmicity, matters of great functional significance (2, 3). Many aspects of the bladder musculature remain obscure; the working of the contractile apparatus, for example, or the links between muscle cells, or the working of neuro-muscular junctions, or the presence of myokines). Functional correlations are still rather tentative, and one cannot even start considering the fundamental questions about morphogenesis, that is, the developmental mechanisms (and learning processes) that create those structures, consistently and uniformly (or, in modern parlance, with high quality control). From the literature one has the impression that physiology and pharmacology of the bladder musculature have been studied to a better extent, and are better understood than the plain structure.

Here, certain aspects of the microscopic structure of the detrusor muscle are described and documented, by conventional transmission electron microscopy, in a single mammalian species, in adulthood and only in a standard condition of organ distension. There is no particular thesis to support or original hypothesis to bring forward, and there is no review of the literature (available elsewhere in adequate amounts). There may be interest in some insights and challenges, and in new morphological details. The use of electron microscopy on thin sections provides only limited views of the tissue; however, they can be strengthened by assembling photographic montages and by collecting serial sections, as is done here. In line with modern practice, some of the negative results are included; even if negatives are always difficult to prove, they may be of interest nonetheless. Various structural components are described individually, having I mind, however, the overarching

conclusion that the muscle is not just an assembly of different cells but is a tissue, where interaction of all its cells is of crucial significance.

An early version of this study was presented at the Japanese Society of Autonomic Neuroscience in Nagoya in August 2017 (4).

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## Material and Methods

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### *Fixation*

Adult albino rats were used (Sprague-Dawley strain, body weight 250–300 g, female). Male rats were also used, and showed no differences; those results were not included. The animals were killed with an overdose of anaesthetic (pentobarbitone 100 mg/kg i.m.). All the procedures involving material from animals complied with the UK Home Office Regulations under a Personal and a Project Licence. Immediately after death (no heart beat) the abdominal and thoracic cavities were opened on the midline. A bulb-tipped needle or a plastic tube was inserted into urethra and bladder; any urine present was washed out and replaced with a measured amount of Krebs' solution (ranging from 0 to 1.2 ml). Chemical fixative was then dripped over the bladder, and, with a needle, was injected at points around the urethra. The fluid inside the bladder was slowly replaced with fixative. Bladder and urethra were then dissected out and immersed in fresh fixative, at room temperature. In some experiments the procedure was preceded by trans-cardiac perfusion of Krebs' solution, to wash out the blood from the vessels. The bladder was dissected out and cut with a razor blade into strips and laminae as large as possible compatibly with the embedding procedure; position and orientation of each specimen was recorded, so that sections could be cut at chosen orientation and sites. The fixative was glutaraldehyde, buffered with 100 mM Na<sup>+</sup>-cacodylate at a concentration of 3% to 5%. The specimens were osmicated, block-stained with uranyl acetate and dehydrated in ethanol and epoxy-propane, before embedding in Araldite.

### *Histology*

Sections about 0.1 µm thick were cut with glass knives, collected on 200-mesh copper grids or on single-hole grids, and stained with uranyl acetate and lead citrate. They were examined and photographed in a transmission electron microscope. Specimens from bladders at various degree of distension were collected. The quantitative work in the microscope was carried out on bladders filled with 0.85 ml of fluid (this is regarded as a full physiologic distension).

### *Morphometry*

The morphometric work was carried out on micrographs (printed and often assembled into montages of 10–50 pictures), digitized with a flat bed scanner and analyzed with the software Micromedia FreeHand version 10 and version MX (both originally produced by Micromedia, but no longer supported by Adobe). This required the use of Apple desktop computers operating with an obsolete operating system (earlier than OX 10.6). The structures being investigated were 'traced' by hand on screen, in order to record and measure their number, outline, perimeter and area. The tracings were made by placing a linear sequence of clicks with the computer mouse along the contour of the objects of interest (muscle cell membrane, mitochondrial outer membrane, external border of nuclear envelope, cell membrane of axons, fibroblast and glial cell). The points around an individual object, joined to each other by straight segments, form a polygon; the software then replaces each segment with a Bezier curve for each point, and a smooth profile of the object thus emerges. All the micrographs used for this study and all the morphometry files are stored digitally and are available for

checking, re-evaluation and further analysis.

For quantitation, transverse sections were preferred because cell profiles are sharper and easier to measure than in longitudinal or oblique sections.

Montages were a favoured approach because they maintain a good resolution even if viewed at low magnification. At least five montages including more than 100 muscle cells each, were used for any of the parameters measured. Micrographs were taken at a magnification of 5,000–10,000 $\times$ , and the prints enlarged 2.5 $\times$ . For the finest features, high magnification micrographs (17,000–60,000 $\times$ ) were obtained, taking them before the low magnification ones, in order to keep the effects of radiation damage to a minimum.

In the morphometric perspective used, because the structure that are measured (cells, mitochondria etc.) have a tapering shape (without a flat bottom) the percentage values of sectional area (which are measured) are assumed to be equal to percentage values of volumes (calculated).

### *Freeze-fracture*

For freeze-fracture, bladder specimens at the end of the fixation in glutaraldehyde were immersed for 1 h in 15% glycerol, then for 1 h in 25% glycerol. The specimens were then mounted on supporting discs and frozen in liquid nitrogen. The specimens were then fractured at  $-105^{\circ}\text{C}$  and rotary shadowed with platinum-carbon at  $45^{\circ}$  followed by carbon at  $90^{\circ}$ . The specimen was brought back to room temperature and the tissue removed with Na hypochlorite, leaving behind a replica (of carbon and platinum) which was collected on a copper grid and examined in a transmission electron microscope.

### *Histochemistry*

For immunohistochemistry, the bladder was dissected out after perfusion with Krebs solution. It was slit open and pinned down, and progressively distended with pins on a Sylgard base. The mucosa was removed by manual dissection under a dissecting microscope and the musculature was immersed in a fixative made of 2% formaldehyde and 0.2% picric acid in PBS at  $4^{\circ}\text{C}$  for 1 h. After washes the specimen were incubated with primary antibodies (anti-synaptophysin raised in rabbit against synthetic human synaptophysin, from DAKO, Glostrup, Denmark; and anti-CGRP raised in rabbit against synthetic rat alpha calcitonin gene-related peptide, from Affinity, Exeter, UK), diluted 1:1,000, for 18–24 h, in a moist chamber at room temperature in the dark, on a mixing table.

The specimens were then incubated with biotin-conjugated donkey anti-rabbit immunoglobulins (Amersham, Little Chalfont, UK) diluted 1:250 for 1 h at room temperature.

Then, after a 45 min wash in PBS, the specimens were incubated with streptavidin-fluorescein reagent (Amersham) diluted 1:100 for 1 h at room temperature, mounted in Citifluor (Canterbury, Kent) and examined in a fluorescence microscope as whole-mount preparations.

Other bladder specimens were fixed in 2% formaldehyde and 0.2% picric acid in PBS at  $4^{\circ}\text{C}$  for 1 h, then frozen in Freon cooled in liquid nitrogen; frozen section of 10–15  $\mu\text{m}$  were cut and processed for histochemical staining as above.

### *Nomenclature*

In histologic sections, cells are never seen in their entirety. The term ‘cell profiles’ is more correct than ‘cell’, which is used only when there is no ambiguity.

Strictly speaking, ‘nerve fibre’ is used to refer to a single axon and its glial sheath and basal lamina, and, again, what one sees in the microscope is an ‘axonal profile’.

Cell proximity, cell contact, cell junction are used as explained in the Results, to refer to degrees of as-

sociation between adjacent cells.

‘Collagen fibre’ is used to refer to a large fibrous structure of collagen (a chemical element) visible by light microscopy, and formed by the assembly of vast numbers of ‘collagen fibrils’.

‘Chondrioma’ is the collective term used for the ensemble of all mitochondria in a cell.

‘Areas’, ‘regions’ and ‘domains’ of the cell membrane (or ‘plasmalemma’) are used interchangeably.

The rendering of visual features into words is often an intriguing task, except for the cases when certain details can be quantitated and averaged. In this paper and only within its restricted context, the following rather arbitrary terms of reference are employed.

All / Invariably: >99%

A great majority: ~90%

A majority / Mostly: ~80%

Many / Often: ~70%

More than half / Frequently: ~60%

About half: ~50%

Commonly: ~40%

Sometimes / A few: 30%

Occasionally / Infrequently: 20%

Rarely: ~10%

Very rarely: <1%

Never: 0%

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## Results

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### *Light microscopy*

In the conditions of distension examined here (0.85 ml of fluid into the lumen) the bladder wall measures ~160  $\mu\text{m}$  in thickness, of which the detrusor muscle takes up about half (Fig. 1A–F). The shape of the rat bladder is characteristic, and, unlike the size, it does not change with the amount of fluid inside. The width of

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#### **Fig. 1.**

A. Transverse section through the full thickness of the bladder wall, unstained, examined by phase contrast microscopy (photographic montage). The lumen is at the top, lined by the urothelium. The darkest parts of the wall are musculature. The large blood vessels are distended by the vascular perfusion. Width of field of view: 460  $\mu\text{m}$ .

B. Sketch of the rat urinary bladder seen from the front (right) and from the right side of the body, with the dimensions of its inner axes.

C. Transverse section of the detrusor muscle, by light microscopy. Dark is muscle cells, light is extracellular space Width of field of view: 300 $\mu\text{m}$ .

D. Detail of C. At higher magnification the muscle cells of the detrusor begin to show some of their cytologic details, such as nuclei, mitochondria (black dots, barely visible). Width of field of view: 55  $\mu\text{m}$ .

E. Light microscopy of bladder wall in full thickness. The urothelium is at top with superficial, umbrella cells (none showing the nucleus), and epithelial cells beneath, many with a large round nucleus. Underneath, in the lamina propria are fibroblasts and blood vessels (not perfused, and containing some red blood cells). The musculature of the detrusor is partly in longitudinal section (showing muscle cell nuclei cut lengthwise), partly in oblique section. Some fibroblasts are visible in the adventitia at bottom. Width of field of view: 85  $\mu\text{m}$ .

F. Whole-mount preparation of the detrusor muscle stained for acetylcholinesterase (used here as a general enhancer of the optical density of parts of the tissue) showing bands of musculature (muscle bundles) running in all directions. The dark lines, from the left edge and the bottom right corner are heavily stained nerves. Width of field of view: 1 mm.

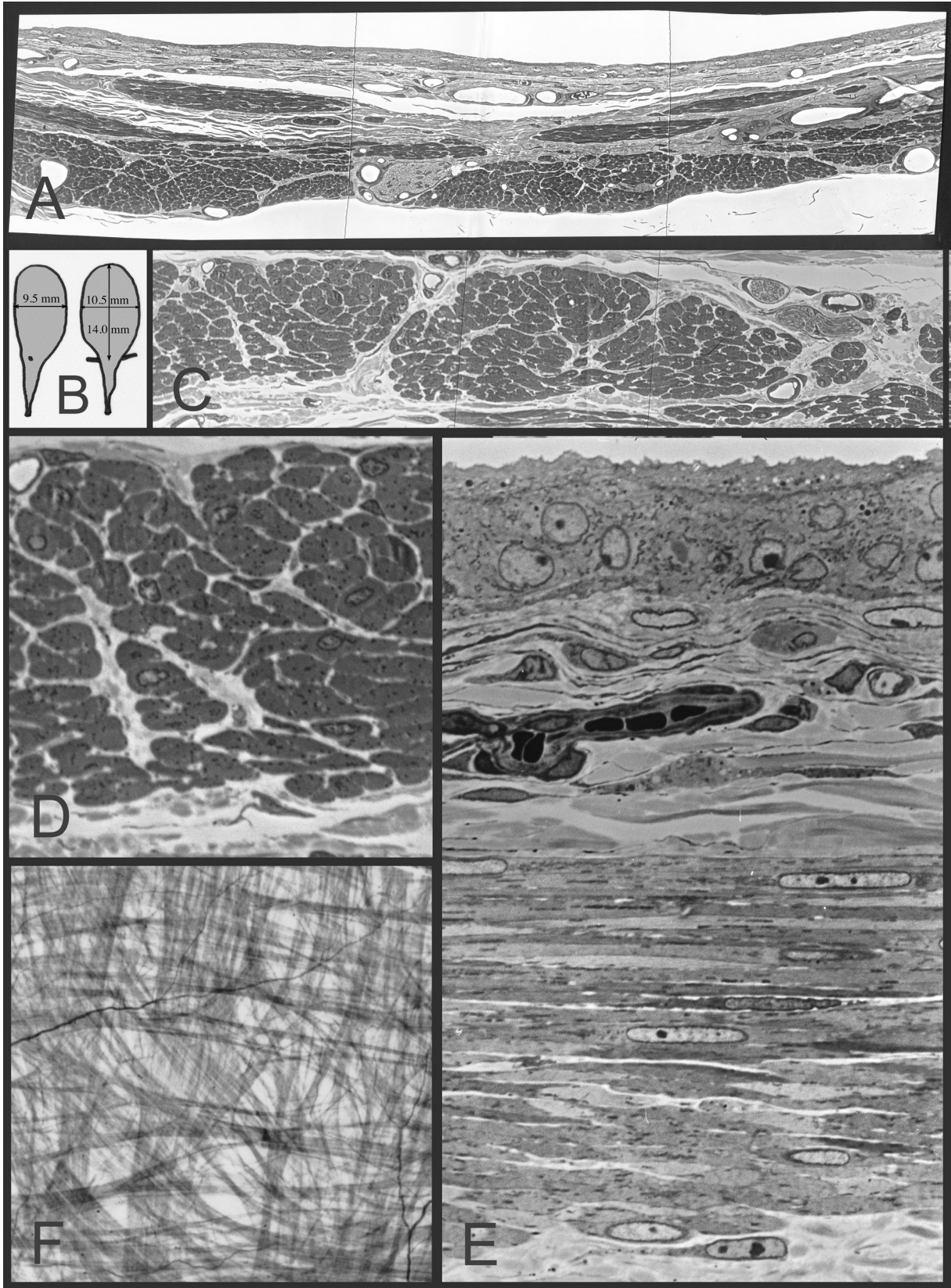


Fig. 1.

the organ in full physiologic distension is larger on the lateral axis (10.5 mm) than on the dorso-ventral axis (9.5 mm), and the cranio-caudal axis is the largest (14 mm, excluding the neck). The ventral wall is slightly more curved than the dorsal wall (Fig. 1B). The muscle (detrusor muscle) is continuous around the bladder, leaving openings for ureters and urethra, and is traversed by large vessels and nerves that reach the mucosa, predominantly in the lower (caudal) third of the organ (Fig. 1B).

The muscle is made of bundles, straight or slightly curved, variable in size (width) and of various orientations, extensively connected with each other (Fig. 1F). In some regions the bundles run predominantly parallel to each other (ventral wall), in other regions they run mainly at right angle to each other (posterior wall) or without a predominant orientation (sides and cranial pole). They always lie parallel to the bladder surface. The bundles are roughly circular in profile or somewhat flattened as if by radial compression within the wall. Each bundle is relatively uniform in size along its length, that is, it is cylindrical in shape, then it frays out or it merges with other bundles at both ends.

### Overview of ultrastructure

At the electron microscope, the first impression is that the detrusor is compact, dense, fully occupied by structures, hence lacking any empty spaces. It appears quite solid, even when we know that it is highly deformable, both actively and passively (Fig. 2A–D). There is no binding structure around a muscle bundle, such as a capsule or a fascia (Fig. 1C and D).

An immediate visual distinction in the micrographs is between elements that are cellular, that is part of cells, and all the rest, which is non-cellular. The non-cellular component, referred to as the stroma, is modest in extent, spread thinly between the cellular elements, and by definition it occupies the intercellular or extracellular space. The cellular component is predominant in extent and it is a straightforward task to identify the cells present. Smooth muscle cells, fibroblasts, glial cells, axons and vascular cells are readily recognized, and there are no cells falling outside these five groups (Fig. 2A–D). The muscle cells are by far the largest component, but they lie within a mixed population, highlighting the fact that a smooth muscle is an association of several cell types, and suggesting much interaction between its cells.

### Amount of cellular elements

To document the cell classification and to carry out counts and measurements, micrographs, assembled in large montages, are scanned and analyzed on screen. The membrane of all cellular elements is traced for measuring perimeter and area, and is colour coded for display (Fig. 2D). One of the largest of the montages obtained (Fig. 3A–F), covering an area of 2,075  $\mu\text{m}^2$ , comprises 202 full muscle cell profiles and 130 axons; there

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#### **Fig. 2.**

A. Low power electron micrograph of a muscle bundle of the detrusor sectioned transversely (part of a montage), giving an overview of the tissue. Muscle cells (muscle cell profiles), axons, glial cells and a fibroblast are visible. Width of field of view: 43  $\mu\text{m}$ .

B. Detail of A. showing some muscle cells (all rich in mitochondria), two of which are joined by a finger-like process. A small axonal bundle and a single axon (with two small mitochondria) make a junction with a muscle cell. Between the cells there are abundant collagen fibrils mainly cut transversely. Width of field of view: 8.2  $\mu\text{m}$ .

C. Another detail of A. showing six axons amongst the muscle cells. Width of field of view: 8.2  $\mu\text{m}$ .

D. A coloured tracing of the same area as in A, superimposed on it. The envelope of nuclei and the cell membrane of muscle cells (light grey profiles), fibroblasts (brown), axons (red), glial cells (yellow) and vascular cells are traced with a back line. All the elements can be identified in the micrograph in A. The structure marked with a pointer (bottom left of centre) is not included in this analysis.

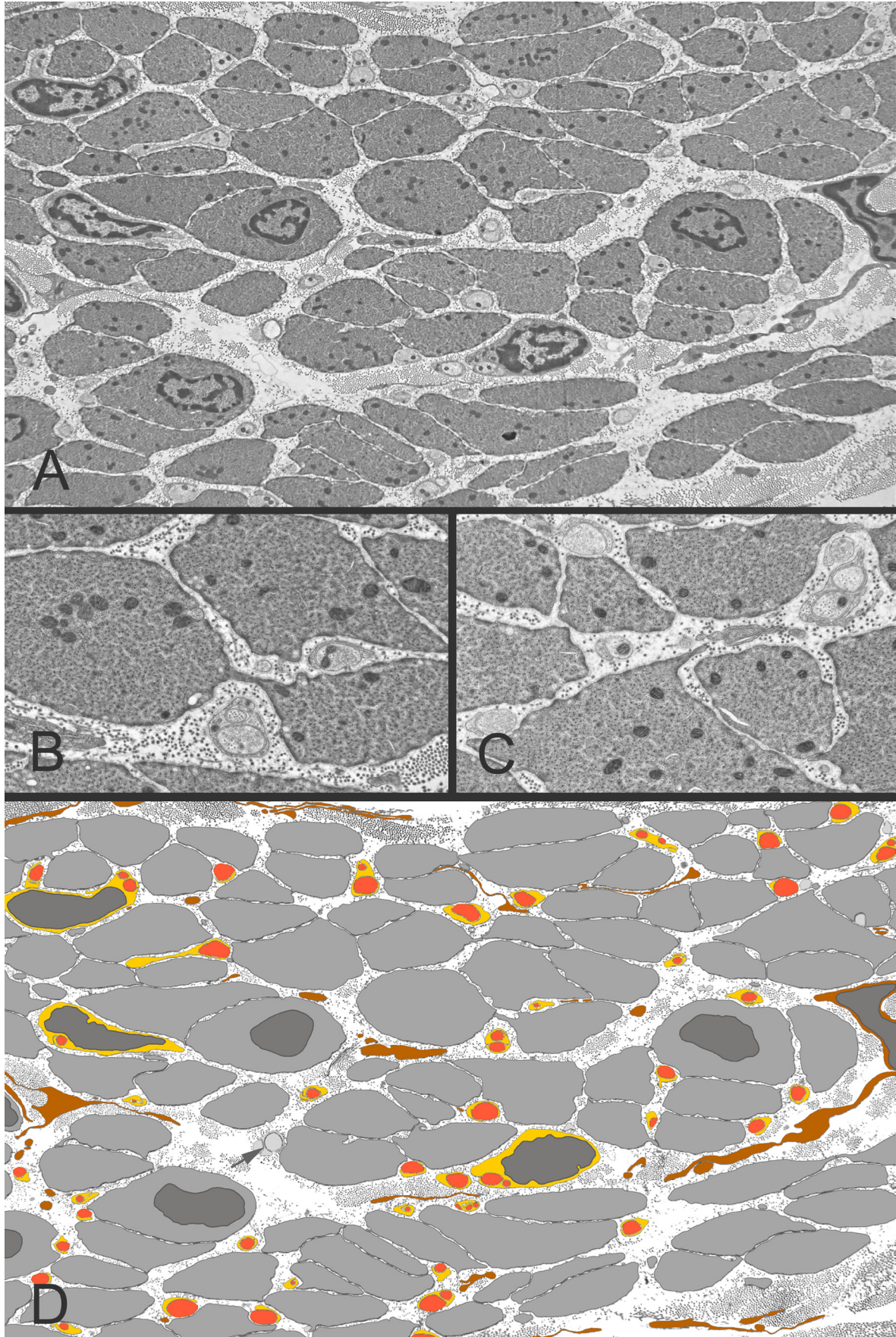
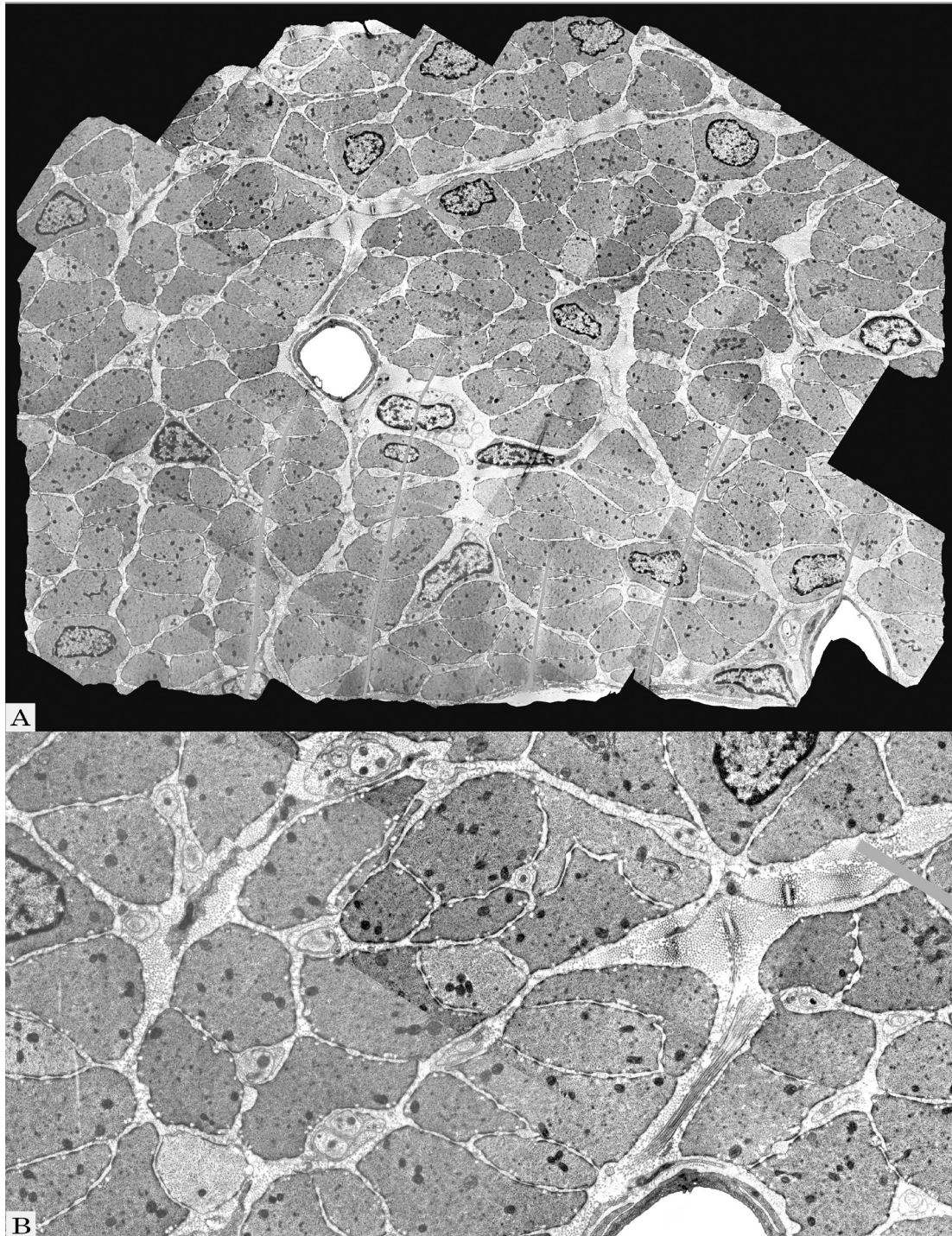


Fig. 2.





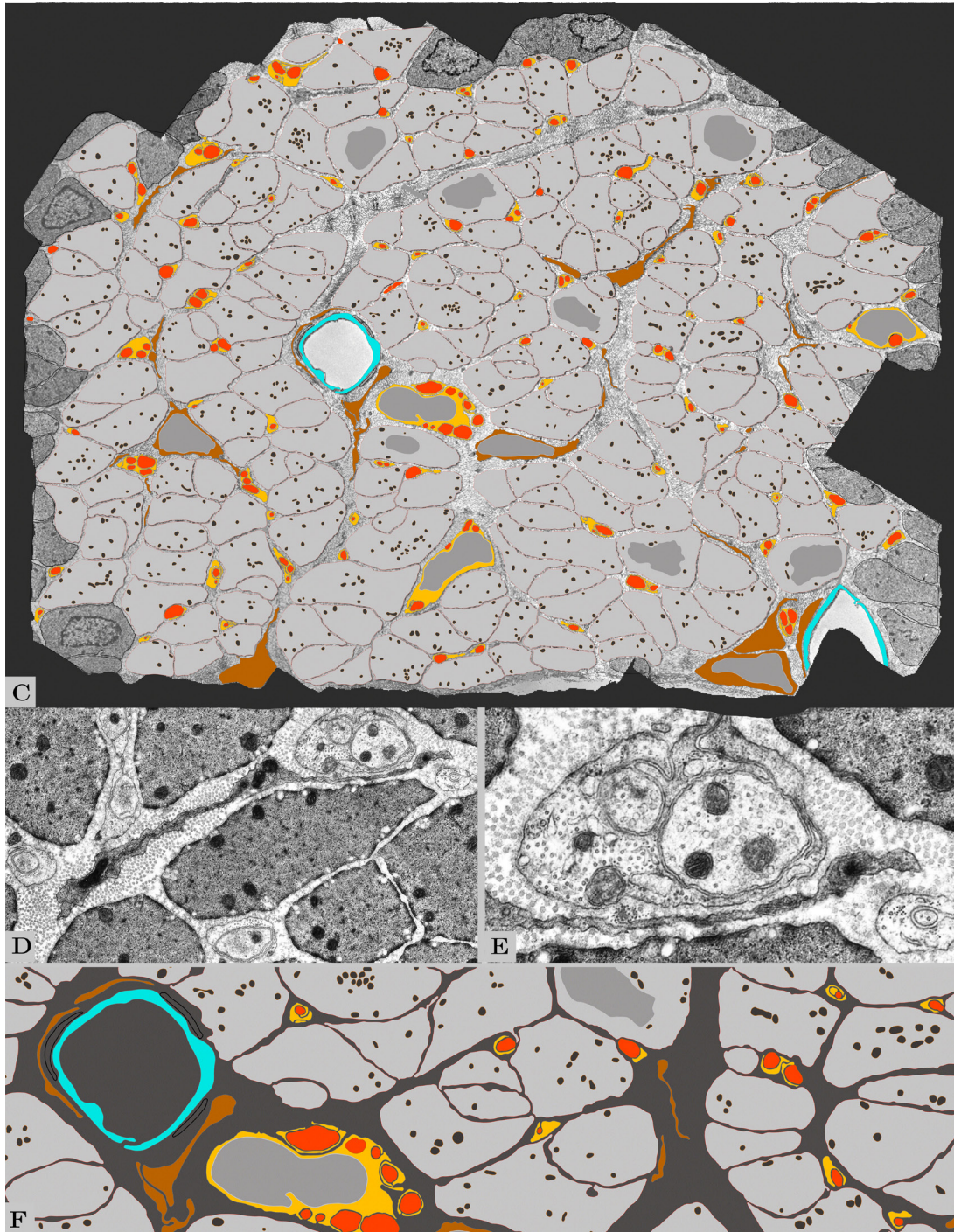
**Fig. 3.**

A. Electron micrograph (an assembled montage of about 30 micrographs) of part of a muscle bundle of the detrusor transversely sectioned. Width of field of view: 60  $\mu\text{m}$ .

B. Detail of an area by the top left corner of the montage, with a field  $\sim 30 \mu\text{m}$  wide. Even at low magnification many structural details of muscle cells, axons and glia, fibroblasts and blood vessels are in evidence. They are traced and colour-coded in C.

C. Scanned, traced and coloured version of A.

All the nuclear envelopes, muscle cell membranes, mitochondria of muscle cells, cell membrane of axons and glia, cell membrane of fibroblasts, endothelial cells and pericytes were traced manually on screen, and then analyzed morphometrically. The area covered measures  $\sim 2,100 \mu\text{m}^2$ . All the nuclei are in dark grey, the muscle cell profiles are in light grey (and their mitochondria in black), the axons (all 130 of them) are in red, the glial cells are in yellow, the fibroblasts are in brown, and the endothelial cells are in blue. The partial muscle cell profiles at the edge of the montage are not coloured in.



**Fig. 3.**

D. Detail from an area by the top left corner of the montage. Amongst the muscle cells there are 7 axons, all sheathed by some glial cell process, and a long, slim, irregular process of a fibroblast. Width of field of view: 10  $\mu\text{m}$ .

E. A detail from the same area (field width: 35  $\mu\text{m}$ ) shows a small nerve bundle with two axons and their complete glial sheath. Axonal vesicles, mitochondria and microtubules can be seen in the axons. In the glial process, typically, there is a large number of microtubules all in sharp cross section. By the bottom right corner is a very small axon, an intervaricose segment, a little over a tenth of a micrometer in diameter, occupied by a microtubule and a filament and entirely sheathed by a glial process. The intercellular space is full of collagen fibrils.

F. A detail from the centre of the tracing of the montage, with the cellular elements colour coded; all the non-cellular spaces are filled-in in black. The black dots in the muscle cell profiles are mitochondria (the mitochondria of axons, glia, fibroblasts and vascular cells are not shown).

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are also 2 capillaries (not included in the area measurement). Of the total sectional area  $1,486 \mu\text{m}^2$  or 72% is occupied by muscle cells (including the partial profiles at the edges of the montage). Axons occupy 1.3% of the total area, while glia and axons together occupy 4% of it. Fibroblasts (3 nucleated cell bodies and innumerable lamellar processes) occupy 2.8% of the sectional area. All the cellular areas together in this montage amount to  $1,638 \mu\text{m}^2$  or about 79% of the total area, the remaining 21% being non cellular space.

### *Smooth muscle cells*

The muscle cell transverse profiles range widely in size, up to 5–6  $\mu\text{m}$  in width and down to less than 1  $\mu\text{m}$ , in line with the fusiform shape of these cells. In the experimental conditions used an area of transversely sectioned muscle should contain more than 90,000 muscle cell profiles (a very approximate figure, given the uncertainty about the inclusion in the count of the smallest cell profiles). The bulk of the cell is made of the contractile apparatus (cytoskeleton and myofilaments) (Figs. 2 and 4). By subtracting the space occupied by nuclei (about 2.5% overall in large populations of muscle cell profiles) and by mitochondria (3.1%, see section below on Mitochondria and Other Organelles) it is calculated that on average over 93% of the muscle cell is occupied by the contractile apparatus and cytoskeleton. The rest (nucleus, mitochondria, other organelles in small amounts) makes up less than 7% of the cell volume.

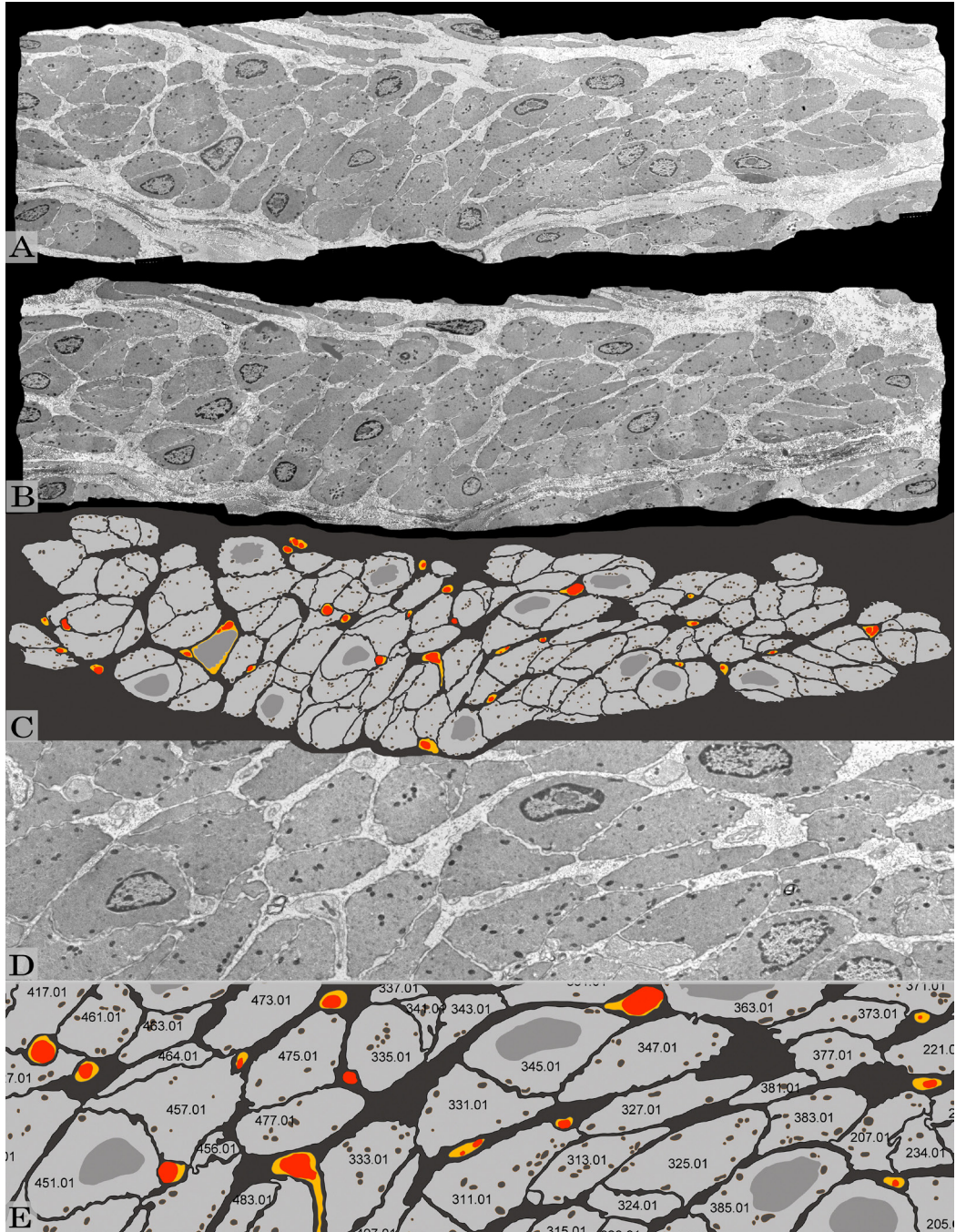
At low magnification there is a remarkable homogeneity in the appearance of these cells (not unlike the unstructured appearance of smooth muscle cells in the light microscope (Fig. 1D), except for nucleus and mitochondria (Fig. 2A–C). Even at high magnification, where the myofilaments and the scattered dense bodies become visible, their distribution does not suggest any clear pattern or compartmentalization (in contrast with the chequered appearance of striated muscles in transverse section); the distribution of myofilaments hardly varies from one region of the cell to another, across the cell's width or along its length (Fig. 4A–C). Only the smallest profiles (less than  $1 \mu\text{m}^2$  in area), rich in dense bands incrusting the cell membrane, are different in that they are devoid of mitochondria and contain no thick (myosin) filaments.

### *Muscle cell nucleus*

Between 3.5 and 3.9% of all the muscle cell profiles are nucleated (based on measurements on 5 montages comprising more than 100 cells each). The nucleated profiles are among the largest, although some of the largest profiles are not nucleated (Fig. 3C). The nuclear length, at the degree of bladder distension used here, is 20–23 micrometres (Fig. 1E). In transverse section the shape of the nucleus mirrors that of the cell; however, it is always smoother and closer to a round shape than the cell itself (Figs. 2A and 3A). In the majority of cases the nucleus is not quite at the centre of the cell profile. Binucleate muscle cells are very rare.

On account of the nucleus being 20–23  $\mu\text{m}$  long, and the nucleated profiles being 3.7% of the total, an average cell length of about 600  $\mu\text{m}$  can be estimated. This value makes the muscle cells about 100 times longer than they are wide at their widest.

Looking at micrographs that cover wide areas, the immediate impression given by the detrusor is of an assembly of cell profiles, irregular but roughly rounded up, and this is a misleading impression: the cell profiles appearing on microtomic sections hide the great length of these cells. The full shape of a muscle cell is rather that of a thin graphite pencil or a chopstick. Likewise, the visible axon profiles, a micrometre or less in diameter, are intersections of cable-like structures whose length can be measured in millimetres.



**Fig. 4.**

A. Electron micrograph of part of a detrusor muscle bundle sectioned transversely (photographic montage). The bundle was followed in serial sections over a distance of about 100  $\mu\text{m}$ , and this is level #1. Width of field of view: 120  $\mu\text{m}$ .

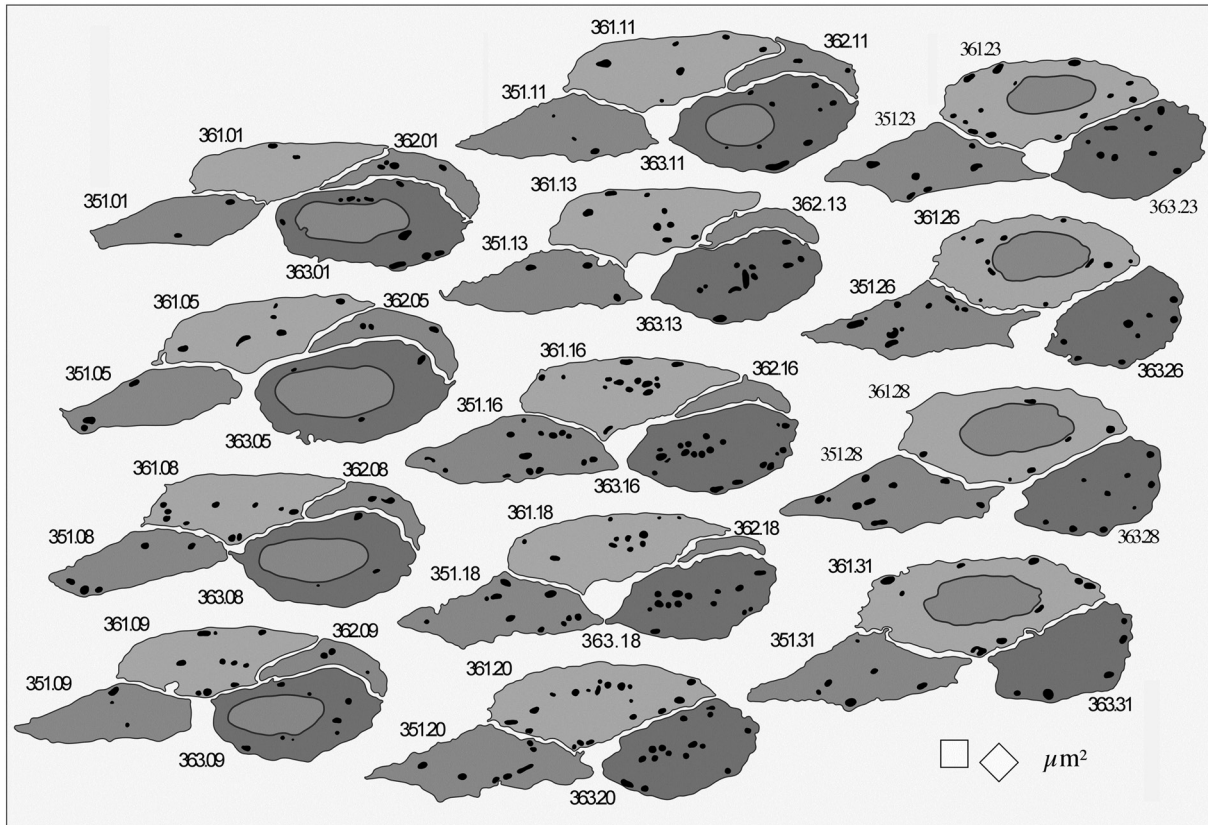
B. The same bundle at level #9, about 30  $\mu\text{m}$  along the series, documenting the extensive morphological changes of the cells along their length. Some fifteen nuclei in the montage at level #1 help to identify the same cells at level #9, although some disappear over that distance and some new ones appear. A comparison of the bundle at the beginning and at the end of the series gives the impression that they are two distinct bundles.

C. Tracing of the membrane of all the cells in the montage in A (muscle cell, axons, glia, nuclei and mitochondria of muscle cells). Colour-coding is the same used in Fig. 3F.

D. A higher magnification detail from the montage in A. Width of field of view: 60  $\mu\text{m}$ .

E. The line drawing is a tracing of the micrograph in D. (which is a detail of A.) and is a detail of C. Each muscle cell profiles has a numerical code: the first part is the number of the individual cell (constant through the series of sections); the second part is the level of the section (here is section level #1.).

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**Fig. 5.**

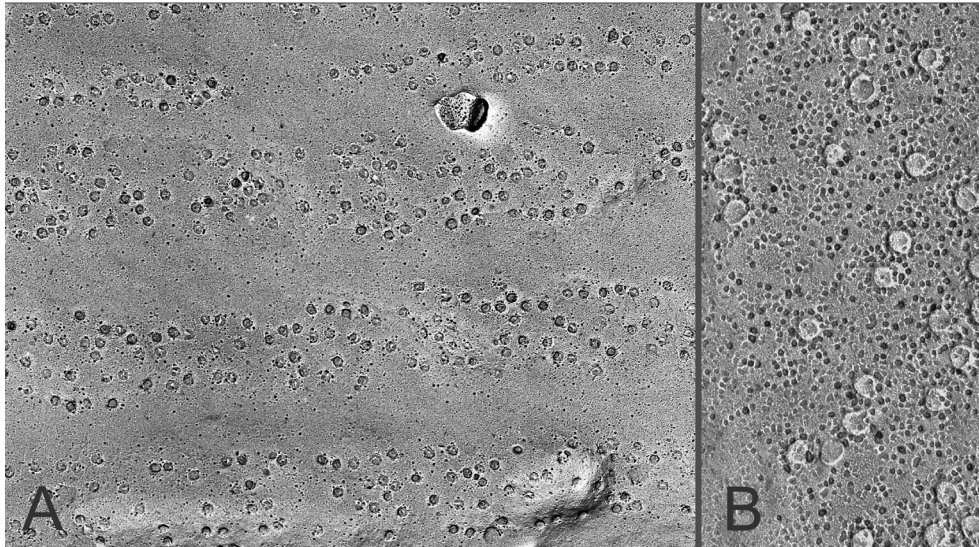
Five muscle cells of a muscle bundle are followed through in serial sections (from level #1 to level #31 displayed here in three sequential columns, left to right). Each cell has its individual number within the population, followed by a two-digit figures that indicates the level along the series. All mitochondria (small black dots) and nuclei are also traced. The series documents the considerable variation in shape of every muscle cell and the changes in their position and relationships. Calibrations: square and rhombus of 1  $\mu\text{m}^2$ .

### *Muscle cell shape*

By comparison with other visceral muscles, notably those of the intestine, the detrusor muscle cells, in their transverse profile, are more irregular in outline and their profiles more angular, less iso-diametric and far from an ovoid or circular shape. In this way they achieve high compactness and great reduction of the spaces between cells. Frequently some concave portion in a cell is moulded onto the convex portion of an adjacent profile, and some profiles partly encircle an adjacent one (Figs. 3B and 4A–C).

Very irregular muscle cells profiles are found in all preparations, and more irregular still they become in isotonic contraction (not examined here). In spite of what must be a highly structured cytoskeleton and contractile apparatus, the muscle cell appears highly deformable; it shortens and fattens with isotonic contraction, but the shape of its profile can also change extensively under the effect of external forces impinging on the cell. For this study, data on the three-dimensional aspect of the cells of the detrusor were obtained from serial sections covering sequentially several micrometres along the length of a muscle bundle (Fig. 4A–E).

From the analysis of serial sections, the shape of the muscle cells, quite variable in a single transverse section, appears markedly variable also along the length of each cell (Fig. 5). Round profiles can turn into flattened or polygonal ones within a few micrometres along the cell's length. Pairings between two muscle cells persist for some distance over the bundle length, but new pairings can be formed, a few times over a cell's length. Therefore, the impression given by longitudinal sections of the muscle that the cells are exactly parallel to each



**Fig. 6.**

A. Freeze-fracture preparation of a muscle cell of the detrusor (E-face). Rows of caveolae, exposed at the neck, lie along the length of the cell. A small cell process, probably penetrating into the cell from another cell, is visible near the top right corner. Width of field of view: 4.5  $\mu\text{m}$ .

B. At higher magnification this freeze-fracture preparation (rotary shadowed) shows the neck of caveolae, on the P-face of the cell membrane, with a characteristic array of intramembrane particles. Width of field of view: 0.6  $\mu\text{m}$ .

other is not borne out by the serial transverse sections. The area of the profiles of a muscle cell measured over serial sections does either increase, rather regularly in spite of the irregularity of the shape of the profile, or it does decrease (indicating whether the series moves towards, or away from, one of the ends of the cell); however, there is never a mixture of the two, that is, the profile is irregular but the solid shape is that of a cone (or rather two cones joined at the base).

Near the tapering ends, the cell's profile often becomes highly convoluted, with deep grooves and folds of the cell surface. While the sectional area decreases approaching the cell's end, the cell perimeter remains large because of these convolutions. In addition, inter-digitations with similar expansions in other muscle cells are particularly common in the terminal regions. At this level the membrane bears expanded dense bands, presumably with the effect of increasing the cell's bond with the stroma.

### ***Muscle cell surface***

The surface of the muscle cells, seen in outline, is predominantly convex, but it also presents concave regions (something unusual in the muscle cells of the gut), an arrangement that increases the compact fitting together of the cells within a bundle. Volume-to-surface ratio, based on measurement of over 600 muscle cell profiles in montages, is about 1: 2.4 (there is 2.4  $\mu\text{m}$  of cell perimeter per 1  $\mu\text{m}^2$  of cell profile area). Again, the same value of 1: 2.4 applies also, in the solid tissue, to the ratio of cell volume to cell surface. This value would amount to about 230  $\text{cm}^2$  of muscle cell membrane (muscular plasmalemma) per gram of tissue. Except for some points of intercellular contact (described below) the muscle cell surface is fully lined by a basal lamina (with fuzzy density and ill-defined outer border), sometimes shared between adjacent muscle cells, or between a muscle cell and an axon or a glial cell.

### ***Muscle cell membrane***

The cell membrane of the muscle cells is far from smooth and is rich in structural features (Figs. 6–8).

Firstly, the membrane is studded with the invaginations known as caveolae, very uniform in size and

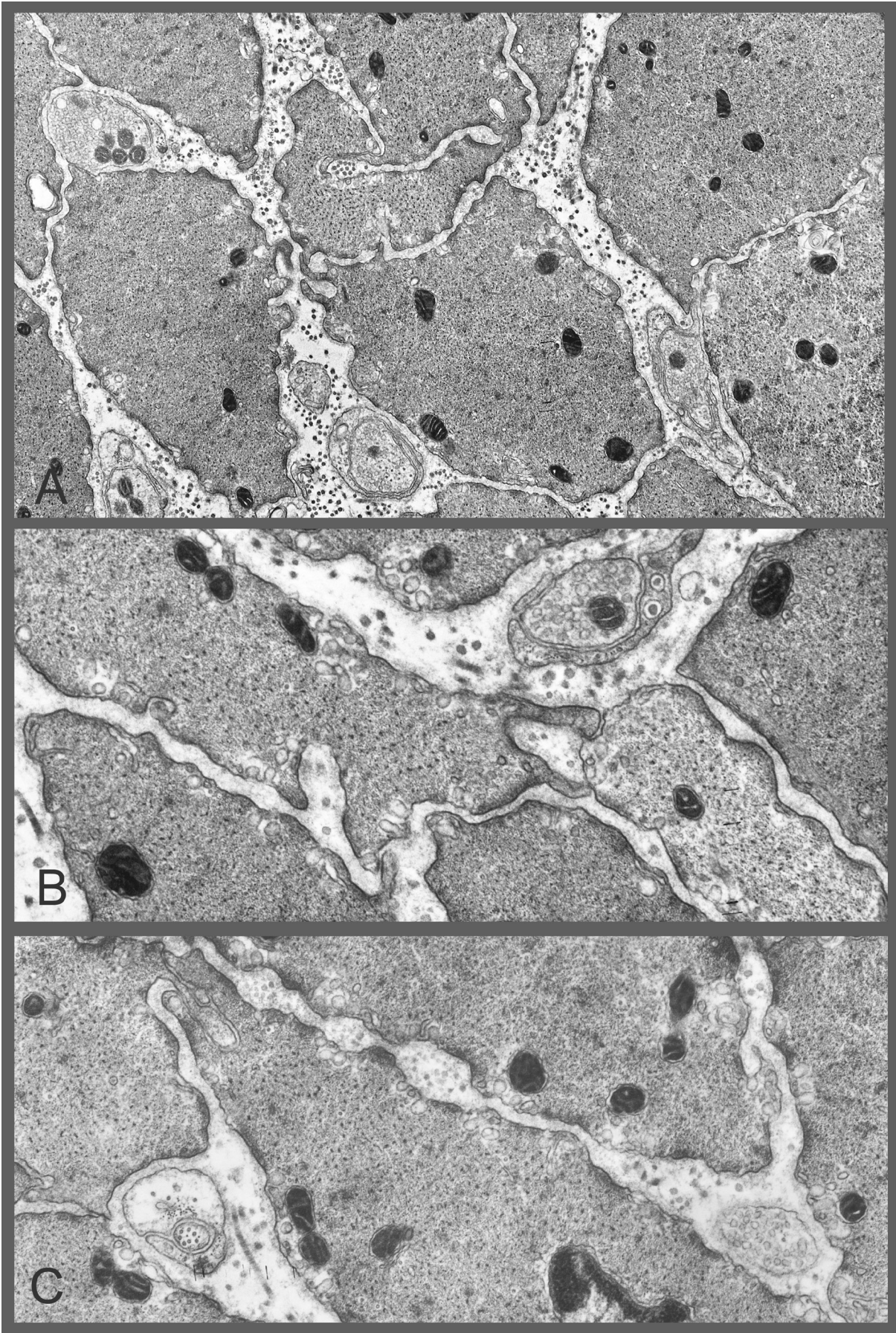


Fig. 7.

appearance, and common in all smooth muscles. The spatial density of caveolae, their assembly and their arrangement in longitudinal rows are better visible in freeze-fracture preparations than in sections (Fig. 6A and B). A ring of intramembrane particles surrounds the neck of every caveola (Fig. 6B). These caveolae do not differ in size and distribution from those of other muscles, including their occasional association with an underlying, concave, elongated cisterna of endoplasmic reticulum. Estimates of number of caveolae on thin sections have led to interesting claims about a fall in number in the rat detrusor of aged rats (5) and in mouse detrusor muscle after genetic manipulation (6).

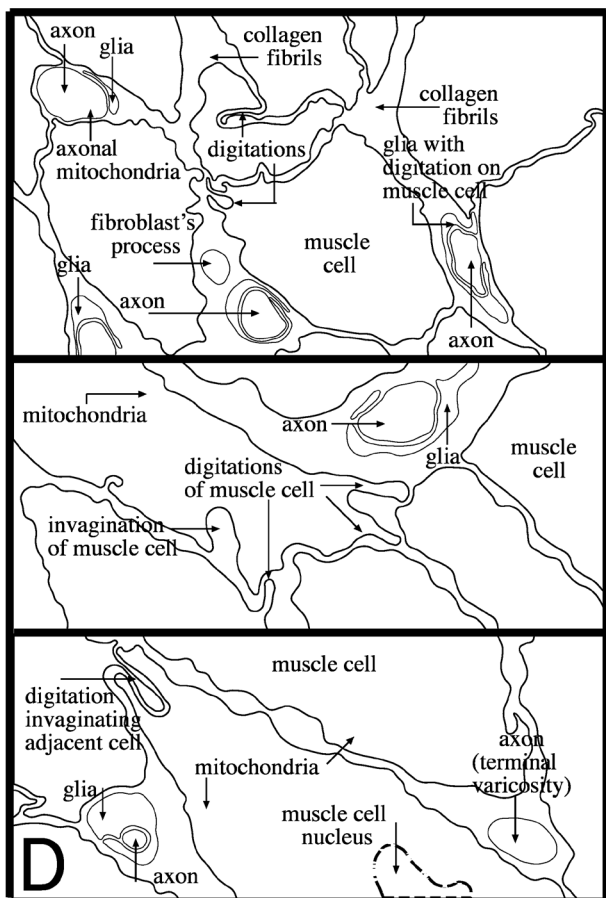
Secondly, there are small indentations, or small tubular invaginations, of the cell membrane, lined by the basal lamina and with an amorphous content or one or two fibrils (presumably) of collagen (Fig. 7B). They rarely exceed a tenth of a micrometer in length.

Thirdly, there are areas (or domains), in the form of patches or of bands, where the muscle cell membrane is incrustated with electron-dense material and often bears the attachment of actin filament cables. These areas are matched by a basal lamina of increased density and are often associated with collagen fibrils, an arrangement suggesting a cell-to-stroma bonds (Fig. 7C).

Fourthly, the cell membrane is involved in specialized junctions with other muscle cells, including adherens type junctions (see, Muscle cell junctions).

Fifthly, there are also some specialized junctions where a muscle cell comes in contact with cells of another type, notably axons (see, Axonal contacts and neuro-muscular junctions).

Sixthly, almost every muscle cell profile has some fine, short processes directed outwards, usually con-



**Fig. 7.**

A. Electron micrograph showing several muscle cell profiles, of irregular and diverse shapes. Abundant collagen fibrils occupy the extracellular space. Four varicose axons are visible, all associated with glial processes and two of them making a junction with muscle cells. Mitochondria (dark roundish structures) are found both in the muscle cells and in the axons. Width of field of view: 17  $\mu\text{m}$ .

B. The muscle cell profile at the centre has large digitations contacting three adjacent muscle cells, and a deep invagination that is lined by a basal lamina and is occupied by some collagen fibrils. Width of field of view: 5  $\mu\text{m}$ .

C. At top left a digitation from a muscle cell penetrates and expands into an adjacent muscle cell. At bottom left an intervaricose segment of an axon is fully wrapped by a glial process. Width of field of view: 7  $\mu\text{m}$ .

D. In these drawings all the cell membranes in the three micrographs are traced (highlighting the complexity of the cell outlines) and the key elements are labelled.



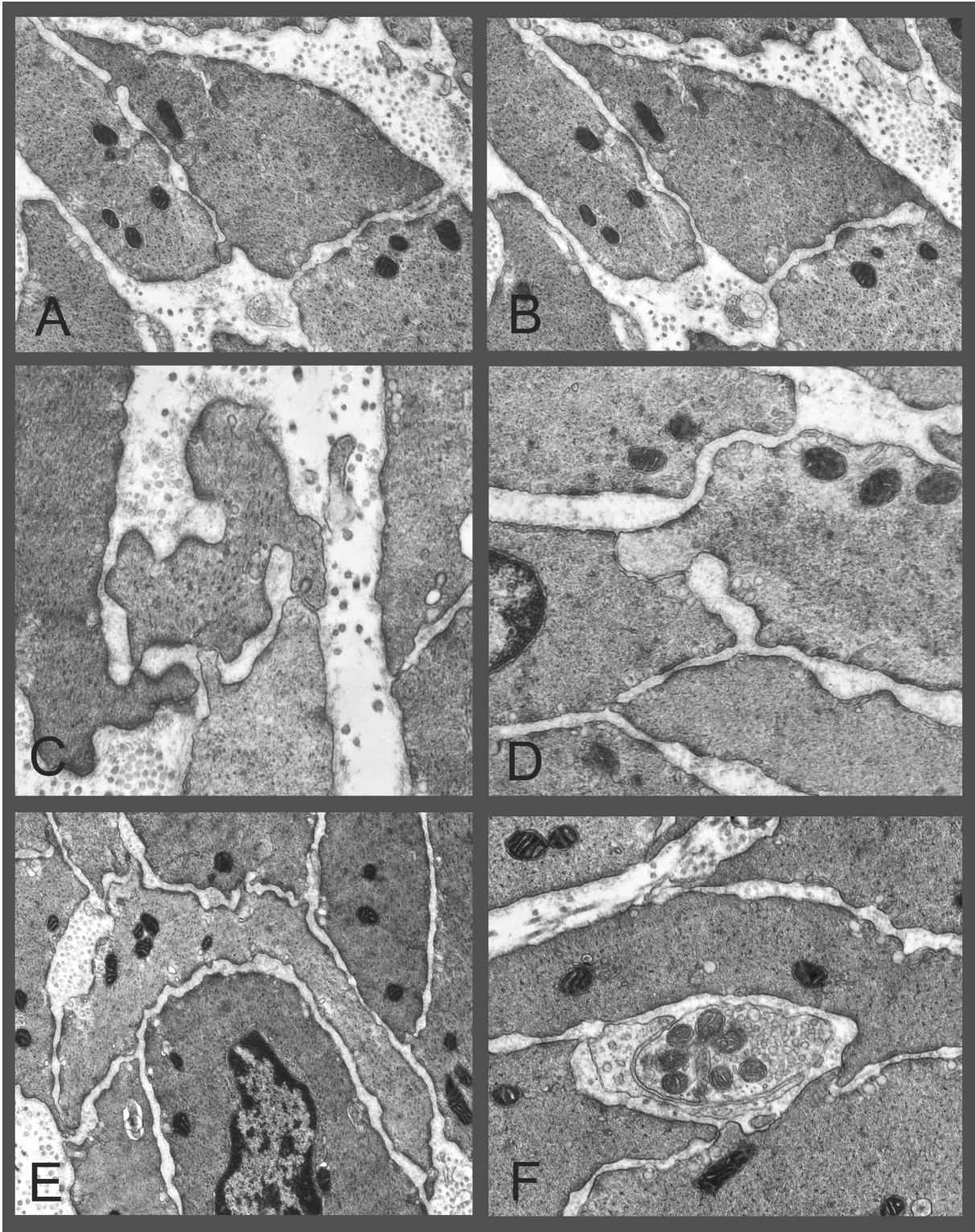


Fig. 8.

tacting another muscle cell (or, less commonly, an axon or a glial cell) (Fig. 7). These digitations of the muscle cell membrane are described below (see, Muscle cell digitations).

Seventhly, there are areas of extensive muscle cell contact, involving fusion or adhesion of basal laminae and exclusion of other extracellular materials, between adjacent muscle cells (Fig. 8). They seem to form a sealed region between two muscle cells. They are described below (see, Muscle cell sealed appositions).

Lastly, there are areas where the membrane of a muscle cell simply lies in the proximity of other structures (with no closeness and no indication of some link or some interaction): these are quite rare. Similarly, muscle cells at the edges of a bundle have a large area that is not related to other cellular structures, is always convex and in general keeps to a minimum the outer surface of the bundle.

### *Muscle cell digitations and invaginations*

Characteristically in the detrusor muscle cells, the cell membrane presents innumerable small projections or digitations (Figs. 2B and C, 7, 8A–C). The longest ones extend out of the cell profile or along the cell's length by about 1 micrometre, while the majority are only a fraction of a micrometer (most commonly about a fifth of a micrometer). Some are bulbous or mushroom shaped (Figs. 7B and 8D), but the majority are finger-shaped, tapering and issue transversely or at a slight oblique angle (the term 'digitation' is used to refer to all of them) (Figs. 7 and 8). Almost all of them make a membrane-to-membrane contact with another muscle cell, or, occasionally are joined by a similar process issued by the adjacent muscle cell. A small number of them, less than one in twenty, abut on an axon or, more rarely, on a glial cell (none were found abutting on a fibroblast). Some penetrate tightly for some distance in an invagination of an adjacent muscle cell (Fig. 7C). At the point of contact or interpenetration there is no basal lamina and the membrane-to-membrane separation is 10–12 nm, without any visible specialization. These projections, very common though they are, are so irregular in shape, size, position and frequency, that it is impossible at this stage to provide a morphometric evaluation. Their cytoplasmic content appears amorphous, without filaments and without densities attached to the membrane. The one feature that they all share is the interruption of the basal lamina in the small area where a process contacts another cell. Again, the shape and distribution of these processes is better understood in the serial sections, which document their high frequency, their appearing and disappearing within a few sections (Fig. 8A and B), and their remarkably heterogeneous shape. The serial sections also show that only very few of these processes are unattached to another cell, even when this is the appearance in a single transverse section.

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#### **Fig. 8.**

A. & B. Electron micrographs of two sections of the detrusor less than half a micrometer apart (the same mitochondria appear in both pictures). In A. the muscle cell at the centre (with a triangular profile) has two digitations abutting on the cell to the left. The digitations are no longer present in B., where there is another digitation onto the cell at bottom right. Width of field of view: 7.5  $\mu\text{m}$ .

C. The muscle cell profile at the centre is cut at a level close to the end of the cell and is highly irregular in outline; it has contacts with two adjacent muscle cells and extensive membrane-bound dense bands linking it to the stroma. Width of field of view: 3  $\mu\text{m}$ .

D. In the centre a large process from a muscle cell at right abuts on an adjacent muscle cell (with nucleus) forming an extensive membrane-to-membrane contact with an intercellular gap of 10–12 nm. The process has an amorphous content, without filaments or organelles. Width of field of view: 4  $\mu\text{m}$ .

E. Electron micrograph showing one of the many muscle cells with irregular, flattened shape and corrugated surface. Width of field of view: 7  $\mu\text{m}$ .

F. An axon sectioned at the level of a varicosity, packed with mitochondria and axonal vesicles and partly wrapped by a glial process (at bottom left). The axon is situated in a confined space between three muscle cells and forms what is regarded as a neuro-muscular junction with the muscle cell at top. Width of field of view: 4  $\mu\text{m}$ .

### *Muscle cell sealed appositions*

There is another form of contact between muscle cells that is consistently and widely present, and looks structurally almost the opposite of the minute discrete contacts provided by the digitations described in the previous section. This form of contact involves large areas, up to a micrometres wide around the cell profile and several micrometres along the cell length, where the two membranes lie parallel to each other and there is a gap (Fig. 7A–C), fairly regular, of 50–200 nanometer in width. The intercellular gap is occupied by amorphous material of the same electron density as the basal lamina; collagen fibrils are usually excluded from it or present in very small number.

### *Muscle cell junctions*

Contacts between muscle cells involving visible membrane specializations are referred to as cell junctions. Dense bands under the cell membrane, bearing insertion of bundles of actin filaments, are matched by a similar structure in the adjacent cells. These are adherens type junctions, with membrane-to-membrane gap of 30–50 nanometer occupied by electron dense material. They are abundant, but also rather variable in appearance, and they are not always associated with actin filaments. They can be point-like or elongated and extending a micron or two along the cell length, with rows of caveolae on either side. Adherens junctions become more abundant in the proximity of the cell's ends, where adjacent muscle cells appear even more closely associated (Fig. 8C).

Gap junctions (abundant and readily recognized in some other smooth muscles) are not observed in the present preparations. A negative result is obtained also in freeze-fracture preparations (which give unequivocal images of gap junctions).

### *Muscle cell contacts and junctions with other cell types*

Adherens junctions are symmetrical and are found only between muscle cells. In contrast, digitations from muscle cells are occasionally seen abutting not onto another muscle cell but on a glial cell or an axon. At their level the basal laminae of the two cells are interrupted, and the membrane-to-membrane separation is 10–12 nm. In addition, muscle cells have specialized contacts with the axons (see Neuro-muscular junctions).

### *Mitochondria and other organelles in muscle cells*

Conspicuous elements in the muscle cells are mitochondria, rather electron-dense and visible also at low magnification (Figs. 2A–C, 3A, B and F, 7). They are even faintly by light microscopy (Fig. 1D).

In the montages prepared for morphometry, the mitochondria were traced digitally and their number, size (sectional area) and overall volume obtained. In muscle cells mitochondria occupy 3.13% of the sectional area of muscle cells (2,155 mitochondria in 419 muscle cell profiles from 3 animals; the average in each animal is 3.24%, 3.04% and 3.16%), in line with the results already published (7). Mitochondrial spatial density varies greatly between individual cell profiles, and some profiles have no mitochondria. Mitochondria are not observed in muscle cell profiles smaller than 1  $\mu\text{m}^2$ . However, when followed in serial sections for some of their length all cell profiles have a mitochondrial density close to the average for the entire muscle, and there is no indication of variation between cells (Fig. 5). Also, there is no variation between different sectional levels of a muscle bundle (an observation that simply confirms the validity of the morphometric method used). Mitochondria are roughly spherical or more commonly elongated along the cell's length, frequently arranged in columns. The largest group of mitochondria is in the conical cytoplasmic domains near the poles of the nucleus.

Other organelles are poorly represented in these muscle cells: endoplasmic reticulum, lysosomes, ribo-

some, microtubules. Microtubules are in small number but they are found in all muscle cell profiles except the smallest ones. The endoplasmic reticulum (or sarcoplasmic reticulum) appears less abundant than in other visceral muscles, such as those of the gut.

### Nerves

The innervation of the detrusor is particularly abundant, consisting of tightly coupled axons and glial cells. Nerves (or nerve trunks), originating bilaterally from each pelvic ganglion penetrated the wall around the area of insertion of the ureters. They branch repeatedly (usually dichotomically) and issue side branches, reaching every region of the bladder; they are always wrapped by a perineurium that isolates the nerve fibres completely from the other bladder tissues. When subdivisions reduce a nerve to bundles of 10–12 axons, their perineurium ends abruptly and the fibres become located directly in the extracellular space of the tissues; these structures are referred to as axon bundles (8). The intramuscular innervation is provided by small axon bundles, of 2–6 axons and their glia, and by individual axons, deriving by subdivision of the axon bundles. In a transverse section of the detrusor the individual axons are more than a third of the entire axonal population. Except for a small number of terminal varicosities, the axons are all associated with glial cells.

The density of innervation of the detrusor can be estimated by visualizing, colour-coding and counting all the axons in large montages. In the montage of Fig. 3 the 200 muscle cells are accompanied by 130 axons (axon profiles), apparently with a uniform distribution. This value would amount to more than 50,000 axons per square millimetre of muscle sectional area.

In volumetric terms, more than 1/20 of the cellular component of the muscle is nerve tissue and of this one third is axons and two thirds glia.

In the representative montage of Figure 3, the summation of all the axonal membranes sectioned is about 53  $\mu\text{m}$ , and that of the glia is about 153  $\mu\text{m}$ . By comparison, in the same area the muscle cell membranes together measure 541  $\mu\text{m}$ , that is less than three times the membranes of axon and glia. Indicative values (given the difficulty of precise measurement of the perimeter of cell profiles, especially the smallest ones) show that of all the cell membranes in the detrusor, about 7% is axonal membrane and about 70% is muscle membrane; nearly 20% is glial.

### *Histochemical data on nerves*

In frozen sections, the detrusor muscle immune-stained for synaptophysin (an axonal vesicle marker) appears occupied by innumerable fluorescent spots, each representing an axon (Fig. 9A). On these preparations large areas of the detrusor can be surveyed. The density of this innervation is uniform through the thickness of the muscle and in all regions of the bladder. Muscle bundles are innervated uniformly through their length.

Whole-mount preparations (the detrusor of almost an entire bladder lying flat under the microscope) confirm the richness of the innervation and allows individual axons to be examined in their full length (although the stain is limited to the vesicle-containing varicosities) (Fig. 9B–D). The varicose or beaded pattern is the paramount feature, complementing the data from electron microscopy (see Axons).

In preparations stained for CGRP (revealing afferent axons, known to originate from dorsal root ganglion neurons) the detrusor muscle shows some positive fibres, indicating the presence of an afferent innervation (sensory) (Fig. 9E–G). These fibres are varicose, consisting of chains of twenty or more varicosities. These varicosities are irregular in frequency, size and separation to an even greater extent than those of efferent axons (motor), as has been described for the afferent fibres in the bladder mucosa (9).

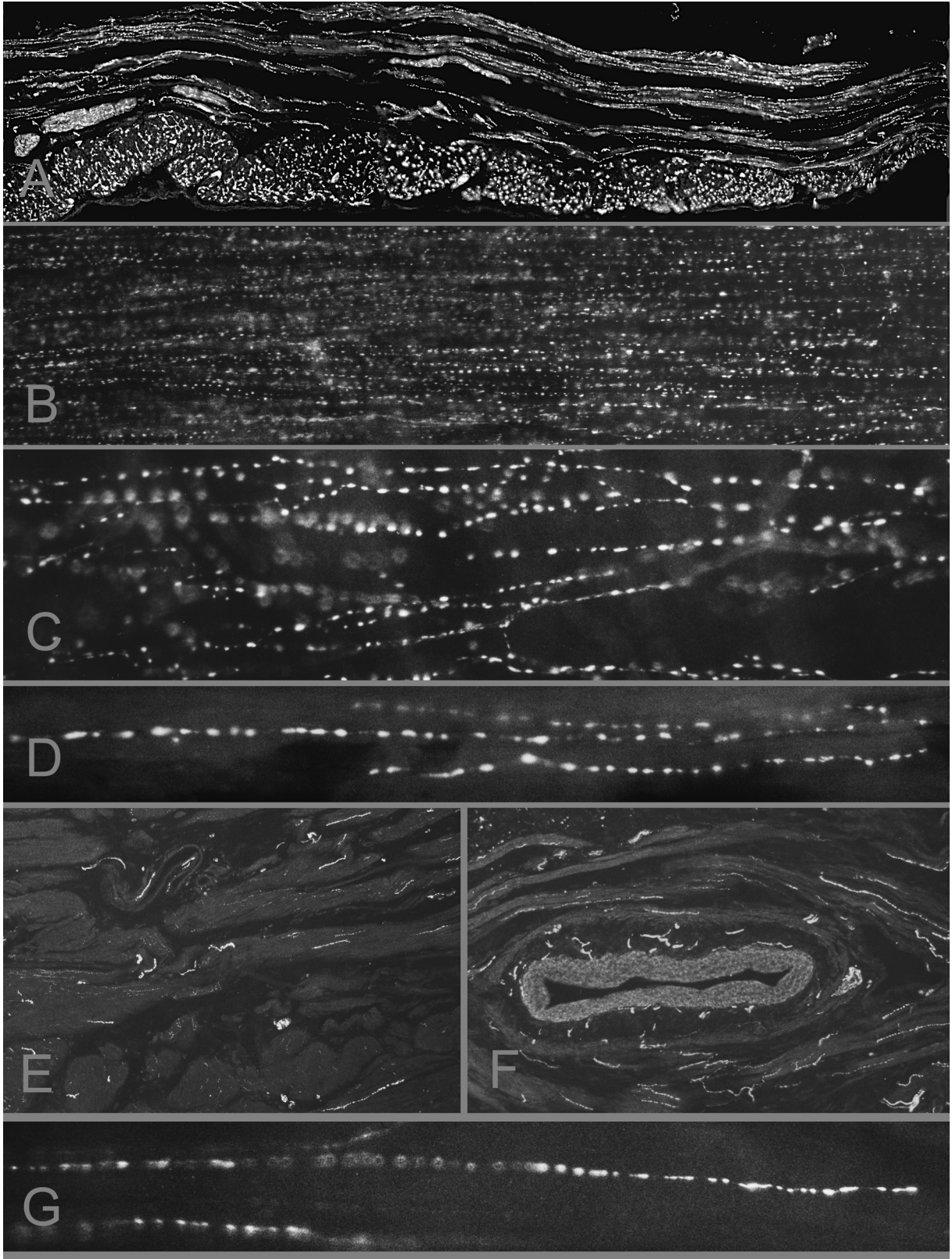


Fig. 9.

## Axons

In transverse section the axons usually have a circular, smooth profile (occasionally they are slightly flattened), whatever their size and their relation with the glia. An exception is the very terminal varicosity at the end of each axon (in the few case when that varicosity is recognized), which is wrinkled and often of irregular shape.

In a transverse section of the muscle all the axons appear transversely sectioned. Followed for some of their length, as can be done by serial sections, some axons are indeed parallel to the muscle cells, while others are at an angle of up to 5 degrees even when associated with the same muscle cell. Some large axon bundles lie oblique to the muscle cells, within a large space with collagen—a pattern already observed in the vas deferens (10). The heterogeneity in the size of axon profiles is mainly accounted for by the great majority of axons being varicose, presenting a sequence of expanded (sometimes greatly expanded) and constricted (sometimes extremely constricted) segments.

The axonal varicosities of the rat detrusor were investigated and characterized in a previous study (11). One of its conclusions was that a classification into different types of axon was not yet possible, even when many varicosities were followed through their full extent by means of serial sections. That negative conclusion is confirmed by further data obtained with the present study. There are, however, a few consistent observations. About half of the varicosities make some specialized contact with a muscle cell (described in the next section, Axonal contacts and neuro-muscular junctions). The largest varicosities can be up to 1  $\mu\text{m}$  in diameter; the intervaricose portions can be as small as 50 nm, in which case their content is reduced to a single microtubule.

All varicosities are packed with axonal vesicles, of uniform appearance: about 40 nm in diameter, with a lucent content. The vesicle packing is very dense, especially in the large varicosities, and there is no space left except for a few mitochondria and some microtubules.

The varicose or beaded pattern is also documented by immunohistochemistry in fluorescence microscopy, confirming the considerable variation in separation and apparent size of the varicosities, even along an individual axon.

The varicose pattern is already present when the axon is in a bundle. The single axons, varicose, can still divide, and the branching point is always at the level of a varicosity. The pattern extends over tens of micrometres along the terminal portion of an axon and comprises several tens of varicosities.

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### Fig. 9.

A. Frozen section through the detrusor muscle, immuno-histochemically stained for synaptophysin (the section is  $\sim 10 \mu\text{m}$  thick). Part of the musculature is in transverse section (bottom half) and each fluorescent spot is the intersection of an axon (whose fluorescence is due to its content in axonal vesicles). Above, the musculature is sectioned approximately longitudinally and short strings of dots are varicose axons included in the section for part of their length. Width of field of view: 1,400  $\mu\text{m}$ .

B. Whole-mount preparation of the detrusor muscle, stained for synaptophysin (the preparation is  $\sim 50 \mu\text{m}$  thick; only part of its thickness is in focus). The high spatial density of fluorescent spots (axonal varicosities, in linear chains) illustrates the density of innervation of the muscle. Width of field of view: 720  $\mu\text{m}$ .

C. A similar preparation as in B., at higher magnification, showing individual axons in the form of chains of fluorescent varicosities. Width of field of view: 360  $\mu\text{m}$ .

D. At higher magnification individual axons can be identified with confidence in a whole-mount preparation, and some axons display a chain of a few tens of varicosities (even when their full length is not in sight). Width of field of view: 270  $\mu\text{m}$ .

E. Section (partly longitudinal, partly oblique) through the detrusor, stained for CGRP. Short stretches of fluorescent axons (afferent) are visible inside the musculature. Width of field of view: 1 mm.

F. Section of bladder wall and ureter at the point of entry, stained for CGRP. Fluorescent axons (afferent fibres) lie close to the epithelium of the ureter; some are associated with muscle bundles of the detrusor. Width of field of view: 860  $\mu\text{m}$ .

G. At high magnification, CGRP-stained axons display their varicose pattern, which is characteristic but also rather irregular. Width of field of view: 225  $\mu\text{m}$ .

All the axons, whether they are single or within a bundle, are tightly wrapped by glia (exceptions are describe below). Even within a bundle the axons are individually wrapped by the glial cell; at no point axons in the detrusor muscle come into membrane-to-membrane contact with one another (in contrast, axon-to-axon contacts are common in the nerves of the bladder mucosa). The association of axons and glia is very close and constant, and a single basal lamina surrounds the two.

One or two of the axons in a bundle, and the majority of the axons that run singly, show an interruption of the glial wrapping, a kind of 'window' where the axonal membrane is not covered by glial but is in direct contact with the basal lamina and frequently also with a muscle cell. Rarely, a digitation from a varicosity abuts or invaginates a muscle cell. Some other varicosities (generally only the smaller of them) are fully wrapped by glia through their entire length. Some varicosities become exposed through a 'window' while lying within the stroma, far away from a muscle cell over their entire extent.

The terminal varicosity at the very end of an axon (identifiable only by means of serial sections) is often devoid of glial wrapping, that is, an axon may extend peripherally by a varicosity or two beyond the glia. Glia never extends beyond the axons.

### ***Axonal contacts and neuro-muscular junctions***

There are two types of contact between an axon and a muscle cell. One is infrequent and it involves an area of a varicosity lying flat on the surface of a muscle cell, with a gap, which can be extensive and regular, of 10–12 nm.

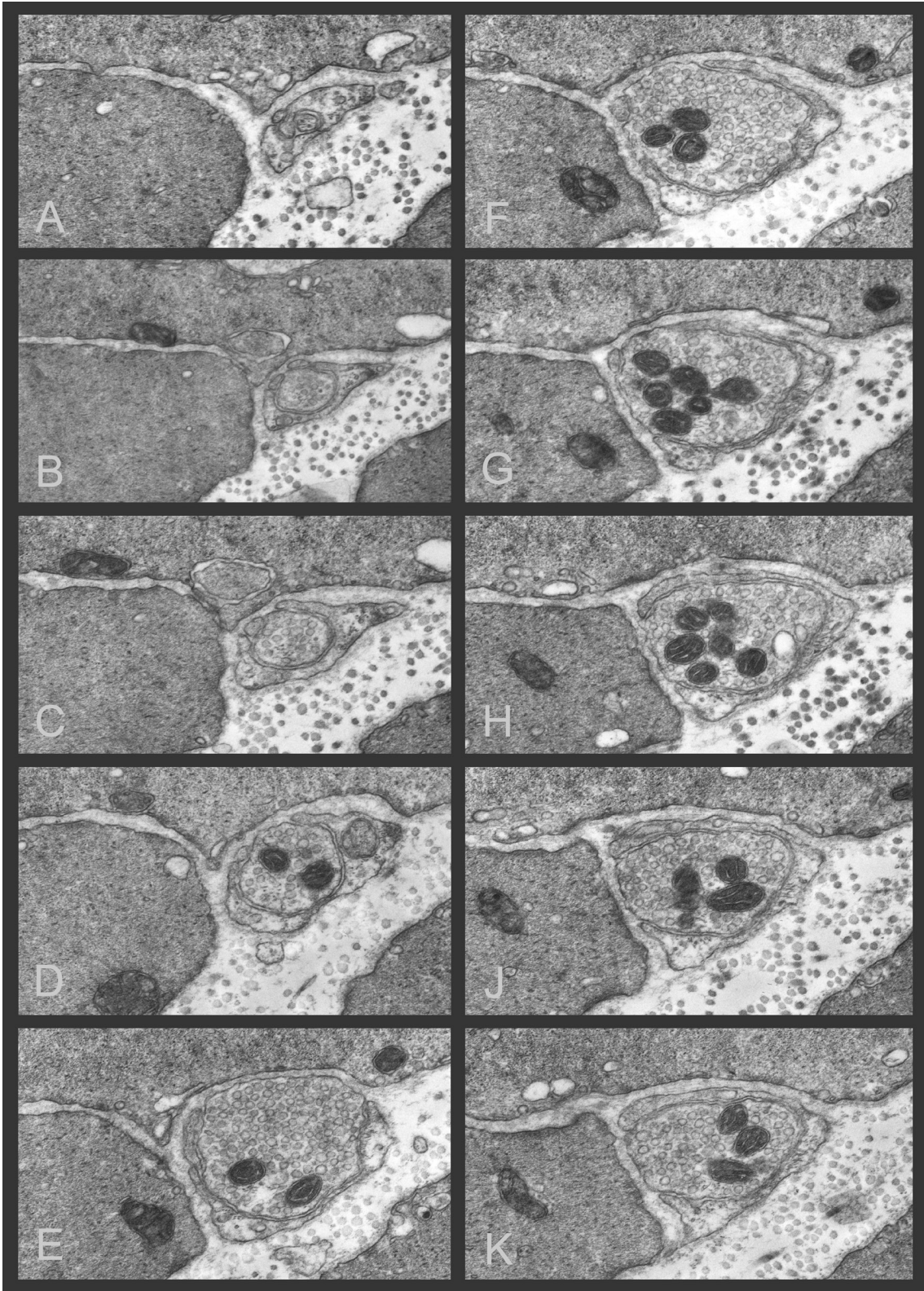
The second type is frequent, somewhat variable, consisting of the surface of a varicosity, exposed by a 'window' (that is, uncovered by glia), parallel to an area of a muscle cell's surface, usually concave. There is an intercellular gap of about 40 nm, occupied by a basal lamina shared by the two cells and excluding collagen or other fibrillar materials (Fig. 8F). There are no structural specializations on the membranes involved in these junctions. On the axonal side densely packed vesicles are invariably present, filling all the space close to the junction. The structure of these junctions is strongly suggestive of their being a site of transmission between axon and muscle cell, that is, a neuro-muscular junction both in the descriptive and the functional sense.

Even on a single section some varicosities make a junction with two or three muscle cells (Fig. 10). Over its length, studied on serial sections, every muscle cell has several neuro-muscular junctions, both from the same axon or from two or more axons (which may or may not originate from the same ganglion neuron). No muscle cells were found without neuro-muscular junctions. An occasional digitation issued by an axon makes a contact with a muscle cell; it could make an indentation within the muscle cell.

### ***Glial cells***

The nucleated part of glial cells (cell body) extends to the very periphery of the nerve and can be found even around very small nerve bundles or around single axons. Glial cell bodies (nucleated profiles) are roughly in the same number as fibroblast, about one among a hundred muscle cell profiles. They have many long laminar processes that are invariably associated with axons, forming a partial or complete sheath around each of them individually (Figs. 7, 8F and 10). A glial process was never found that was not associated with an axon.

The main elements in the glial cytoplasm are microtubules and gliofilaments and small amounts of rough endoplasmic reticulum. A distinctive feature of glia (in addition to the association with axons) is a basal lamina (Fig. 10). The basal lamina of glial cells is continuous with that of the exposed surface of the axons. The membrane-to-membrane separation between axon and glia is highly regular, uniform and without discontinuities, the gap measuring about 12 nm, with minimal variance, both around varicosities and around intervaricose



**Fig. 10.**

A selection of micrographs of a varicosity followed through in serial sections. Approximately every tenth sections is shown (each  $\sim 0.1 \mu\text{m}$  thick). The axon is at an intervaricosity level in A., fully surrounded by a glial process. In the following levels the axons is progressively wider; it is packed with vesicle; it has several mitochondria (2-6 dark bodies in D through K); at two levels it loses part of the glial covering, leaving two 'windows' beneath the basal lamina and making a neuro-muscular junction with the muscle cell at top (in D through G) and with the muscle cell at left (in G through H). Collagen fibrils are abundant in the extracellular space; they are absent between the axon and the two muscle cells. Width of field of view:  $3 \mu\text{m}$  in each frame.



segments (Fig. 7). There is also adhesion between parts of the same glial process (self-adhesion) producing a meso-axon that can be straight or highly sinuous, with a uniform gap of about 12 nm.

### ***Fibroblasts***

About one cell in a hundred in the detrusor muscle in transverse section is a fibroblast (Fig. 11). Their cell body is slightly larger in profile than the muscle cells, and more than half of it is occupied by the nucleus. The nucleus is around 3 times as long as it is wide, and its length is more than half the length of the cell, which lies parallel to the muscle cells. Mitochondria, cisternae of rough endoplasmic reticulum and Golgi units are the main organelles. The endoplasmic reticulum is usually abundant, making up more than half of the cytoplasm. From the cell body issue laminar processes, branching, without organelles, extending several tens of micrometers, both radially and along the length of the muscle bundle. These processes are variable in thickness but always tapering down as they move away from the cell body.

Fibroblasts are always devoid of a basal lamina (Fig. 11B) and there are only a few points of contact between the processes of two fibroblasts, without distinct specialized junction, either of the adherens type or as gap junctions. Occasionally, some axonal varicosity lies in the proximity of a fibroblast, without showing any of the features of the contacts between axons and muscle cells described above (Fig. 11C).

### ***Blood vessels***

The largest vessels of the bladder are in the lamina propria, where they arrive by passing through the detrusor after reaching the bladder wall. The blood supply to the detrusor is through small vessels located between the muscle bundles. In addition, some capillaries run directly inside the muscle bundles. These vessels comprise endothelial cells and pericytes, and are sheathed by a thin layer of collagen fibrils that lie parallel to the length of the vessel.

Lymphatic vessels are found at the outer (adventitial) and inner (mucosal) surface of the detrusor, but not within the muscle. This agrees with observations on human bladder (12).

### ***Stroma***

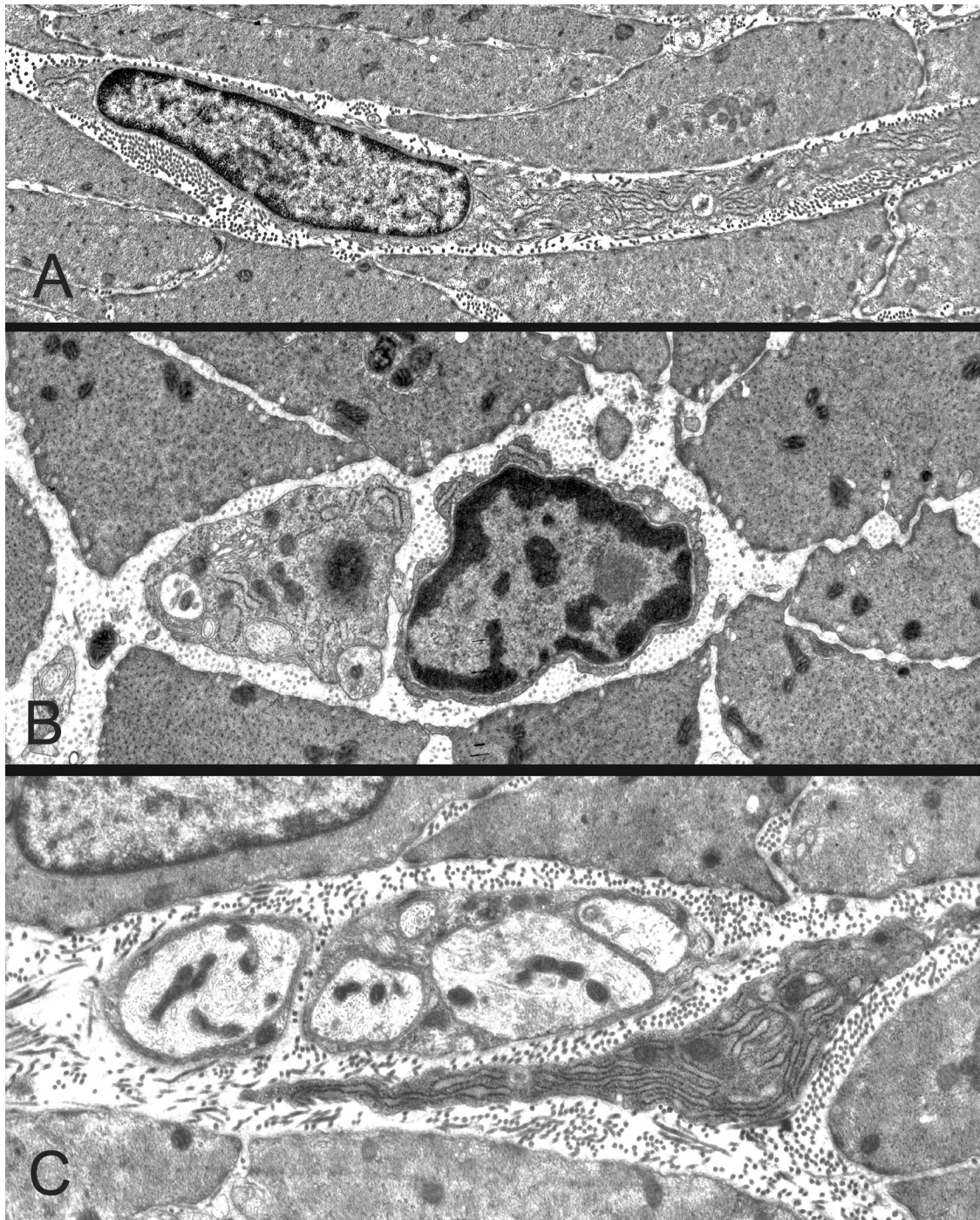
The stroma is a complex assembly of several molecular components, some in fibrillar form. In the electron microscope only collagen fibrils, elastic fibres and microfibrils can be identified.

Elastic fibres are consistently present but only in small numbers. When a fibre lies in the proximity of a muscle cell, the cell issues a laminar extension that partially engulfs the fibre; or the fibre may indent the muscle cell's surface and the cell membrane is in contact with half or more of the fibre. These configurations suggest a specific molecular and mechanical link between elastic fibre and muscle cell membrane. In contrast, there is nothing similar between an elastic fibre and glia or fibroblasts.

Intramuscular collagen fibrils measure 50–200 nm, the thinner ones being predominant. The serial sections have not produced data on the fibril length, and the heterogeneity in fibrillar size cannot yet be correlated with biochemical studies showing the presence different types of collagen in the detrusor (13, 14).

### ***Extracellular and intercellular space***

The spaces around the cells (non-cellular space), which amount to just over a fifth of the muscle volume, is occupied by the basal laminae of muscle cells and glial cells, by collagen and elastic fibers and other microfibrillar elements, and by amorphous substance (The assignment of the basal laminae to the extracellular space rather than to the cells themselves is arbitrary).



**Fig. 11.**

A. Electron micrograph of a fibroblast located between muscle cells of the detrusor, showing its nucleus and a cytoplasm with large amounts of rough endoplasmic reticulum. The fibroblast has no contacts with the adjacent muscle cells and it is surrounded by collagen fibrils, mostly transversely sectioned. Width of field of view: 18  $\mu\text{m}$ .

B. In the centre is a fibroblast whose profile is almost entirely occupied by the nucleus. To the left of the fibroblast is a nerve bundle with three axons and a glial cell; its cytoplasm shows mitochondria, a Golgi complex and rough endoplasmic reticulum; the dense mass in the cytoplasm at the right is the nucleus, just grazed in this microtomic section. The muscle cells profiles around are linked by many small processes, abutting on each other. The extracellular space is dotted with collage fibrils. Width of field of view: 32  $\mu\text{m}$ .

C. A few axons with glial wrapping lie between four muscle cell profiles (one nucleated, at top left). In the lower and right part of the picture there is a fibroblast, packed with cisternae of rough endoplasmic reticulum. Abundant collagen fibrils surround all the cellular elements. One of the axons (right of centre) has a cluster of axonal vesicle and an opening of the glial wrapping (a 'window') opposite the fibroblasts, with collagen fibrils between the two elements. Width of field of view: 11  $\mu\text{m}$ .

It is possible to recognize two separate aspects within the space around cells. Part of the space—that is here labeled intercellular space to distinguish it from the extracellular space at large—is an apparently specialized area situated between pairs of closely associated muscle cells. This includes the contacts described as muscle cell sealed apposition (see section above) and contacts where the separation between the two cells is wider but confined and delimited at the edges. These areas between muscle cells are very common, affecting virtually every muscle cell over a substantial part of their surface. The separation between the two cells is fairly regular; the gap, which measures 100–200 nanometer, is occupied centrally by a lamina of fuzzy material similar to a basal lamina (occasionally duplicated) and it usually excludes collagen and other fibrillar materials (in effect it excludes the stroma).

The remaining of the non-cellular space, or the extracellular space proper—that could be labeled stromal space to distinguish it from the intercellular space—consists of larger spaces between the muscle cells, where there is no contact between them and where all the elements of the stroma are located.

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## Discussion

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Points for discussion arise from the observations at several levels: technical, analytic, anatomical and functional.

### *Fixed, embedded and sectioned material*

Electron microscopy has strength in bringing to light aspects of the muscle structure, at high resolution (15). This point must be qualified, in view of concurrent limitations, which are not always fully taken into account. For example, the procedure for microscopy fixes the tissue at a single instant in time. Strong chemical fixation is used, which denatures, extracts and displaces some tissue components. Fixation and embedding cause shrinkage, which is not always uniform and is hard to estimate. Sectioning can produce compression of the material in one direction. High energy electron beam can damage and deform the section under examination. A two-dimensional perspective dominates the analysis of microtomic sections.

On the positive side there are two factors. First, the presence of artifacts and other shortcomings may be easier to detect in microscopy than with other techniques that rely on purely numerical outputs. Second, the observations are recorded in full and filed away, in the form of micrographs, which can be re-examined at any time.

### *Animal species*

This work was carried out on a group of animals as uniform as possible, of a single species and a limited range of body sizes. The smaller and possibly simplified musculature of the rat bladder could be analyzed better than with the data from several species; however, the rat may lack structural complexities found in other species and may have some unique features.

Comparative studies on several animal species are valuable. However, when the data available are not abundant and when they are predominantly qualitative, then comparative work has both benefits and risks. Here, it was felt that generalizations are premature, and certain extrapolations across species may not be useful. Critical considerations about allometry when dealing with such a range of body sizes as is found in mammals, and about the range of life spans among species, suggests caution. There also different behavioural roles of the bladder in many species. In the case of bladders of humans, the large body size, bipedalism and erect position, large pharmacological interventions, and long life span (to say nothing of the problem of sampling) create special concerns.

### *Morphometry and cell types*

Morphometric work on electron micrographs of sections of the muscle has generated many data. Their potential interest lies in the possibility of comparisons with other conditions (age, pathology, stimulations, experiments) and in other species. The morphometric approach has its own limitations; nevertheless, it offers some valid addition to biochemical approaches.

The identification of cell types turns out to be quite straightforward and unambiguous. Every cell profile can be confidently assigned to one of five groups (based on standard histological criteria): muscle cells, fibroblasts, axons, glia and vascular cells. The key figures are a partition of 79% to 21% of cellular vs non-cellular components, and the cellular component being partitioned into muscle cells (~72%), nerves (~4%, of which 2/3 are glia and 1/3 axons) and fibroblasts (~3%). The muscle cells comprise more than 93% of contractile apparatus, ~3% mitochondria, ~2.5% nuclei, and an estimated 1% of other organelles. From these figures one can calculate that the contractile apparatus occupies 2/3 of the volume of a muscle bundle.

The presence of several cell types, thoroughly mixed, and the compactness of the tissue are remarkable aspects. Muscle bundles have smooth borders, and their cells are packed together with a most economical use of space. The detrusor as a whole has a smooth surface both at its inner and outer side, both in distended and empty bladders. In the absence of external constraining structures (such as a capsule) the compactness must arise from intrinsic properties of the bundles, including adhesion between the muscle cells. The connections between cells are complex and extensive, and they have a critical role in the mechanics of this tissue. The motor (contractile apparatus) of each cell is bound to the others via the muscle cell membrane with its cell-to-cell and cell-to-stroma connections.

### *Muscle bundles*

The subdivision of the musculature into bundles is well visible in the detrusor, and bundles are regarded as the functional units of the muscle, large or small as they can be. The transverse size of a bundle is constant along its length, that is, its solid shape is cylindrical, as documented by serial sections. Mechanically, this is an optimal structure: its effect is that the amount of force generated upon activation is uniform along the length of the bundle, and force or pull can be transmitted additively and uniformly, over the full length. If the cross section of a muscle bundle were not of constant area, then a thick part of the bundle would pull (and possibly stretch) a thin part of the bundle, even when there is a uniform activation of the entire bundle.

How this uniformity of width is achieved in the course of development, and how it is then maintained, is obscure. There must be within the muscle some kind of ‘sensory’ mechanism—a form of mechano-transduction—that responds to any arising irregularity of tension distribution, and that activates some compensatory growth or changes in the cell-to-stroma localized attachments—of which we know nothing.

### *Muscle cell content*

The inside of muscle cells is dominated by contractile apparatus and cytoskeleton, which occupy ~93% of cell volume, the rest being nucleus and mitochondria.

The remarkable uniformity of appearance of the muscle cells on sections—in a population of cell profiles, or along a muscle cell’s length—is difficult to reconcile with the current models of myofilaments and cytoskeleton spatial arrangement. Apart from the shape of their profile (which is as irregular as it is variable) muscle cells seem to constitute a uniform population (without subtypes) in terms of size or cytological features.

### ***Muscle cell surface***

Under the microscope the dullness and uniformity of the muscle cell content contrast with the abundance and variety of features at the cell surface, adding to the rather complex shape of the cell outlines. It should also be recalled that with isotonic contraction the cell shortens and fattens (geometrically, by the same amount), and the perimeter of the muscle cell profile can double, involving extensive and reversible rearrangements in the membrane.

The compactness of a muscle bundle derives from the shape of the cell profiles adapting to each other (to a greater extent than in other smooth muscles, such as those of the gut) as well as from various cell-to-cell contacts. The membrane is also bendable, and can be folded, producing narrow projections and invaginations at the cell surface. In outward bendings (as at the tip of some digitations) the radius of curvature can be as little as 30 nm, whereas at the inward bendings, usually bearing a basal lamina, the radius of curvature is rarely less than 300 nm.

### ***Contacts between muscle cells***

Several forms of cell-to-cell contact, involving different levels of intimacy, communication, bondage are visible in the electron micrographs. Each configuration derives from some molecular architecture, presumably in some case a highly specific one. Microscopic observations are of interest mainly because data on these molecular structures are not yet available.

In turn, microscopy is hindered by the excessive variability encountered in the tissue itself (while we are still at a stage where the more observations one makes, the more complex the interpretation of the structure becomes).

### **Adherens junctions**

The contacts between muscle cells range from simple proximity (with no suggestion of interaction) to adherens junctions, a well recognized site of attachment between two muscle cells. Actually, the adherens junctions are rather heterogeneous even when they fit the general notion of mechanical links between the contractile apparatus of two muscle cells. They are not always symmetric, and the amount of electron-dense material is variable.

### **Gap junctions**

The failure to observe gap junctions between muscle cells of the detrusor is remarkable in that it contrasts with many observations reported in the literature on the presence of gap junctions, the sites of electrical (ionic) and metabolic (small-molecule) coupling between cells (e.g., Ref. 16, 17).

The negative results also contrast with the general notion, well received in the literature, that electrical coupling is universally present in smooth muscles, being provided in most cases by gap junctions (18). Electrical coupling between muscle cells of the detrusor is well documented, at least in the guinea-pig (17, 19), although the spread of the injected current is somewhat limited (20). Gap junctions are observed in the guinea-pig detrusor even if there is only a limited electrical coupling (16). More extensive data supporting the occurrence of gap junctions are based on connexin-43 histochemistry in guinea-pigs (21); however, levels of connexin-43 mRNA transcript and protein expression are barely detectable in the detrusor of control rats (22), while connexin-45 is expressed there (23). Even if one assumes possible species differences and the effect of other physiological factors, the present data on lack of evidence of gap junctions (which is interpreted as an absence of gap junctions in the rat detrusor) is difficult to explain, and the current study casts no new light on the matter.

### **Digitations**

Two types of muscle cell contact stand out as of potential functional significance, for which the tentative

labels of digitations and sealed appositions are used here. Short finger-like projections or digitations are an intriguing form of muscle cell contact; they are prominent in the bladder musculature, although present, but less common, in other visceral smooth muscles. Small though they are, digitations are encountered almost on every muscle cell profile; there must be hundreds on a single muscle cell of the detrusor. Their content is amorphous and the membrane itself has no distinctive features.

At their tip—point-like, usually, but sometimes wider, or expanded, or involving another digitation—these processes lose the basal lamina and the membrane-to-membrane apposition leaves a visible gap of about 10–12 nm. The ability to perforate the basal lamina seems an important property, given the continuity of the basal lamina around the muscle cell. Digitations are rare in the muscle cells at birth, and so it is not the case that they are formed before the appearance of a full basal lamina.

### Membrane dynamics

The impression given by the morphological data, highlighting the exuberance of contacts and of projections from the cell surface, is that many of these processes are transient projections, caught in the microscope at various transitional states of formation and withdrawal. They reveal an intense activity at the cell surface, a notion that is referred to as membrane mechanics, parallel to the contractile mechanics, but probably less intermittent.

### Sealed appositions

Sealed appositions are extensive areas of the cell surface where the membranes of two muscle cells, flat or curved, are roughly parallel. The geometrical regularity of these appositions, the presence of fused basal laminae, and the almost complete exclusion of fibrillar extracellular materials, suggest not only a specialized inter-cellular space, but also a private, narrow, chamber-like, space shared by two cells. There is a clear distinction between these narrow intercellular spaces and the extracellular (or stromal) space at large.

### *Tissue space*

The muscle cells are a semi-fluid material and they are embedded in a stroma that provides a rigid but pliable frame. The combination of the two materials favours reversible changes in shape of the tissue, and it also imposes specific mechanical conditions. The compactness of the muscle and its being incompressible are accompanied by the absence of any empty space that would assist the movement of its parts. In the absence of empty spaces, and given that all the elements have constant volume, any movement or any change in shape of any part of the muscle involves a displacement of another element.

There is, therefore, a particular type of space (without empties where components could move into), sometimes referred to as a naught-space (N-space) that has specific mechanical characteristics.

### *Mechanical considerations*

Contraction of the musculature, when allowed to develop isotonicly (bladder outlet open) rearranges the muscle bundles—their length, width, profile, orientation—to a limit point, when the lumen of the bladder is obliterated. In contraction, the shape of the bladder, unlike its size, does not change markedly; both inner and the outer surface of the detrusor remain relatively smooth. A mechanical account of these processes is not yet within reach. However, the mechanical properties are not best understood by the standard linear mechanics that is used to account for passive and active movement of objects in space.

Within the range of smooth muscles there are different arrangements: roughly linear configurations (tra-

cheal muscle, tenia coli), tubular configurations (blood vessels), tubular layered configurations (gut) and ovoid configurations (bladder), the latter being the most difficult to understand.

### *Mitochondrial spatial density*

The morphometric method has produced a reliable estimate of the extent of the chondrioma, that is the spatial density of mitochondria in muscle cells. The merit of microscopy in the evaluation of the chondrioma, in addition to more popular biochemical methods, has been discussed elsewhere (7). According to these results, when measurements are carried out on sufficiently large samples, the extent of the chondrioma in muscle cells: i. it is the same in different individuals; ii. it remains constant over the length of a muscle bundle; and, iii. there is not significant variation between cells. The quantitative differences observed between cells are so small (in addition to being partly accounted for by technical reasons) that they indicate a mitochondrial spatial density kept within strict limits (below  $\pm 5\%$ ). This in turn points to regulatory mechanisms that can achieve constancy of a dynamic organelle, presumably as an effect of processes of synthesis of new mitochondria and destruction of old mitochondria, processes that have been studied in many tissues (24–26), including the detrusor (27).

The current data and those in the literature (7) obtained with the same method, confirm the quantitative differences in the chondrioma in different smooth muscles of the same species. For example, gut muscle cells (in the ileal circular muscle) have about 30% more mitochondria than the bladder ones. This substantial difference indicates a different access to energy in the two muscles, with a higher anaerobic energy production via glycolysis and a lesser reliance on oxidative metabolism in the detrusor muscle cells.

### *Innervation*

The innervation of the detrusor of the rat is impressive for its extent and for an excess of contacts and junctions between axons and muscle cells. In the absence of direct studies, most of what is known about the electrophysiology of this type of neuro-muscular junction is found in early studies (28–30). The large number of terminal axons, already documented in a previous study (11), is a sign of the strong control that nerves exert on the bladder; however, it also presents several intriguing aspects.

The varicose, or beaded, pattern of the great majority of intramuscular axons is obvious and distinct, but of uncertain origin and significance. Varicosities are found only when the axons are no longer bundled by a perineurium and lie directly between muscle cells; at this level they have become richly endowed with axonal vesicles. The pattern is a segmentation of the axon so that the abundant axonal vesicles are distributed in clusters at intervals, and the intervening regions of the axon collapse into intervaricose segments. Varicosities tend to become larger distalwards along the axon (although not so regularly), while the intervaricose portions usually become thinner and thinner. The observations by electron microscopy can usually not be extended beyond a single varicosity, while the observations by immunohistochemistry have limited resolution. Additional limitations are that sizes can not be measured in fluorescence microscopy and the procedure stains the axonal vesicles rather than the varicosity itself. However, fluorescence microscopy documents that the varicose pattern extends for many tens of micrometres and comprises chains of tens of varicosities. The varicose axon can still divide, in which case the division takes place at a varicosity.

The number of varicosities is extraordinary. One can count scores of them along a single stretch of an axon (which must be just one of the branches of a nerve fibre issued from a ganglion cell). The scarcity of true terminal varicosities indicates that the great majority of varicosities are *de passage* or in passing, with an intervaricose segment on both sides. There must, therefore, be hundreds of varicosities on each axon.

An important question is what makes the detrusor so attractive to nerves that they grow so extensively

into it. So-called trophic factors may play a role, including NGF, which is already been studied in the bladder (31, 32). It seems likely that there is something very special in the way the innervation is formed in the detrusor, involving several factor, and conditions particularly favourable for nerves.

Contacts between varicosities and muscle cells are numerous, and some of them are identified as neuromuscular junctions (11). The data suggest that every muscle cell has junctions with an axonal varicosity, and probably several of them for each cell. On the axonal side, a varicosity can make junctions with several muscle cells (even two or three of them in a single transverse section).

Afferent fibres are also seen throughout the muscle. They could not be identified ultrastructurally among the vastly more numerous efferent fibres.

The glial has a tight relationship with axons and it is never found without a close membrane-to-membrane appositions to an axon. There is always some glia between axons, and axons are never in membrane-to-membrane contact with each other (unlike what is observed in other autonomic nerves, including those of the bladder mucosa (9). The role of the glia at this level is mostly obscure. It is uncertain, for example, what their role is in the formation of 'windows' at the axonal surface, whether the glia withdraws or is unable to grow over it.

### *Fibroblasts*

From a descriptive point of view the fibroblasts in the detrusor do not present problems. Their fine structure is not at variance from that of the fibroblasts in almost every tissue of the body (33). Fibroblasts in the detrusor are not often mentioned, and yet, in spite of their small number, their role is important in laying down most of the elements of the muscle's stroma. Amount and distribution of collagen are major factors in smooth muscle mechanics. The collagen within the detrusor and the much more abundant collagen of the lamina propria are produced by resident fibroblasts. Presumably, also the mechanically critical orientation of the collagen fibres (and even of the individual fibrils) is directed by the fibroblasts.

Beside the production of collagen, the significance of fibroblasts should not be underestimated. Additional functions, actual or potential, have emerged for these cells in recent studies. Especially interesting is the potential transformation of fibroblasts into stem cells, capable of differentiating into endothelial cells or striated muscle cells in the heart, for example (34). There is no evidence that fibroblasts have such roles in the bladder, but the possibility is not excluded, among the potential roles of these cells. Attractive suggestions on the critical role of the stroma in bladder pathology have been made (14).

The fibroblasts of the detrusor may resemble cells amply described in the literature and identified as a new cell type, interstitial cells of Cajal. The interstitial cells of Cajal are better known than the fibroblasts, even if their ultrastructure in the bladder is not well documented (with some important exception, e.g., Ref. 35), and there are many claims about their functional properties and their subtypes (36). The issue of interstitial cells of Cajal in the bladder is presented in several review articles (e.g., Ref. 34, 37) and is not raised here, where none of the cells observed in the electron microscope falls outside well established cell types, which do not include interstitial cells.

### *Intercellular and extracellular space*

A suggestion emerged from the micrographs is that within the space outside the cells there are areas that seem private to the two adjacent cells. There the separation between the cells is 100–200 nanometers and the regularity in width and uniform material and exclusion of stroma fibrillar elements indicates a specialized, even if not close, contact.

The term inter-cellular space is used to distinguish these sites around muscle cells from the extracellular space at large or stromal space. The distinction of two components of the non-cellular space is structurally evi-



dent; whether the inter-cellular space constitutes a functionally distinct space is purely speculative. However, it is possible that the intercellular space forms some selective chambers, private to two apposed muscle cells, and less accessible to exogenously administered chemical than other areas. Some of these secluded areas may contain an axonal varicosity.

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## Conflicts of interest

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The Author declares that there are no conflicts of interest.

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