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Detailed morphology of the stapedius muscle of the rat. An integrated light microscopical, morphometrical, histochemical, immunohistochemical and electron microscopical study in relation to function*

H. VAN DEN BERGE AND P. WIRTZ†
Department of Otorhinolaryngology, University of Maastricht, The Netherlands

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INTRODUCTION

The middle ear muscle reflex is an important input control mechanism for the inner ear. By modulating sound transmission over the chain of ossicles the tensor tympani and the stapedius muscle can protect the inner ear from noise-induced hearing loss and increase the sensitivity of the hearing system (Borg, Counter & Rösler, 1984). The reflex is elicited by self-phonation and loud acoustic stimuli, but it can also be activated by certain tactile stimuli (Djupesland, 1964). Electrophysiological studies show considerable interspecies differences and it seems that the function pattern is adapted to the special needs of the individual mammal. Despite the investigations of the middle ear muscle reflex in the past, many questions remain. For example, when considering the role in the protection against noise, more detailed information about the fatiguability, the latency time and the contraction time of the reflex has to be obtained. To obtain clues in answering these physiological questions a thorough knowledge of the functional morphology of the muscles is necessary.

Anatomical studies of the muscles in the past were mostly fragmentary, i.e. without integration of the various morphological aspects in a single animal species. (For references see Berge & Wirtz, 1989.) Such integration is important since major morphological interspecies differences appear to exist (Kobayashi, 1956; Vegetti, Mascarello & Carpenè, 1982; Mascarello, Vegetti, Carpenè & Rowlerson, 1983).

The aim of our study is to create an integrated insight into structure and function of the middle ear muscles in the rat. Results of the functional morphology of the tensor tympani muscle of the rat and preliminary electrophysiological studies have been previously published (Jong *et al.* 1988; Berge & Wirtz, 1989). In this article the gross anatomy, microscopic anatomy, muscle fibre morphometry, enzyme histochemistry, immunohistochemistry and electron microscopy of the stapedius muscle of the rat are presented and the functional implications are discussed.

MATERIAL AND METHODS

Following examination of the anatomy *in vivo*, ten stapedius muscles were removed from five 12 weeks old male Lewis rats during sodium pentobarbitone anaesthesia. Serial cryosections of 10 μm were made and the following stainings were applied:

* Reprint requests to H. van den Berge, University Hospital of Maastricht, Department of Otorhinolaryngology, Postbox 1918, 6201 BX Maastricht, The Netherlands.
† Formerly: Department of Cell Biology and Histology, University of Nijmegen, The Netherlands.

(*a*) Sirius red; (*b*) myofibrillar ATPase (preincubation pH: 9·4, 4·6, 4·3, 4·0 and 3·9); (*c*) succinic-dehydrogenase (SDH); (*d*) alpha glycerophosphate dehydrogenase (GPox); (*e*) immunohistochemical reactions against myosin heavy chain isotypes (anti-embryonic, anti-neonatal, anti-slow, anti-slow plus IIA, anti-IIB and anti-slow tonic). Reference staining of the extensor digitorum longus muscle (EDL) and soleus muscle of the rat were included in the same series of incubations for the enzyme histochemical and immunohistochemical reactions of the stapedius muscles (for references and detailed information about the technical procedures see Berge & Wirtz, 1989).

Histochemical characterisation of the fibres was carried out on four muscles using serial sections stained for ATPase, SDH and GPox (Berge & Wirtz, 1989).

The cross-sectional area (in μm^2) and the smallest diameter of the individual fibres were determined with a MOPP videoplan 2000 digitiser. Sirius red-stained cross-sections from the mid-belly of the muscle were used for this purpose. In most cross-sections some of the muscle fibres were not cut exactly in a transverse plane and care had to be taken to prevent bias in the measurements of the cross-sectional area. Therefore as well as the measurements of the cross-sectional area, measurements were made of the smallest diameter. To check the results, measurements were made in three different muscles.

For a reconstruction of the anatomical structure of the muscle 10 μm serial frozen cross-sections from the origin to the tendon of insertion were collected from three different muscles and stained with Sirius red.

For electron microscopy, three muscles were dissected following perfusion fixation through the heart (Berge & Wirtz, 1989).

To study and present the *in situ* orientation and localisation of the stapedius muscle in relation to other structures in the middle ear serial sections of decalcified middle ear bullae were made.

RESULTS

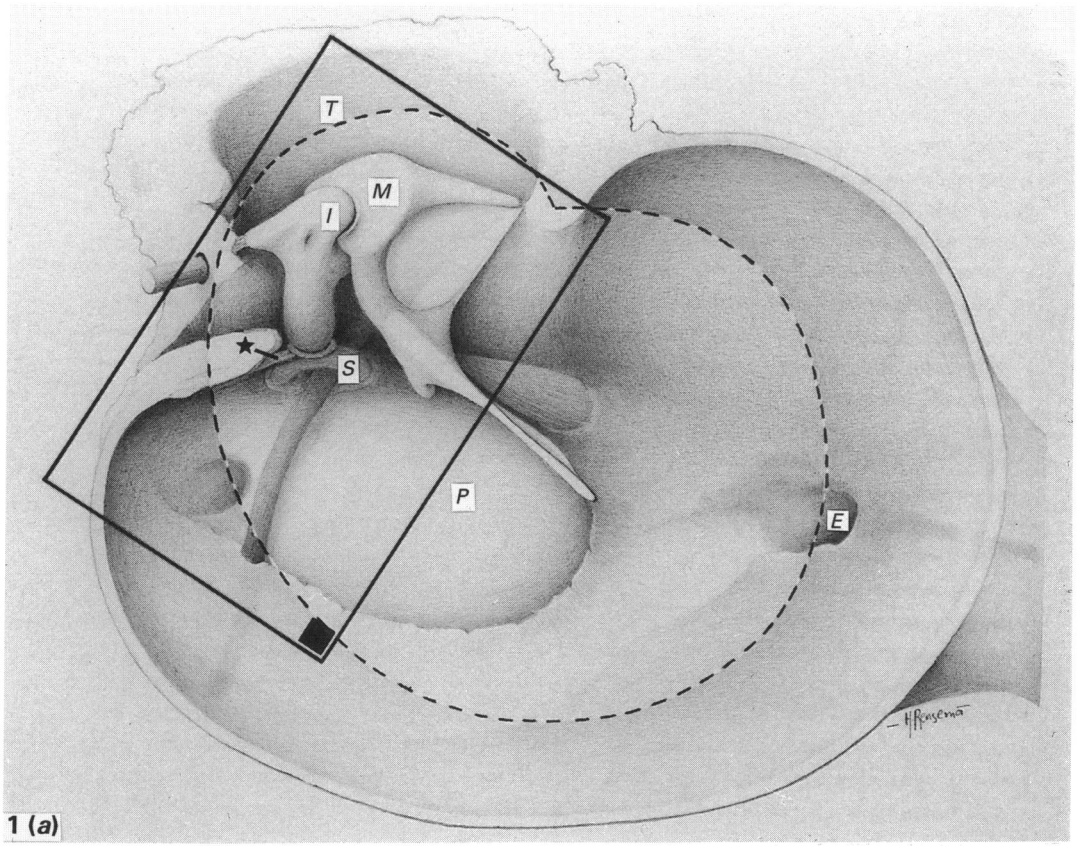
Gross anatomy

The stapedius muscle of the rat is about 1·2 mm long and has a diameter of approximately 0·6 mm. Figure 1 schematically shows the anatomical relations. *In situ* the muscle has a hemi-ellipsoid configuration. It is embedded in the dorsomedial wall of the middle ear bulla and the dorsocranial aspect of the muscle is in close relation to the facial nerve. A small longitudinal part of the muscle is exposed to the tympanic cavity by a slit in the bony dorsomedial wall. The very thin tendon of insertion enters the bulla through this slit and is attached to the stapes at an angle of 180° to a plane through both crura of the stapes. On the site facing the tympanic cavity the muscle is covered by a relatively thick fibrous tissue plate onto which the epimysium is attached. On the opposite site, the muscle fibres are attached via the epimysium to the bone of the concave cavity in which the muscle is situated.

Microscopic anatomy

Figure 2 shows cross-sections, stained with Sirius red, at successive levels from origin to insertion. As can be seen in the Figure the muscle is surrounded by a thin perimysium which is attached to a relatively thick fibrous tissue plate on the bulla side.

Fig. 1 (*a*, *b*). Schematic representation of the middle ear bulla of the rat. (*a*) Lateral view. (*b*) Section of the area indicated in (*a*). *B*, bone; *C*, connective tissue; *E*, orifice of Eustachian tube; *F*, facial nerve; *I*, incus; *M*, malleus; *mf*, muscle fibres; *P*, promontory; *S*, stapes; *T*, tympanic membrane; *TC*, tympanic cavity; Asterisk, tendon of the stapedius muscle.



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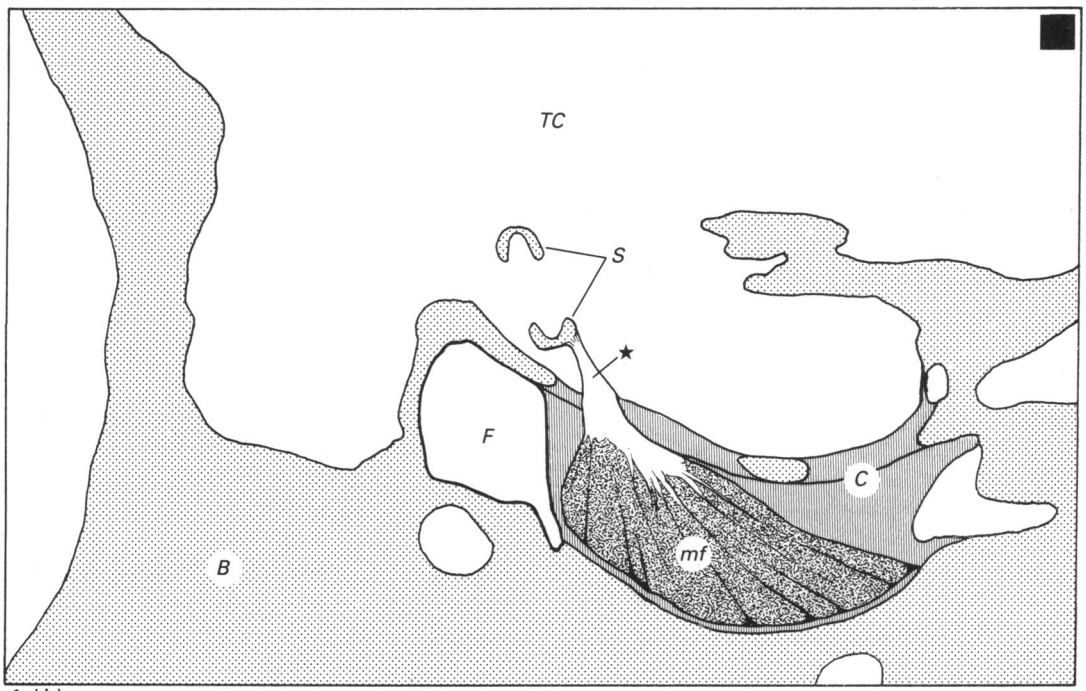

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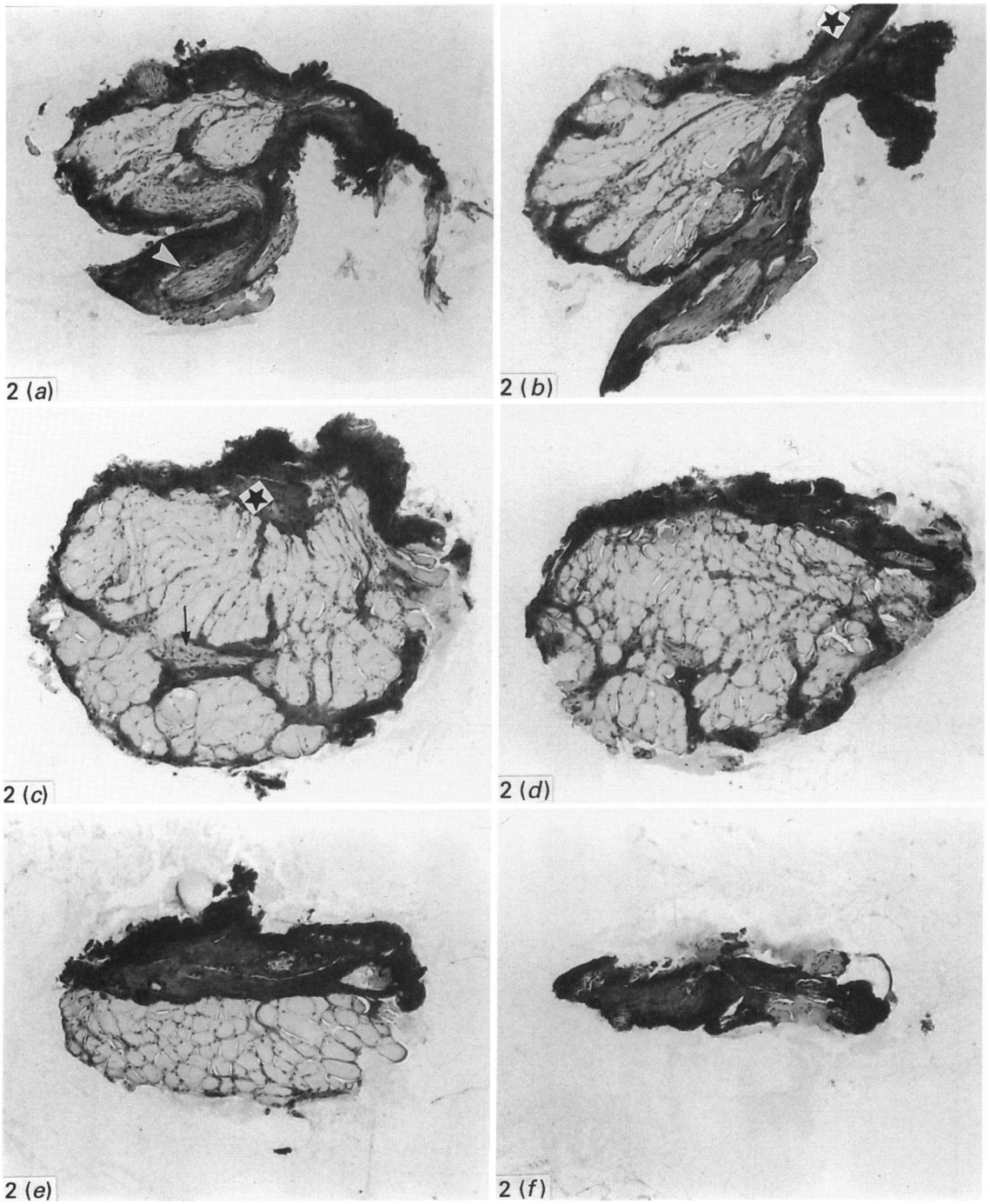


Fig. 2(*a–f*). Frozen cross-sections of an excised stapedius muscle of the rat. Sirius red stainings. From (*a*) to (*f*): sections at successive levels from origin to insertion. (*a*) White arrowhead, stapedial nerve; (*b*, *c*) asterisk, tendon; (*c*) arrow, branched nerve, ×81.

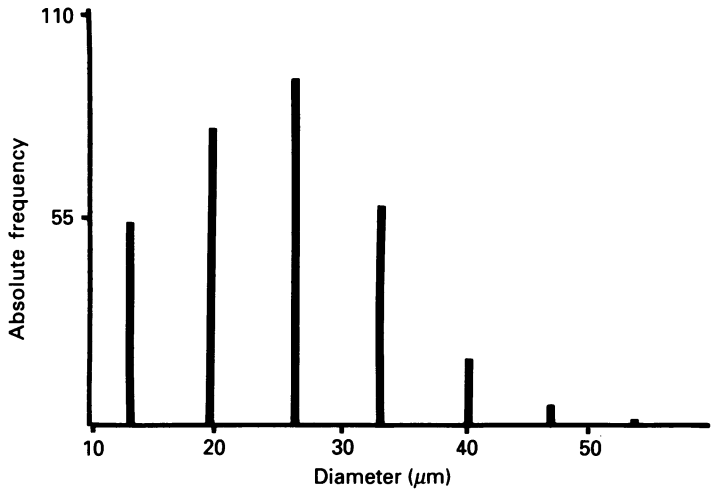


Fig. 3. Histogram of the smallest diameter of single muscle fibres of the stapedius muscle of the rat. N = 315. Mean single smallest diameter 25 μm; median 24·7 μm; S.D. 8·4.

The muscle is divided into compartments by irregular strands of fibrous tissue. These contain blood vessels and an elaborate network of nerve fibres (Fig. 2*c*), the latter arising from the stapedial nerve that enters the muscle near the tendon of insertion (Fig. 2*a*).

The muscle fibres are arranged mainly in parallel but not in a very regular way because of the strands of fibrous tissue. At the insertion site the muscle fibres converge to the paracentrally situated tendon. As a result, in cross-sections, the muscle fibres in this area are cut obliquely (Fig. 2*b*, *c*). A very thin tendon of insertion connects the muscle to the junction of the posterior crus and the neck of the stapes. In contrast to findings in the tensor tympani muscle of the rat, no fat cells were encountered.

Morphometry

The largest cross-section of the muscle contains about 300 muscle fibres. Figure 3 shows the histogram of the smallest diameter of individual muscle fibres. The mean smallest diameter of a single muscle fibre was 25 μm with a standard deviation (S.D.) of 8·3 indicating much variation in fibre size. The mean cross-sectional area was 782 μm^2 (S.D. 443). Measurements in two other muscles were in good agreement with those of the muscle presented.

Enzyme histochemistry

Figure 4 shows serial cross-sections stained for, respectively, ATPase, SDH and GPox, while in Figure 5 the occurrence of the various fibre types is indicated schematically. The enzyme histochemical figures presented below are mean figures derived from three different muscles. All three muscles showed a similar fibre type composition.

Upon ATPase (pH 4·35) staining the majority of the muscle fibres reacted negatively (87%) and thus can be considered to be fast twitch fibres. A small number revealed a weak to moderate reaction (8%) (Fig. 4*a*, *b*). All these fibres showed a moderate to strong reaction on both the SDH and the GPox staining (Fig. 4*c–f*), indicating a high oxidative as well as a high glycolytic capacity respectively. Qualitatively the SDH and

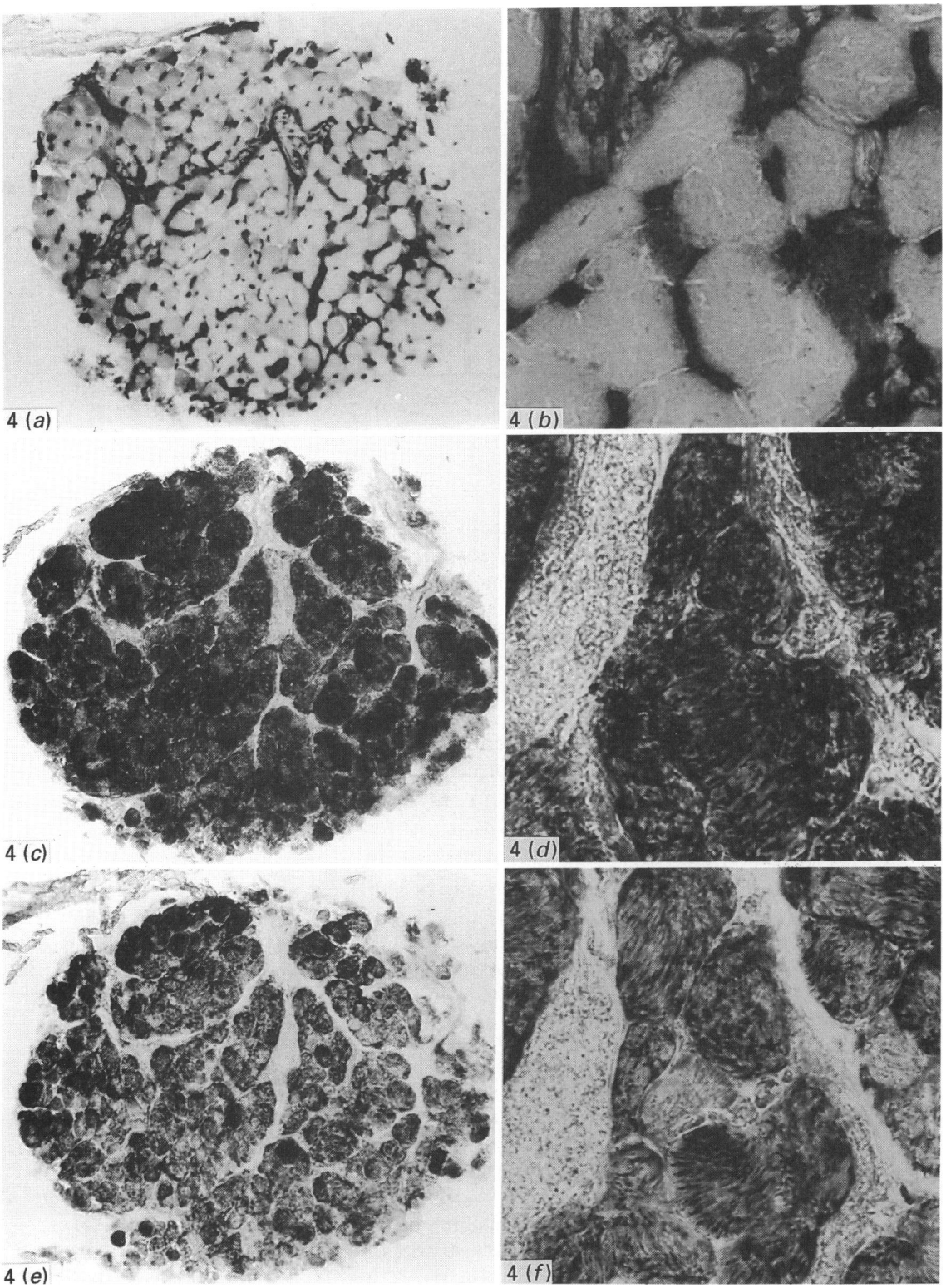


Fig. 4 (*a–f*). Frozen cross-sections of an excised stapedius muscle of the rat. On the left: serial sections of respectively ATPase (pH 4·35) (*a*), SDH (*c*), and GPox (*e*). On the right (*b*, *d*, *f*): details of the corresponding sections. (*a*) (*c*) (*e*) × 95; (*b*) (*d*) (*f*) × 495.

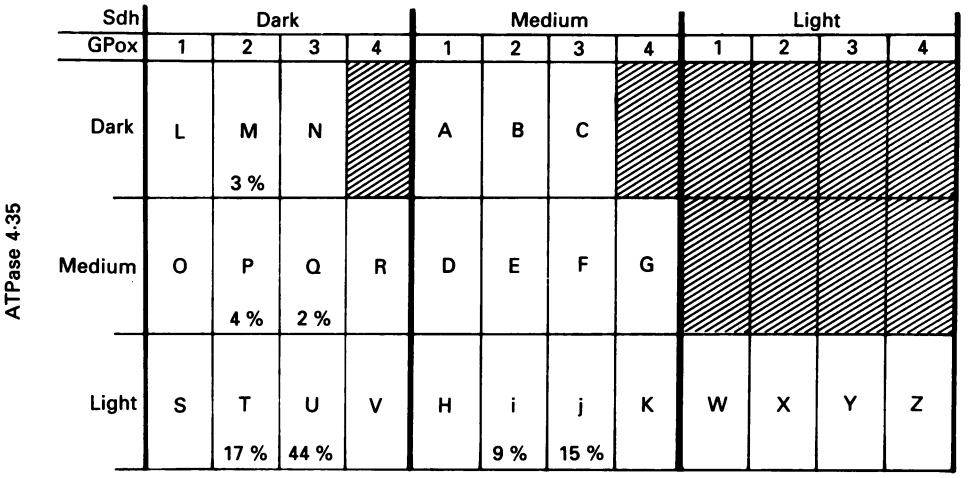


Fig. 5. Combination scheme of histochemical reactions (ATPase pH 4·35, SDH and GPox) indicating the different fibre types (Wirtz, Loermans, Peer & Reintjes, 1983). A–C, slow fibres, equivalent to slow oxidative fibres* (SO); D–G; transitional fibres; L–N and O–R, rare fibres; S–V, fast-red fibres, of these U and V equivalent to fast oxidative glycolytic* (FOG); H–K, intermediate fibres; W–Z, fast white fibres, of these Y and Z equivalent to fast glycolytic fibres* (FG). The shaded blocks are non-occurring combinations. (*According to Peter *et al.* 1972.) The mean percentages of the fibres of three stapedius muscles of the rat are indicated in the figure. NB: fibres that occurred in a percentage less than 2% are not presented.

GPox reactions showed a patchy staining pattern (Fig. 4*d*, *f*), indicating an uneven distribution of formazan.

Only a small number of the fibres reacted positively on the ATPase (pH 4·35) reaction (5%). With the ATPase pre-incubation at pH 3·9 and pH 9·4 these fibres reacted negatively. As a result they should be considered as slow twitch fibres. In the acid pre-incubation (pH 3·9 and 4·0) no indication of the presence of IIc fibres could be found. Like the fast twitch fibres, the slow twitch fibres reacted in a moderate to strong way with both the SDH and GPox staining.

In summary, enzyme histochemically the stapedius muscle is mainly composed of fast twitch fibres with a relatively high oxidative and glycolytic capacity most closely resembling Type IIA fibres (95%). A small proportion of the muscle fibres is slow twitch (5%). These fibres, too, have a high oxidative and glycolytic capacity. Therefore they are not classical Type I fibres (= slow oxidative).

Immunohistochemistry

Figure 6 shows serial cross-sections stained with the various anti-heavy chain myosin antibodies.

With the anti-slow antibody all fibres stained above the background level and a range of staining intensities could be distinguished (Fig. 6*g*, *h*). Of all fibres, 33% showed a strong reaction when compared with reference sections of rat hindlimb muscles and therefore those fibres were slow, heavy chain myosin-positive. When compared with the ATPase (pH 4·35) all the dark fibres in the ATPase staining (slow fibres) also appeared to react strongly with the anti-slow antibody (Fig. 6*i*, *j*). However, not all the fibres that reacted strongly with the anti-slow antibody showed a strong reaction in the ATPase staining.

Many of the fibres that stained intensively with the anti-slow antibody, also contained the anti-neonatal antibody (Fig. 6*a*, *b*); 28% of the fibres were found to

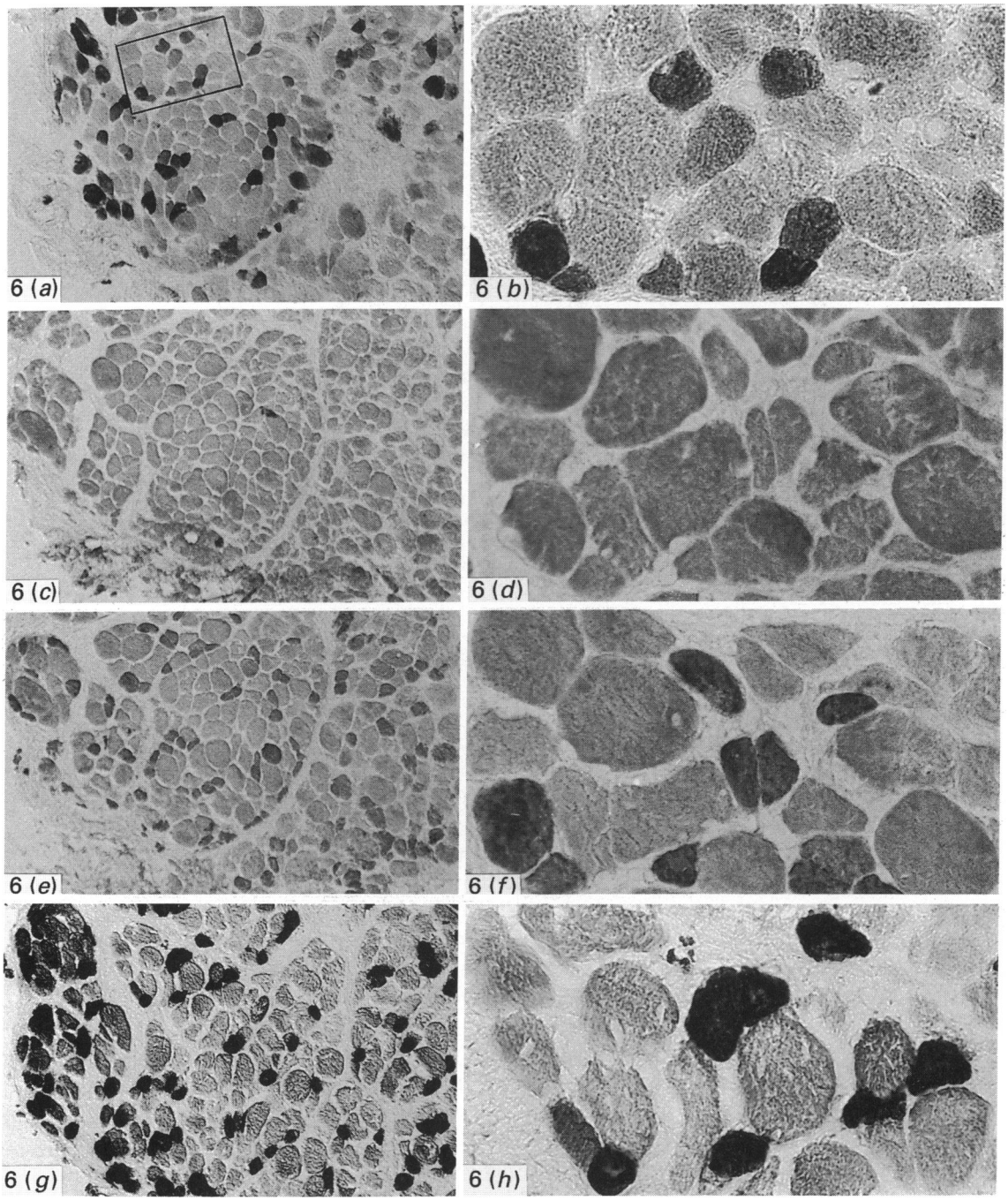


Fig. 6(*a–j*). Anti-heavy chain myosin stainings of serial cross-sections of the stapedius muscle of the rat. On the left: serial sections of, respectively, anti-neonatal (*a*), anti-IIb (*c*), anti-slow plus IIA (*e*), anti-slow (*g*). On the right (*b, d, f, h*): details of the corresponding sections of the area indicated in (*a*). (*i, j*) Detail of ATPase staining (pH 4·35) (*i*) and corresponding area stained with the anti-slow antibody (*j*). See text. (*a*) (*c*) (*e*) (*g*) × 117; (*b*) (*d*) (*f*) (*h*) (*i*) (*j*) × 495.

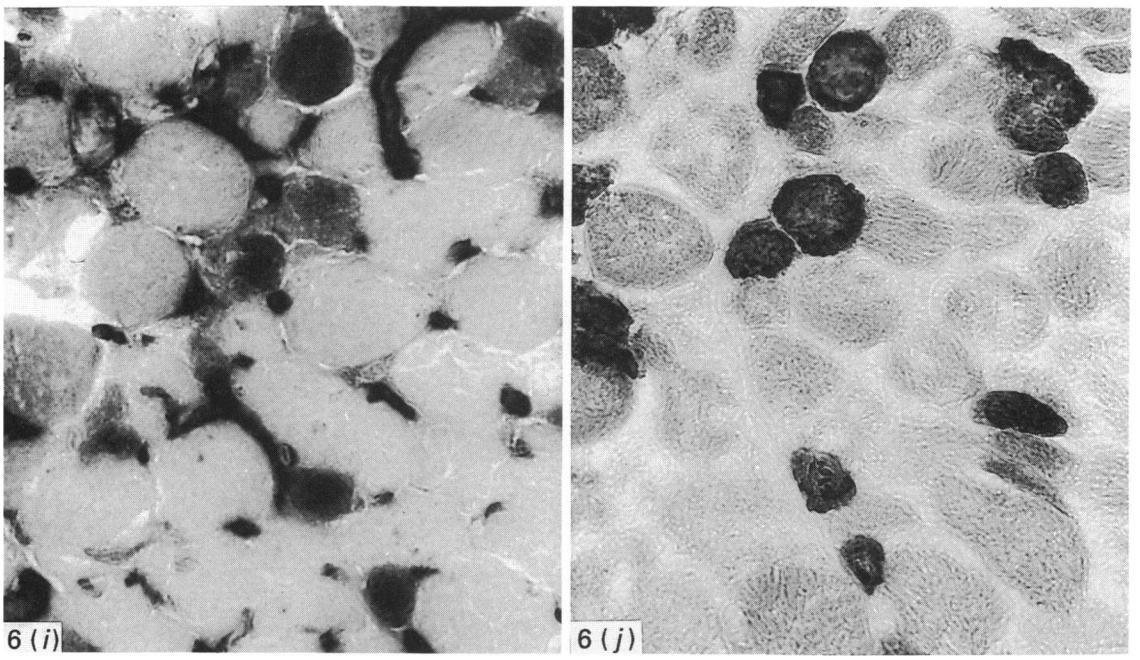


Fig. 6(*i*, *j*). For legend see opposite.

react positively with this antibody. However, not all of these neonatal myosin-positive fibres were positive for anti-slow and *vice versa*.

With the anti-slow plus IIA antibody, all fibres reacted positively. The majority of the fibres showed a moderate staining intensity (63%), while 37% stained strongly (Fig. 6*e*, *f*). Most of the latter fibres also reacted strongly with the anti-slow antibody.

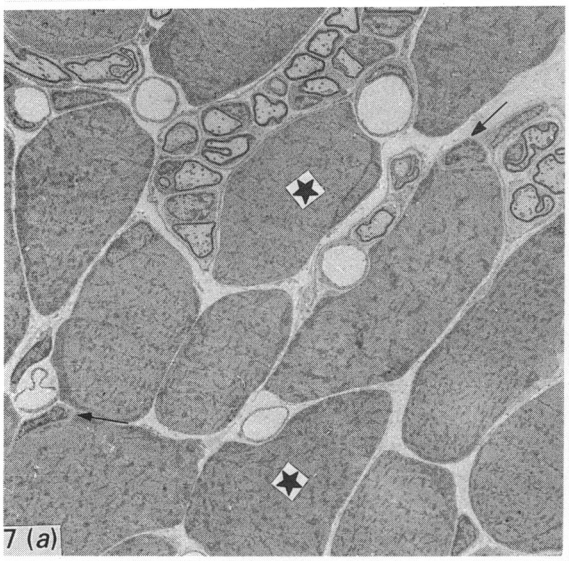
With the anti-IIB antibody all of the fibres showed a weak reaction as compared to the background level.

Staining with the anti-embryonal and the anti-slow tonic antibody did not reveal any positive fibres. However, results with the latter are not fully conclusive since reference stainings of a tensor tympani muscle of a dog, which is believed to contain slow-tonic fibres (Mascarello *et al.* 1982), did not show any positive fibres either.

Electron microscopy

Ultrastructurally the stapedius muscle fibres showed a rather atypical aspect compared to normal skeletal muscle. Several fibres were composed of relatively small bundles of myofilaments as are often found in slow fibres of normal skeletal muscle. Apart from the irregular course of the fibres the mitochondria had an atypical arrangement. Many fibres demonstrated longitudinal clusters of large mitochondria, preferentially localised between bundles of myofilaments. Others showed a less irregular arrangement of mitochondria. However, in general, the regular localisation of mitochondria next to the I-band, as often present in normal skeletal muscle, was not found. Furthermore, aggregations of mitochondria were observed in a subsarcolemmal position and often close to capillaries.

Satellite cells were encountered and an extensive nerve and blood supply was present. The motor end plates showed extensive branched foldings of the postsynaptic



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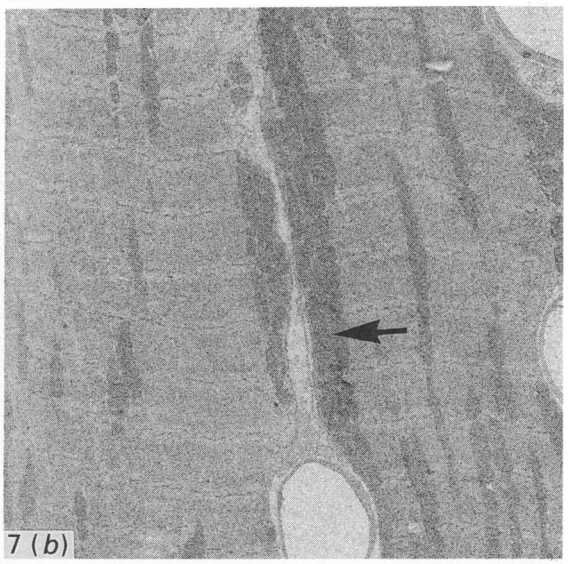

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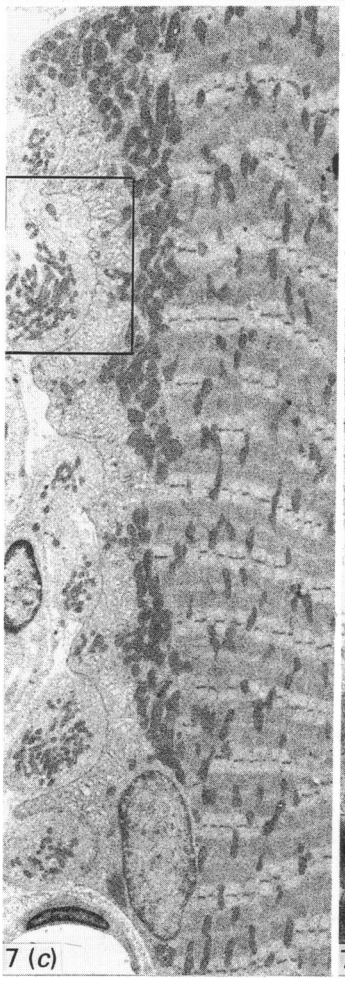

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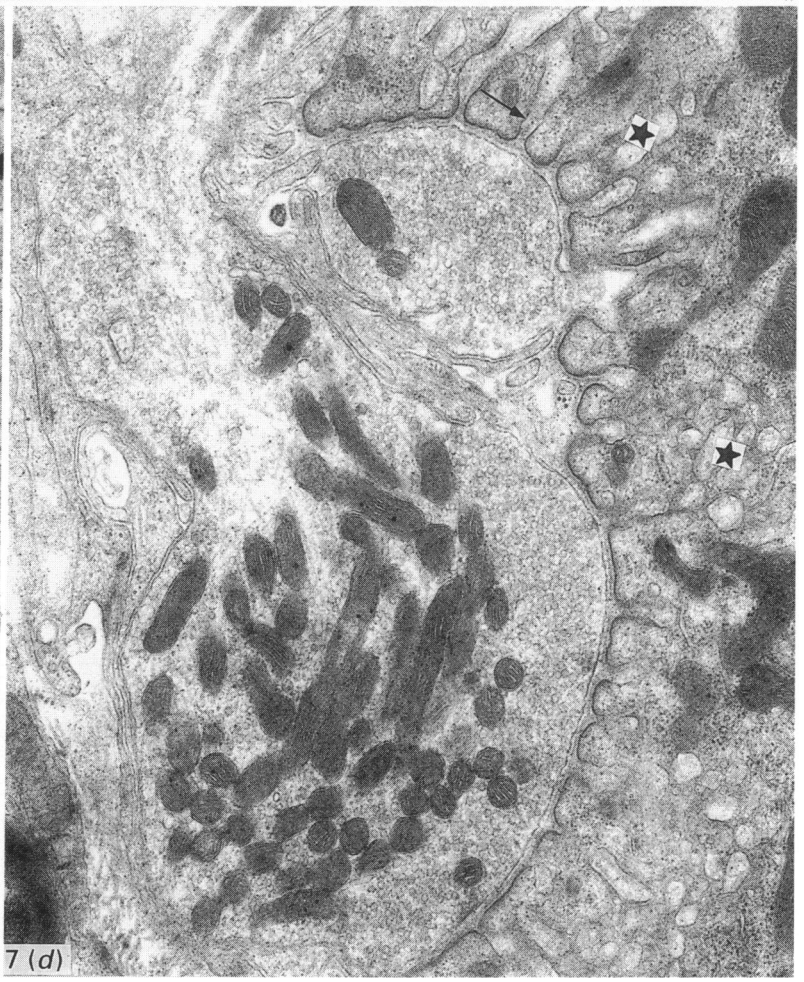

7 (*d*)

membrane resulting in a labyrinthine pattern. The presynaptic area contained many vesicles and mitochondria indicative of a distinct neuromuscular activity.

DISCUSSION

The stapedius muscle of the rat is a very small muscle. Not much is known about its gross morphological structure. The small size and the localisation of the muscle in the bony wall of the bulla render dissection of the whole muscle *in vivo* difficult.

The various morphological features may indicate interesting physiological aspects such as the contraction pattern, contraction force, contraction velocity, and fatiguability. As far as the contraction pattern is concerned, the reconstruction, using serial cross-sections, showed that the muscle is circumpennate and the insertion tendon is situated paracentrally. Based on this structural arrangement, the range of action on contraction must be limited, resulting in a contraction pattern that will be nearly isometric. Considering the fragile position of the stapes in the oval window niche an isometric contraction pattern is indicated.

Compared to the other middle ear muscle, the tensor tympani, the contraction force of the stapedius muscle is probably small. The muscle consists of approximately 300 muscle fibres, whereas the tensor tympani has approximately 1000 fibres (Berge & Wirtz, 1989). However, the mean cross-sectional area of the fibres of the stapedius muscle is greater than that of the tensor tympani muscle fibres, respectively 782 and 259 μm^2 (Berge & Wirtz, 1989). This tendency is also found in sheep, cats, dogs, rabbits, horses, cows and pigs (Vegetti *et al.* 1982). The difference in fibre area between the two muscles is smaller when calculating the stapedius muscle fibre area from the smallest diameter: 490 μm^2 ($\pi r^2 = 3{\cdot}14 \times (25:2)^2$). This may indicate bias in the measurements of the fibre area, possibly caused by measurements on partially obliquely cut muscle fibres.

Next to the number of fibres and the area of the fibres, the ultimate resulting contraction force also depends on the course of the fibres. The serial cross-sections showed the fibres to run radially at the insertion site. This would reduce the ultimate contraction force. However, definite conclusions cannot be made as deformation of the muscle on excision may alter the course of the fibres. To evaluate this, an *in situ* three dimensional reconstruction would be necessary.

Submicroscopically, the structure of the muscle fibres is atypical. The muscle fibres were found to be irregularly shaped, and the bundles of myofilaments were often found to be small. Bundles of myofilaments that interconnect, as was found in the tensor tympani muscle of the rat (Berge & Wirtz, 1989), were not observed. In summary, the morphological findings indicate a smaller maximal contraction force for the stapedius muscle than for the tensor tympani muscle. Indeed, electrophysiological studies in the cat and rabbit confirmed a smaller contraction force for the stapedius muscle (Teig, 1972). Despite this, the attenuation of sound transmission caused by contraction of the stapedius muscle is found to be large (Møller, 1965; Teig, 1973). An explanation for this could be the larger moment developed on contraction of the

Fig. 7 (*a–d*). Electron microscopy of the rat stapedius muscle. (*a*) Transverse section. Note the irregular shape of fibres (asterisks) and extensive nerve supply. Arrows, satellite cells. (*b*) Longitudinal section. Note the longitudinal clusters of mitochondria between the bundles of myofilaments. Arrow, subsarcolemmal mitochondria. (*c*) Longitudinal section of a large motor end plate. (*d*) Detail of the area indicated in (*c*). Note the great number of presynaptic vesicles and mitochondria and the labyrinthine foldings of the post-synaptic membrane (arrow and asterisks). (*a*) × 1250; (*b*) × 3479; (*c*) × 3776; (*d*) × 20089.

stapedius muscle (Teig, 1973). Also as a result of its insertion onto the stapes, the stapedius muscle influences directly the sound transmission from middle to inner ear.

The enzyme histochemical findings indicate the muscle to be adapted for fast contraction as the majority of the fibres appeared to be fast twitch. Only 5% of the fibre population were classified as slow twitch. The ATPase reactions at different pre-incubation pH and the staining for GPox and SDH showed that the fast fibres are Type IIA, i.e. fast oxidative glycolytic fibres. The slow twitch fibres, however, cannot be classified properly as the classical Type I fibres, i.e. slow oxidative, since these fibres were revealed as slow oxidative glycolytic. The immunohistochemical staining also showed atypical findings. Firstly, with the anti-slow antibody, 33% of the fibres reacted positively, in contrast to the 5% slow fibres with the ATPase staining. Furthermore all fibres reacted positively with the anti-slow plus IIA antibody, all fibres reacted positively with the anti-IIB antibody and 28% of the fibres were found to be anti-neonatal-positive. The latter myosin subtype is absent in adult skeletal muscle (Schiaffino *et al.* 1988). It may be considered that cross-reactions cause all these findings. However, in the same series of incubations, stainings of cross-sections of the soleus muscle and EDL muscle of the rat, with a well known enzyme histochemical and immunohistochemical fibre type composition (Zuurveld, Wirtz, Loermans & Veerkamp, 1985; Reggiani, in preparation), reacted as predicted. Therefore it is most likely that in the stapedius muscle of the rat different, but fully differentiated, i.e. heavy chain myosin types, do occur in one fibre. As has been previously suggested (Berge & Wirtz, 1989) the explanation of this could be that genes coding for the various myosin heavy chains continue to be activated, and possibly do so in different nuclei that are contained in the syncytium.

Concerning the fatiguability of the muscle, SDH and GPox stainings showed the muscle to have a relatively large aerobic and anaerobic capacity. The patchy appearance of the fibres in these stainings is caused by the mitochondria being clustered. A relatively rich blood and nerve supply, the latter combined with large end plates containing many presynaptic vesicles, suggests that the muscle has a relatively high resistance to fatigue and an active neuromuscular junction.

On reviewing the overall morphological data it appears that the stapedius muscle of the rat has certain atypical morphological characteristics of which the biological significance is not yet completely understood. However, it becomes clear that the smallest skeletal muscle has properties which possibly bear important physiological consequences; the muscle is presumed to have an almost pure isometric contraction pattern, it is able to contract rapidly and with a relatively great endurance. As comparable findings were made in the tensor tympani muscle, it emerges that the middle ear muscles of the rat are adapted for active functioning over longer periods of time. Both are important properties in the concept of the middle ear muscles being protective to loud impact noise over longer periods. Electrophysiological studies are in progress to evaluate this concept.

SUMMARY

The stapedius muscle of the rat, a very small muscle, appeared to have several atypical morphological characteristics. The muscle fibres were found to be irregularly shaped and composed of relatively small bundles of myofibrils. Mitochondria were distributed irregularly, i.e. in long strands, between the bundles of myofibrils. Enzyme histochemically, most fibres could be classified as fast oxidative glycolytic fibres (IIA). Only 5% of the fibres appeared to be slow twitch fibres; however, these fibres also had

a high glycolytic capacity and therefore could not be classified as the classical slow twitch fibres. The immunohistochemical staining with monoclonal anti-heavy chain myosin isotypes showed discrepancies when compared to the enzyme histochemical results. Moreover, different adult heavy chain myosin isotypes appeared to coexist in one single muscle fibre.

The results suggest that the muscle has an isometric contraction pattern and that it can contract rapidly with great endurance. This endows the muscle with properties suitable for a function in the prevention of noise-induced hearing loss and also in impact noise over longer periods of time.

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REFERENCES

BERGE, H. VAN DEN & WIRTZ, P. (1989). Detailed morphology of the tensor tympani muscle of the rat. An integrated light microscopical, morphometrical, histochemical, immunohistochemical and electron microscopical study in relation to function. *Journal of Anatomy* **164**, 215–228.

BORG, E., COUNTER, S. A. & RÖSLER, G. (1984). Theories about middle ear muscle function. In *The Acoustic Reflex; Basic Principles and Clinical Applications* (ed. S. Silman). Orlando, Florida: Academic Press.

DJUPESLAND, G. (1964). Middle-ear muscle reflexes elicited by acoustic and nonacoustic stimulation. *Acta oto-laryngologica, Suppl.* **188**, 287–292.

JONG, F. I. C. R. S. DE, KINGMA, H., WIRTZ, P., BERGE, H. VAN DEN & MARRES, E. H. M. A. (1988). Indications of a differentiated regulation of sound transmission by the middle ear muscles of the rat. *Journal of Otology* **9**, 70–75.

KOBAYASHI, M. (1956). The comparative anatomical study of the stapedial muscles of the various kinds of mammalian animals. *Hiroshima Journal of Medical Science* **5**, 63–84.

MASCARELLO, F., CARPENÈ, E., VEGETTI, A., ROWLERSON, A. & JENNY, E. (1982). Tensor tympani muscle of cat and dog contains IIM and slow-tonic fibres: an unusual combination of fibre types. *Journal of Muscle Research and Cell Motility* **3**, 363–374.

MASCARELLO, F., VEGETTI, A., CARPENÈ, E. & ROWLERSON, A. (1983). An immunohistochemical study of the middle ear muscles of some carnivores and primates, with special reference to the IIM and slow-tonic fibre types. *Journal of Anatomy* **137**, 95–108.

MØLLER, A. R. (1965). An experimental study of the acoustic impedance of the middle ear and its transmission properties. *Acta oto-laryngologica* **60**, 129–149.

PETER, P. B., BARNARD, R. J., EDGERTON, V. R., GILLESPIE, C. A. & STEMPEL, K. E. (1972). Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. *Biochemistry* **11**, 2627–2633.

SCHIAFFINO, S., GORZA, L., PITTON, G., SAGGIN, L., AUSONI, S., SARTORE, S. & LÖMO, T. (1988). Embryonic and neonatal myosin heavy chain in denervated and paralyzed rat skeletal muscle. *Developmental Biology* **127**, 1–11.

TEIG, E. (1972). Force and contraction velocity of the middle ear muscles in the cat and rabbit. *Acta physiologica scandinavica* **84**, 1–10.

TEIG, E. (1973). Differential effect of graded contraction of middle ear muscles on the sound transmission of the ear. *Acta physiologica scandinavica* **88**, 382–391.

VEGETTI, A., MASCARELLO, F. & CARPENÈ, E. (1982). A comparative histochemical study of fibre types in middle ear muscles. *Journal of Anatomy* **135**, 333–352.

WIRTZ, P., LOERMANS, H. M. TH., PEER, P. G. M. & REINTJES, A. G. M. (1983). Postnatal growth and differentiation of muscle fibres in the mouse. I. A histochemical and morphometrical investigation of normal muscle. *Journal of Anatomy* **137**, 109–126.

ZUURVELD, J. G. E. M., WIRTZ, P., LOERMANS, H. M. TH. & VEERKAMP, J. H. (1985). Postnatal growth and differentiation in three hindlimb muscles of the rat. *Cell and Tissue Research* **241**, 183–192.