Proceedings of the Anatomical Society of Great Britain and Ireland, and the British Biophysical Society

A joint meeting of the Anatomical Society of Great Britain and Ireland and the British Biophysical Society was held at the School of Biomedical Sciences, University of Leeds, from 5th to 7th January 1999. It included a symposium on 'Structure and function of molecular motors' and the Annual General Meetings of both Societies. The following are abstracts of communications and posters presented at the meeting.

TALKS

1 Microdamage as a stimulus for bone remodelling in sheep. By T. C. LEE [1,2] and D. TAYLOR[2]. [1]*Department of Anatomy, Royal College of Surgeons in Ireland, Dublin; and* [2]*Department of Mechanical and Manufacturing Engineering, Trinity College, Dublin, Ireland.*

Bone adapts to mechanical stimuli causing net formation when loading is increased and resorption when it is decreased. In total hip arthroplasty such resorption contributes to loosening of the femoral stem. This study investigates the relationship between mechanical loading, microdamage and bone adaptation. Thirty five skeletally mature Suffolk crossbred sheep were randomly assigned to 3 groups for a sham procedure to expose the ulna (controls), ulnar osteotomy or ulnar pinning under general anaesthesia using thiopentone and halothane. Postoperatively the animals were given intravenous fluorochrome labels at known intervals and allowed to walk freely before being killed at 3, 6, 12 or 24 wk by intravenous sodium pentobarbitone. Postmortem strain measurement showed that cranial and caudal strains in osteotomies were quadruple those of controls at 6 wk but approached control levels by 24 wk. In pinned specimens cranial and surface strains were of the same magnitude as controls but the caudal cortex was in tension, and neither changed over time. Fluorochrome labels revealed 2 adaptive processes: formation of woven bone de novo on the endosteal and periosteal surfaces or modelling, and resorption and intracortical formation of secondary haversian systems or remodelling. The numbers of microcracks, stained with fuchsin, and of resorption cavities were greater in osteotomies than controls ($P < 0.05$) but no significant differences were found between controls and pins. Crack counts were found to be maximal at 6 wk coinciding with a peak in the number of resorption spaces in the osteotomies. Formation of secondary osteons peaked at 10 wk and a formation Sigma of 7.5 wk was calculated. The spatial and temporal associations of cracks, resorption cavities and refilling osteons suggest that microdamage is a stimulus for bone remodelling.

Grant support from the Health Research Board and Royal College of Surgeons in Ireland is gratefully acknowledged.

2 Acute nonsteroidal anti-inflammatory drug therapy and proliferation in murine small bowel epithelium. By P. KIELY and R. R. ETTARH. *Department of Human Anatomy and Physiology, University College, Dublin, Ireland.*

The clinical benefits of nonsteroidal anti-inflammatory drugs (NSAIDs) are limited by their profiles of undesirable side effects especially in the small intestine. The side effect profiles of this group of drugs was explored further by examining the effects on gut kinetics of indomethacin and flurbiprofen (which has a better safety profile than indomethacin), both of which in sufficient doses can induce ulceration in the gastrointestinal tract. Forty five male CD-1 mice aged 8–10 wk were divided into 3 equal groups and given either indomethacin (1 mg/kg body weight intraperitoneally 12 hourly for 36 h) or flurbiprofen (1 mg/kg body weight intraperitoneally 12 hourly for 36 h), or else received no treatment (controls). Following administration of vincristine (1 mg/kg body weight intraperitoneally), animals were killed at 30 min intervals and samples of the distal quarter of the small bowel removed, stained for the Fuelgen reaction and crypts obtained by microdissection. Counts of accumulated metaphases within the crypts were regressed against time to determine the crypt cell production rate and estimated duration of mitosis. The cell production per hour was greater in the indomethacin treated group compared with the cell output in control or flurbiprofen treated groups (controls 7.8 cells/h; flurbiprofen 7.6 cells/h; indomethacin 9.2 cells/h). Cell production in indomethacin treated mice was faster than in either the flurbiprofen treated group or in controls (controls 64.9 min; flurbiprofen 63 min; indomethacin 50.8 min). These findings suggest that short term administration of flurbiprofen does not alter small bowel crypt kinetics in contrast to indomethacin which accelerates and increases crypt cell production.

3 Morphological changes in luminal epithelium of human endometrium during the luteal phase. By S. A. SARANI[1], M. A. WARREN[1], P. DOCKERY[3] and I. D. COOKE[2]. *Departments of* [1]*Biomedical Science and* [2]*Obstetrics & Gynaecology, Sheffield University, UK; and* [3]*Department of Anatomy, University of Cork, Ireland.*

The luminal epithelium of the endometrium is the first contact between mother and implanting blastocyst and yet there is virtually no published information about its morphology. In the present study endometrial tissue was taken from 21 normal fertile women (aged 18–40 y) between 4 and 13 d after the luteinising hormone (LH) surge. Tissue was taken with informed patient consent and Ethics

Committee approval. Pieces of endometrium were processed for embedding in JB4 or Epon resins. For light microscopy 2 μm thick sections were cut and stained with toluidine blue and acid fuchsin. Electron microscopic studies were made on 50–70 nm thick sections stained with uranyl acetate and lead citrate using a Phillips 301 EM. Systematic random samples of luminal epithelium were taken for both light and electron microscopy and examined morphometrically. Throughout the luteal phase there were remarkably few changes in the volume fraction of nucleus, mitochondria, rough endoplasmic reticulum and 'vesicular system' (Golgi, smooth endoplasmic reticulum and vesicles) to cell. Nuclear profile dimensions and cell height did not change over time. Cell and organelle volume (estimated as volume weighted mean volume) also did not change but showed numerically smallest values at day LH + 13, e.g. 1105 ± 172 μm^3 (mean ± standard error) at day LH + 4 and 628 ± 115 μm^3 at day LH + 13. However the ratio of desmosomes to whole cell, 0.039 ± 0.006, and both arithmetic mean thickness 60 ± 3 nm and harmonic mean thickness 62 ± 5 nm of basement membrane were minimal at the time when implantation would be most likely to occur, i.e. about 6 d after the LH peak. Therefore it appears that luminal epithelial cell size changes little during the luteal phase. However specific cellular changes do occur to the basement membrane and desmosomes, which may facilitate embryo implantation. These changes occurred around day LH + 6 and may be a morphological representation of the 'implantation window'.

4 Morphometric studies on BeWo cells as a preliminary to their use as a model of human implantation. By C. S. ABAIDOO, M. A. WARREN, C. PIGOTT and P. W. ANDREWS. *Department of Biomedical Science, Sheffield University, UK.*

Owing to practical and ethical difficulties there is little published information on implantation in humans. However in vitro studies on human trophoblastic cell lines, such as BeWo cells (Patillo & Gey, *Cancer Research* **28**, 1968), may provide information about the early stages of implantation. As part of a larger study on the interaction between BeWo cells and polarised cultures of human endometrial epithelium we have examined BeWo cells qualitatively and obtained estimates of cell volume and number using design based morphometric methods. Additionally comparisons were made between model based and design based morphometric methods since the former are more common in the literature.

BeWo cells were grown as spheroids in suspension culture for between 1 and 7 d and then fixed in 3% glutaraldehyde and processed for microscopy. Serial sections (0.5 μm thick) of spheroids were cut and stained with toluidine blue. Spheroid cell volume was estimated using Cavalieri's principle and cell number obtained by several methods including the fractionator, a combination of dissector and Cavalieri volume estimates, and model based traditional methods since the latter are most often quoted in the literature. In addition some tissue was processed for ultrastructural examination.

Between 1 and 7 d in culture total spheroid Cavalieri volume increased about 20 times to 0.00257 ± 0.0004 mm^3 (mean ± standard error; 6 spheroids per group); fractionator cell number from 108 ± 9 to 1070 ± 60; and mean cell volume from 1.300×10^{-6} to 2.401×10^{-6} mm^3(all $P < 0.01$). Quantitative data show that BeWo cells grow mainly by hyperplasia and the qualitative observations indicate they have marked similarities to human trophoblast cells. These results provide a basis for further studies on the interaction of BeWo cells with cultured human endometrium.

5 Morphometric analysis of the effects of human recombinant growth hormone (rhGH) on the structure of the renal glomerulus of rats subjected to subtotal nephrectomy. By M. SOLEIMANI MEHRANJANI and G. H. COPE. *Department of Biology, Arak University, Islamic Republic of Iran; and Department of Biomedical Science, University of Sheffield, UK.*

Groups of 16 wk old male Lewis rats (4 per group) and age matched growth hormone deficient (dwarf) Lewis rats were subjected to subtotal (5/6th) nephrectomy (SNX) under deep barbiturate anaesthesia. Control groups of rats underwent sham operations. After 30 d some groups of rats received daily subcutaneous injections of human recombinant growth hormone (rhGH, 1.6 I. U. per day) for 30 d while others received only injections of saline. This protocol generated 4 groups of rats for each strain: control, control + rhGH, SNX and SNX + rhGH. All rats were killed after 120 d and their kidneys were perfused with saline, fixed with glutaraldehyde and embedded in JB4 resin. 2 μm thick sections were stained with Masson's trichrome stain and subjected to morphometric analysis using standard sterological procedures. Results were analysed using an analysis of variance routine (ANOVA) and were considered significantly different if $P < 0.05$.

The response to SNX and/or rhGH administration was the same for both the Lewis and dwarf Lewis strain of rat. There were significant increases in glomerular volume compared with the control group, 2 fold following rhGH administration alone, 5 fold after SNX alone and 13 fold when rats were given rhGH following SNX. rhGH administration alone produced 2 fold increase in the volume of most elements of the glomerulus including tuft cells (podocytes and mesangial cells), capillary endothelial cells and tuft matrix. SNX alone caused a 4 fold growth in the volume of these components and rhGH administration after SNX an 8–10 fold increase.

We conclude that rhGH administration alone stimulates growth of most glomerular components, but not to the extent produced by SNX on surviving glomeruli, and that rhGH administered to rats which have undergone SNX has a potentiating effect. We could find no evidence of a difference in response to rhGH administration between the normal Lewis rats and the dwarf strain whose circulating levels of GH are less than 10% of the parent strain.

6 The time course of scatter factor/hepatocyte growth factor induced morphological changes in 3 dimensional epithelial cell cultures. By M. J. WILLIAMS and P. CLARK. *Division of Biomedical Sciences, Imperial College School of Medicine, London, UK.*

Scatter factor/hepatocyte growth factor (SF/HGF) is a multifunctional polypeptide growth factor that has morphogenetic properties. MDCK epithelial cells have been

used extensively to study these properties. It is known that when these cells are cultured in a matrix gel, they form fluid filled inwardly polarised balls of cells (cysts). These cysts form long branching tubules (tubulogenesis) in response to SF/HGF when grown in type I collagen. The composition of the extracellular matrix (ECM) was found to have a profound modulatory effect on SF/HGF induced responses, with branching tubulogenesis being abolished in cysts cultured in Matrigel (a basement membrane substitute). However the cellular mechanisms of these SF/HGF induced responses and the modulation by ECM components remain unclear. Using cysts grown in type I collagen we have investigated the detailed time course of changes to cellular ultrastructure in response to SF/HGF using transmission electron microscopy, and to the organisation of the cytoskeleton and adhesion plaques using confocal microscopy. We have also begun to look at modulatory effects on this system by ECM components. Our preliminary results show dramatic SF/HGF induced modification of the characteristically closely apposed lateral membranes, which we believe to play an important role in the mechanisms that lead to the formation of basal processes and ultimately hollow tubules in cysts. By 6 h SF/HGF induced responses include increased paracellular spacing that contained cellular protrusions. After 6 h basal processes were apparent, consisting of 2 or more cells extending from the most basal points of contact between neighbouring cells. These basal protrusions, which contain large paracellular spaces that appear to connect to the lumen of the cyst, developed into hollow tubules by 24 h. An SF/HGF induced redistribution of actin microfilaments from the basal surface to the lateral membranes was also seen which correlates with the increased lateral paracellular spacing. However the persistence of junctional plaques indicates that strong cell–cell adhesion is maintained during these morphogenetic changes. Our findings suggest that mechanisms governing the formation of epithelial tubules involve the maintenance of close cell–cell contact at the same time as a loss of cellular polarity.

Supported by an Anatomical Society Research Studentship.

7 The role of epithelial-cadherin in mouse gonadal development: a novel in vitro approach. By M. BRITTAN (supervised by S. MACKAY). *Laboratory of Human Anatomy*, *University of Glasgow*, *UK*.

Immunocytochemical studies have previously shown the presence of epithelial-cadherin (E-cadherin) at various stages of mouse gonadal development. Building on these previous studies a novel approach to studying the role of this cell adhesion molecule in both ovarian and testicular morphogenesis was applied in the present investigation to determine its functional significance. Staged embryos were recovered from timed mated pregnant CBA strain mice killed by carbon dioxide inhalation. In a laminar flow cabinet urogenital complexes were dissected out in Hanks buffer. A disaggregation/reaggregation technique was used: gonads separated from mesonephroi were incubated with trypsin/EDTA for 10 min. Enzyme treatment was then arrested with fetal calf serum and gonads were mechanically disrupted by tearing with needles, centrifugation for 1 min at 1000 rev/min and pipetting 10 times. Dissociated tissue was then allowed to reaggregate over a culture period of 2–3 d in Dulbecco's culture medium which contained a 1 in 500 dilution of anti-E-cadherin antibody (experimental cultures) or rat serum (control cultures). Cultures were monitored by phase contrast microscopy and fixed and processed at the end of the culture period for light and electron microscopy. Initially testes from embryos at 15–19 d post coitum (dpc) and ovaries at 17–19 dpc were utilised; the later stages proved more profitable to use for further experiments with this technique. Control cultures showed evidence of successful reaggregation in vitro: light microscopy showed that the tunica albuginea, testicular cords and ovarian follicles had successfully reformed in vitro. Electron microscopy showed the presence of many cell junctions in these cellular aggregates. Though experimental cultures did reaggregate to form a cell pellet, cord and follicle organisation was absent and cell junctions were rarely seen. In conclusion E-cadherin has a role in cell junction formation and establishment of the morphological structures characteristic of each sex at all stages investigated.

8 A quantitative analysis of mononuclear cells with subplasmalemmal linear densities in a peripheral nerve autoimmune rabbit model. By E. WESTON-PRICE (supervised by C. L. CRAWFORD). *Department of Neuromuscular Diseases*, *Division of Neuroscience*, *Imperial College of Science*, *Technology and Medicine*, *London*, *UK*.

With many granulomatous diseases subplasmalemmal linear densities (SPLDs) have been associated. These SPLDs are characterised by a layer of electron dense material and often have a thin layer of extracellular material, resembling basal lamina in appearance. SPLDs are found in mononuclear cells in diseases such as multiple sclerosis and sarcoidosis, including neurosarcoidosis. They have not been reported in any animal model of granuloma or in models using myelin antigens such as experimental allergic neuritis or experimental allergic encephalomyelitis (EAE). EAE is considered to be the most appropriate animal model for multiple sclerosis. In this investigation various peripheral nerve antigens were injected intradermally into 2 Dutch Bantam rabbits, previously sensitised with injections of human sensory peripheral nerve. The experiments were originally erformed as a model of human tuberculoid leprosy which is a granulomatous disorder. Electron microscopic analysis of biopsies from these skin test sites showed the presence of a large number of SPLD containing mononuclear cells, both with whole suspensions of sensory nerve and especially with a deoxycholate extracted fraction from the non-myelin 'nuclear' pellet in doses of only 1 μg of protein. Skin tests with myelin even in doses of 9 mg of protein only produced a small number of mononuclear cells with SPLDs, as did skin tests with sural nerve in an unsensitised English Lop Rabbit. An additional feature not previously reported was the presence of SPLDs in mononuclear cells which had very long cytoplasmic processes. These were most prominent in the mononuclear cells when the deoxycholate fraction was used as the antigen, and were even observed in the endoneurium of dermal nerves. This study confirms the

result of a previous investigation with the additional inclusion of a control in an unsensitised animal. Thus an animal model in which cells with SPLDs could be demonstrated would in one respect be a more precise model for diseases such as multiple sclerosis, than those such as EAE. A nonmyelin antigen might be implicated in the pathogenesis of this disease. The function of SPLDs is unknown, but they are best regarded as markers of important human diseases such as multiple sclerosis, AIDS encephalopathy and sarcoidosis.

9 Terminal Schwann cells show distinct morphological changes in parallel to nerve terminal withdrawal at axotomised adult C57BL/Wlds mouse neuromuscular junctions. By N. P. GANDHI (supervised by S. H. PARSON). *School of Biomedical Sciences*, *University of Leeds*, *UK*.

Terminal Schwann cells are the nonmyelinating glial cells of the peripheral nervous system which are closely associated with underlying neuromuscular junctions. Recent evidence suggests that these glial cells have an important role in the maintenance, repair and rearrangement of synapses between nerve and muscle.

We have previously demonstrated that nerve terminals remain viable for up to 48 h subsequent to nerve section in vivo and in vitro. This study examines the morphology of terminal Schwann cells using double labelling (S100/SV2 or S100/α-bungarotoxin) immunofluorescence techniques in nerve-muscle organ culture preparations isolated from the slow wallerian degeneration mutant C57BL/Wld^s mouse. In freshly dissected preparations terminal Schwann cells have an altered morphology with an indistinct cell body and cytoplasmic projections closely matching the shape of the underlying endplate. After 2 d in culture, Schwann cells at only 5.00 ± 0.65% (mean $\pm$ S.E.M., n = 200 endplates from 4 animals) of endplates retained this morphology. The majority of terminal Schwann cells had an altered morphology, manifested as withdrawal of processes and migration away from the endplate. When we compared the morphology of Schwann cells to that of the nerve terminal we found that in the majority of cases nerve terminals had withdrawn terminal boutons from endplates and that it was at these terminals where altered Schwann cell morphology was seen. In each case we found that Schwann cells were associated with remaining boutons and did not extend beyond them. This suggests that Schwann cell projections had been withdrawn from areas of endplate which had lost boutons. We have quantitatively assessed these preparations: at 25.60 ± 0.25% of endplates Schwann cell nuclei were more prominent and some projections were withdrawn. While at a further 61.8 ± 1.6% of endplates Schwann cell projections had been withdrawn such that only the cell body was left above remaining boutons. Finally, we did not find any naked boutons. We suggest that changes in Schwann cell morphology are closely linked to the piecemeal withdrawal of nerve terminal boutons in culture, and that this may be as a result of repulsive properties at endplate regions.

N.P.G. was supported by grants from the Wellcome Trust and Wolfson Foundation.

10 Axonal chanes in sensory cutaneous peripheral nerves in an autoimmune guinea pig model using nonmyelinated sensory nerve antigen. By N. FURTADO (supervised by C. L. CRAWFORD). *Department of Neuromuscular Diseases*, *Division of Neuroscience*, *Imperial College of Science*, *Technology and Medicine*, *London*, *UK*.

Thials is an electron microscope study of the axonal pathological changes that are present in dermal nerves of a strain specific autoimmune animal model. Four Strain 13 and 2 Strain 2 guinea pigs were injected at age 3 mo with human nonmyelin nerve antigen and skin lesions, similar in histological appearance to tuberculoid leprosy, were discovered on the animal proximal to the site of injection. These lesions were only present on Strain 13 animals and were absent from the control Strain 2 animals. Skin biopsies were taken 6–12 wk after injection from lesions on the Strain 13 guinea pigs and from similar sites on the Strain 2 animals. All samples were examined using a Philips CM10 electron microscope. This experimental animal model provides a method of studying axonal pathological changes without the use of invasive chemical procedures or direct trauma to the nerve.

This study shows that there are characteristic axonal changes in the experimental Strain 13 guinea pigs that were not observed in the Strain 2 animals. A total 8 biopsies from the Strain 13 animals were examined of which 5 were found to contain nerves. Of these 5, all were found to contain abnormalities within the axons that were not visible in the Strain 2 skin biopsies. Pathological changes were largely limited to myelinated nerves. Central accumulations of microtubules were visible which indicate a disruption of axonal flow. Multiple foci of glycogen granules and neurofilamentous accumulations were observed. Mitochondrial pathology could be seen and previous studies have recognised this change as a nonspecific sign for axonopathy.

The similarities of these findings with recently found acute axonal changes in the pathology of multiple sclerosis lesions suggests that this protocol using nonmyelin nerve antigen is a good peripheral nerve model for the disease. Future studies could be performed using a similar antigen in the central nervous system which would serve as a model for multiple sclerosis.

11 A biomechanical evaluation of orbital blowout fractures. By F. AHMAD (supervised by L. J. GAREY). *Division of Neuroscience*, *Imperial College School of Medicine*, *London*, *UK*.

Since the first description of orbital blowout fractures (Lang, *Trans. Ophth. Soc. UK* **9**, 1889), there has been much confusion as to their aetiology. Two principal mechanisms have been proposed to explain their production: the buckling and the hydraulic mechanisms, caused respectively by trauma to the orbital rim and the globe of the eye. The aim of this study was to evaluate both mechanisms qualitatively and quantitatively. It was carried out as anatomical research on bodies donated under the Anatomy Act. Our protocol uses intact cadavers, quantifiable intraocular pressure, variable and quantifiable force, and quantifiable bone strain distribution with strain gauge analysis in the evaluation of

the 2 proposed mechanisms. Trauma was directed onto the globe or onto the rim of the orbit using a specially designed drop tower. Strain gauges were applied to the roof of the maxillary sinus through an anterior antrostomy. We present the results of a study undertaken on 6 female and 3 male fresh human cadavers between 60 and 80 y of age, with one orbit of each cadaver used to simulate 1 of the 2 mechanisms. Fractures produced as a result of the buckling mechanism were limited to the anterior part of the orbital floor in each one of the 9 orbits. In contrast, the hydraulic mechanism produced fractures that were much larger, always involving the posterior part of the orbital floor and medial wall of the orbit. Data from the strain gauges supported these qualitative findings. Following simulation of the buckling mechanism strain in the anterior part of the orbital floor was in excess of 3756 $\mu\epsilon$, while posteriorly distributed strain did not exceed 221 $\mu\epsilon$. In contrast the hydraulic mechanism resulted in strain distributed both anteriorly ($> 3756\ \mu\epsilon$) and posteriorly ($> 3756\ \mu\epsilon$) in the orbital floor. Furthermore we have demonstrated that the average kinetic energy required to fracture the orbital floor by the buckling mechanism is 1.54 J while an average of 1.22 J is needed to produce this fracture by the hydraulic mechanism. Our results suggest that efforts to establish one or other mechanism as the primary aetiology might be misplaced. Both mechanisms produce orbital blowout fractures, but with different and specific characteristics. We believe this provides a basis for a reclassification of such fractures.

12 Structure and mechanism of myosin-S1. By K. C. HOLMES. *Max Planck Institut für Medizinische Forschung, Heidelberg, Germany*.

Crystallographic studies of chicken myosin by Rayment et al. showed the myosin cross bridge to be pear shaped, with an elongated head (the 'motor domain') containing a 7-stranded β-sheet, and a C-terminal 'neck'. Numerous α-helices which surround the β-sheet form a deep cleft joining the nucleotide binding site to the actin binding site. The active site contains a P-loop and switch 1 and switch 2 regions as in G-proteins. The C-terminal neck which would be connected with the thick filament forms an extended α-helix which binds 2 'light chains'. The neck is joined onto the motor domain via a compact domain, the 'converter'. The neck appears to function as a lever arm. Studies by Rayment's group of a truncated form of the cross bridge (without the neck) from *Dictyostelium* (cellular slime mould) myosin II with an assortment of nucleotide analogues reveals 2 conformations of myosin S1 entailing a movement of 5–6 Å of switch 2. This movement opens and closes the active site around the γ-phosphate. ADP.vanadate (a transition state analogue) produces the closed form and shows a coupled movement of the converter domain (a 60° rotation). In Rayment's experiments ADP.BeF3 (an ATP analogue) produced the open form. However with ADP.BeF3 in the active site we have obtained the closed form. The opening and closing of the switch 2 region in the active site appears to respond to the presence and loss of the γ-phosphate. Building the truncated neck back onto these 2 myosin structures shows that the end of the neck would move about 110 Å in an axial direction in response to the opening and closing of the active site. The neck really is a lever arm and this movement in response to loss of phosphate appears to represent the power stroke. A recent determination of a truncated smooth muscle myosin from Carolyn Cohen's group but with the essential light chain still present fully supports this interpretation.

13 X-ray diffraction studies of the structure-function relation of the myosin motor in muscle. By M. IRVING[1], I. M. DOBBIE[1], J. J. HARFORD[1], Y.-B. SUN[1], M. A. FERENCZI[2], N. KOUBASSOVA[3], P. BOESECKE[4], O. DIAT[4], T. NARAYANAN[4], G. BALDUCCI[5], M. LINARI[5], L. LUCII[5], G. PIAZZESI[5], M. RECONDITI[5], M. E. VANNICELLI[5] and V. LOMBARDI[5]. [1]*King's College London, UK.*; [2]*National Institute for Medical Research, London UK.*; [3]*Moscow State University, Russia*; [4]*European Synchrotron Radiation Facility, Grenoble, France*; *and* [5]*Università di Firenze, Italy*.

The myosin head domains in actively contracting muscle spend part of their ATPase cycle time in a group of conformations that is characterised by a precise periodicity of 14.56 nm along the filament axis, producing the intense M3 x-ray reflection. The properties of the M3 reflection suggest that this population of heads is bound to actin, bears the force of active muscle, and responds to sliding between the actin and myosin filaments. The synchronised motions of these myosin heads produced by imposing rapid filament sliding has an elastic component corresponding to a distortion of each head by 2 nm under the force of isometric contraction, a 'working stroke' component corresponding to the force generating conformational change in the myosin head, and slower components related to detachment from actin and subsequent steps. The elasticity of the head can be quantitatively explained by bending of its light chain domain, and the working stroke by a 30° tilting of the light chain domain of the head with respect to its catalytic domain, bringing it close to the rigor conformation. The combination of this ATP-driven tilting and the passive elasticity of the myosin head explains force generation in isometric muscle, when filament sliding is prevented.

Supported by MRC (UK); CNR, Telethon (Italy), EC, Intas; many of the experiments used the non-crystalline diffraction facilities at CLRC Daresbury Laboratory.

14 Structure and mechanism of ATP synthase. By J. E. WALKER. *MRC Laboratory of Molecular Biology, Cambridge, UK.*

The atomic structure of F_1-ATPase suggested that the concerted interconversion of the 3 catalytic sites through the 3 states 'open', 'loose' and 'tight' defined by Boyer's binding change mechanism is mediated by the rotation of the central γ-subunit. This rotation has been visualised by microscopy. In the lecture the following questions will be addressed. Does the solved atomic structure represent an intermediate in the catalytic cycle? What is the nature of the transition state? How is rotation generated and coupled to the γ-subunit? Which parts of ATP synthase rotate and which are static? How did ATP synthase evolve?

15 F_1-ATPase: a rotary stepper motor that can work at near 100% efficiency. By K. KINOSITA, Jr. *Department of Physics, Keio University, Yokohama and CREST 'Genetic Programming' Team* 13, *Teikyo University Biotechnology Research Center, Kawasaki, Japan.*

A single molecule of F_1-ATPase is by itself a rotary motor in which a central γ subunit rotates against a surrounding cylinder made of $\alpha_3\beta_3$ subunits. Driven by the 3 βs that sequentially hydrolyse ATP the motor rotates in discrete 120° steps, as demonstrated in video images of the movement of an actin filament bound as a marker to the central γ (Yasuda et al. *Cell* **93**, 1998). Over a broad range of load (hydrodynamic friction against the rotating actin filament) and speed the F_1 motor produces a constant torque of ~ 40 pN·nm. This torque multiplied by $2\pi/3$ radians (= 120°), ~ 80 pN·nm, is the work done in a 120° step. Because one ATP molecule is hydrolysed in each step, the work per ATP is also ~ 80 pN·nm. On the other hand the free energy of ATP hydrolysis in cells is ~ 80 pN·nm per molecule of ATP. Thus, the F_1 motor appears to work at near 100% efficiency. We confirmed in vitro that F_1 indeed does ~ 80 pN·nm of work under the condition where the free energy per ATP is 90 pN·nm. The high efficiency may be related to the fully reversible nature of the F_1 motor: the ATP synthase, of which F_1 is a part, is considered to synthesise ATP from ADP and phosphate by reverse rotation of the F_1 motor. Interestingly the F_1 motor occasionally makes back steps. The work done in each back step is also ~ 80 pN·nm suggesting that the back step also consumes one ATP molecule.

16 Nucleotide-dependent movement in kinesin and related proteins. By L. A. AMOS, K. HIROSE, J. LÖWE, U. HENNINGSEN, M. SCHLIWA, M. ALONSO and R. A. CROSS. *MRC Laboratory of Molecular Biology, Cambridge, UK.*

In order to understand how the microtubule motor proteins kinesin and ncd move along microtubules in opposite directions we are using cryo-electron microscopy and 3D reconstruction methods to study the structures of dimeric molecules, including chimaeras of the 2 proteins, bound to microtubules in different nucleotide states. In the presence of ADP, AMP.PNP (assumed to be ATP like) or without nucleotides, all the 3D maps showed 2 heads (motor domains) associated with each tubulin dimer, one attached directly and the other tethered to it. In the case of kinesin the positions of the tethered head were on the upper right of the bound head with AMP.PNP but on the upper left without nucleotides or with ADP. The way the bound head attaches to tubulin is different for ADP, a weakly bound state, compared with the other 2 states. The bound head of dimeric ncd shows the same interactions with tubulin. The ncd tethered head always sits to the right of the bound head though it moves up and down as the bound nucleotide varies. One important difference is that ncd heads appear to be connected via their topmost points in the strongly bound states but in the ADP state the connection, via the N-terminal neck domain, is one third of the way down one side as in the crystal structure of dimeric ncd complexed with ADP. A chimaeric homodimer consisting of ncd heads attached at their C-termini to kinesin neck domains resembles kinesin in the AMP.PNP state but in the empty state the tethered head remains on the upper right of the bound head. The results indicate that interactions between motor domains and either the ncd or kinesin neck domains vary according to the nucleotide present. But the ncd motor domain cannot substitute for kinesin's in all interactions.

17 Conserved molecular motors in DNA replication and recombination. By E. H. EGELMAN. *Department of Cell Biology and Neuroanatomy, University of Minnesota Medical School, Minneapolis, USA.*

The prokaryotic RecA protein has been the most intensively studied enzyme in homologous genetic recombination and is active in the catalysis of strand exchange between 2 DNA molecules that share sequence homology. We now understand that eukaryotic cells employ a related protein, Rad51, in DNA recombination and repair. It has become clear over the past several years that the nucleotide binding core of the RecA protein is structurally homologous to the core of a large number of seemingly unrelated proteins, from the F_1-ATPase to DNA helicases. We have been studying both RecA/Rad51 helical filaments and hexameric ring helicases using electron microscopy and computed image analysis. We have been able to show that the hexameric rings can act in different manners in many aspects of DNA metabolism including replication, recombination, repair and transcription. Most of our structural information has come from the *E. coli* DnaB protein and the bacteriophage T7 gp4b protein (both active in DNA replication), the *E. coli* RuvB protein (active in DNA recombination), and the E. coli rho protein (involved in transcription termination). What all of these hexamers have in common is that they use the energy of ATP hydrolysis to either walk along DNA or pump DNA through the ring. In contrast RecA uses the energy of ATP hydrolysis to undergo a conformational change, switching the protein into a DNA binding state with low affinity. The F_1-ATPase uses ATP hydrolysis to support rotational motions of the γ subunit, employing loops that are topologically equivalent to the loops in the RecA protein that bind DNA. We suggest that all these structures have diverged from a common ancestral protein and that during the course of this divergence there has been an enormous divergence of function. Elements of mechanochemical transduction have been conserved, but a detailed understanding of how the RecA filament works may tell us little about how a structurally homologous ring helicase functions.

18 The role of the unconventional myosins in membrane trafficking. By F. BUSS[2], T. HODGE[1], C. LIONNE[1], J. P. LUZIO[2] and J. KENDRICK-JONES[1]. [1]*MRC Laboratory of Molecular Biology and* [2]*Department of Clinical Biochemistry, University of Cambridge, UK.*

Fifteen classes of myosins have now been identified (at least 14 different myosins are expressed within a single cell) but little is known about their mechanisms or functions. We have focused on the myosins in classes I, V and VI since they are believed to be involved in cells in dynamic membrane reorganisations forming ATP dependent linkages between membranes and the actin cytoskeleton. In mammalian cells

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

there is little precise information about the intracellular localisation and function of myosin VI so we initially raised polyclonal antisera to a variety of bacterially expressed domains of chicken intestinal brush border myosin VI B. Affinity purified antibodies against the unique C-terminal tail domains of this myosin recognise myosin VI in several cell lines including NRK and A431 by immunoblotting and immunoprecipitation. Using these antibodies in immunofluorescence (IF) and immuno EM, it was found that myosin VI is associated with the Golgi complex and the leading ruffling edge of the cell as well as being present in a cytosolic pool. Immuno EM of frozen thin sections of NRK cells showed myosin VI concentrated at the plasma membrane, especially enriched in areas with dynamic membrane protrusions such as lamellipodia or filopodia. When surface ruffling was induced in A431 cells by EGF myosin VI was recruited into the newly formed ruffles together with ezrin and myosin V. EGF stimulation leads to a 3–5 fold increase in phosphorylation of both ezrin and myosin VI. In vitro results suggest that a rac/p21-activated (PAK) kinase maybe responsible for phosphorylating the myosin VI in vivo. The site of phosphorylation is within the head (motor domain) possibly in the region involved in actin binding. Thus the localisation of myosin VI at the Golgi and at the leading ruffling edge of the cell suggests that it might play a role in membrane trafficking in secretory and/or endocytic pathways between these cellular compartments possibly mediated by one of the signal transduction pathways. Similar studies are underway to probe the roles in membrane trafficking of myosin IB, which gives a striking vesicular IF staining pattern in the perinuclear region in NRK cells, and myosin V which is also recruited into membrane ruffles on EGF stimulation of A431 cells.

19 Myosin under the microscope. By P. B. CONIBEAR and C. R. BAGSHAW. *Department of Biochemistry*, *University of Leicester*, *UK*.

In order to quantitate the coupling between ATPase activity and filament sliding in actomyosin in vitro motility assays, we have attempted to measure the sliding of rhodamine-phalloidin actin over rabbit skeletal myosin filaments or heavy meromyosin (HMM) tracks simultaneously with the turnover of the fluorescent substrate Cy3-ATP using total internal reflectance fluorescence (TIRF) microscopy. Silica surfaces give a low TIRF background but are poor for supporting sliding on tracks, suggesting that myosin heads bind to the surface in an inactive conformation or molecules may dissociate to leave a surface which is too sparse to support motility.

To assess the reversible detachment of myosin head fragments from a silica surface Cy3-ADP was trapped at the active site using AlF_4. Single Cy3 fluorophores were visible under the TIRF microscope. Upon illumination most of the fluorophores photobleached but occasionally spots reappeared suggesting some protein molecules detached and reattached elsewhere on the surface on the minutes time scale. The latter would contribute to fluctuations observed in single molecule Cy3-ATP turnover measurements with myosin subfragments. Dissociation may also lead to sparse HMM tracks.

Synthetic filaments support actin sliding but initiation of the process by flash photolysis of caged ATP most frequently led to dissociation. We have therefore examined the properties of these filaments. Myosin molecules trapped with Cy3-ADP and AlF_4 were incorporated into synthetic thick filaments by co-polymerising with an excess of myosin molecules pretrapped with unlabelled ADP. Such copolymers show punctate fluorescence with spots greater than 1 μm apart when the labelling ratio exceeds 1:300, indicating the myosin head density is comparable to that of native thick filaments. In some instances the individual spots within a filament array fluctuate in intensity but they are positionally stable. To explore the reversible dissociation of myosin molecules from a synthetic filament separate samples were labelled with near stoichiometric amounts of Cy3-ADP.AlF_4 and Cy5-ADP.AlF_4. On mixing the 2 filament preparations (in the presence of a large excess of ADP to prevent any reincorporation of fluorescent nucleotide) slow exchange of myosin monomers was observed on the hours time scale throughout the length of the filament. However exchange was incomplete after 48 h suggesting the synthetic myosin filaments comprise a stable core coated with some slowly exchangeable (adventitious?) myosin.

20 Probing actomyosin interactions with actin mutants in the myosin binding site. By J. SPARROW[1], S. SCHMITZ[1, 2], A. RAZZAQ[1], K. MOGAMI[3], U. NONGTHOMBA[1], and M. GEEVES[2]. [1]*Department of Biology*, *University of York*, *UK*., [2]*Max PIanck Institute for Molecular Physiology*, *Dortmund*, *Germany*, *and* [3]*Department of Physics*, *University of Tokyo*, *Japan*.

We use the indirect flight muscles (IFMs) of the fruitfly *Drosophila* for genetic studies of muscle function. One of our aims is to study mutants in the IFM specific actin gene, *Act88F*, to understand the molecular details of the cross-bridge cycle and its regulation. We isolate mutations by either selecting for flightless flies or by in vitro mutagenesis of the cloned gene followed by germline transformation for expression in the IFMs. We report the effects of a number of mutants on actin and arthrin accumulation in vivo, polymerisation of F-actin in vitro and actomyosin interactions.

We have found that (1) a number of actin mutants show reduced in vitro motility, though this group of mutants is not restricted to that part of the actin surface believed to encompass the myosin binding site. This suggests that mutations outside the myosin binding site can influence myosin interactions. This might be through conformational changes occurring within the actin monomer or perhaps through changes in the properties of the F-actin filament. (2) Two mutants, *E93K* and *R95C*, in the proposed secondary myosin binding site affect in vitro motility and the K_d and k_{on} of actomyosin binding, confirming that these residues are involved in myosin binding. (3) E93K actin shows increased salt sensitivity of in vitro motility suggesting a role in weak binding or in weak to strong binding transition, but unlike primary site charge mutants (see Miller & Reisler, *Biochem*. **34**, 1995; Miller et al. *Biochem* **35**, 1996) the effects are not reversed by α-methyl cellulose, indicating that primary site actomyosin interactions precede those at the secondary site. (4) The *E93K* actin also affects the position of tropomyosin in vitro on the thin filament, a finding confirmed by its partial suppression of a TnI mutant in vivo.

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

21 Walking mechanism of the nkin molecular motor. BY I. CREVEL[1], N. CARTER[1], M. SCHLIWA[2] and R. CROSS[1]. [1]*Marie Curie Research Institute, Oxted, UK., and* [2]*Adolph Butenandt Institut für Zellbiologie, University of Munich, Germany.*

We report single molecule mechanical evidence that the unusually fast *Neurospora* kinesin (nkin) microtubule molecular motor can walk, and show using single turnover solution kinetics how the mechanical and chemical kinetic actions of the 2 heads are linked. Two headed recombinant nkin moves in 8 nm steps and stalls at about 5 pN of retroactive optical trapping force. Microtubules (MTs) moving over a single surface attached nkin molecule make processive runs of several hundred steps and spin freely when pushed with an optically trapped bead. The MT-motor link becomes torsionally stiff on freezing the motion with AMP.PNP indicating double headed attachment. The chemical kinetic coordination of mechanical stepping differs from that proposed for kinesin. The repetitive cycle rate in solution is about 38 s^{-1} (20 °C). After labelling nkin with mantATP and mixing with MTs and an excess of non-fluorescent ADP, mantADP is released rapidly and quantitatively from one head (30–53 s^{-1}) but very slowly (4 s^{-1}) from the other head. In the absence of added ADP release from the second head is essentially blocked implying that ADP binding to the first head accelerates ADP release from the second head, but only slightly. The nonhydrolysable analogue AMP.PNP induces second head mantADP release at only 6 s^{-1}, whereas the rate is 60 s^{-1} for ATP and 30 s^{-1} for the more slowly hydrolysed analogue ATPγS. We infer that the coordinated walking action of nkin is a result of leading head attachment awaiting nucleotide hydrolysis on the trailing head, and of subsequent detachment of the trailing nkin.ADP head being accelerated by a tug from the attaching leading head. The mechanism is different from that of the slower kinesin, for which it has been proposed that ATP binding rather than ATP hydrolysis is the trigger for leading head ADP release.

22 DNA tracking by type I and type III restriction endonucleases. By M. SZCZELKUN. *Department of Biochemistry, University of Bristol, UK.*

The type I and type III restriction endonucleases are large molecular weight oligomeric proteins which protect bacteria from invasive DNA particles. Both types of endonuclease recognise specific asymmetric DNA sequences but subsequently cleave DNA at nonspecific loci separate from the recognition sites. For type I enzymes, this can be up to 7000 bp away from the site, whilst the type III enzymes cleave ~25–27 bp 3′ to the site. In each case DNA restriction relies on both ATP and Mg^{2+} cofactors. The long range interaction between recognition and cleavage sites was examined on interlinked rings of DNA (catenanes). The results demonstrated that the communication cannot stem from random looping through 3D space but must follow the 1D DNA contour between sites in a process called 'DNA tracking'. During tracking an enzyme remains bound to its recognition site whilst simultaneously translocating adjacent nonspecific DNA past itself, thus extruding an expanding loop of DNA. Subsequent DNA cleavage is triggered in different ways. Type I reactions on linear DNA require a minimum of 2 sites with cleavage occurring wherever a pair of translocating enzymes collide. However on circular DNA a single site is adequate suggesting that an increase in DNA supercoiling generated by tracking eventually arrests motion, triggering DNA cleavage. On the other hand the type III enzymes have an absolute requirement for 2 sites in 'head-to-head' orientation, with cleavage occurring when the tracking enzymes collide.

To provide an unequivocal demonstration of motion along DNA by the endonucleases, an optical tweezers assay is being developed in collaboration with Dr Justin Molloy (University of York). Long DNA substrates (11–15 kbp) are produced by PCR using forward and reverse primers labelled with biotin and digoxygenin (DIG) respectively. The resulting substrates are suspended between 2 latex beads (0.5–1.5 μm diameter) labelled with streptavidin or DIG antibodies. With the bead-DNA-bead complexes held in twin beams of an optical tweezers transducer, any tracking events which shorten the DNA contour length can be monitored by displacement of the attached beads in the optical traps. In the long term the aim is to equate rates for DNA tracking to those for ATP hydrolysis, in order to provide a detailed insight into the coupling of chemical energy to physical motion along DNA.

23 Tubulin mutants in the yeast *Schizosaccharomyces pombe*. By D. R. DRUMMOND and R. CROSS. *Marie Curie Research Institute, Oxted, UK.*

Kinesin and kinesin related motor proteins move by a process involving attachment and detachment from the surface of the microtubule in a cycle which depends on the energy derived from the motor's ATPase activity. A complete description and understanding of how a kinesin motor protein works must include the role of the microtubule in the process. Since molecular structures have now been determined for both kinesin and tubulin it is possible to begin modelling the interaction of the 2 proteins at the molecular level. To enable us to test such models we are developing a system in which it is possible to examine the role of individual tubulin residues in the motor-microtubule interaction. We are utilising the fission yeast *S. pombe* which contains only 2 isoforms of α tubulin and a single β tubulin. This coupled with the ability to manipulate the yeast genetically makes it possible to determine the effect of single amino acid changes on the function of tubulin in vivo.

Our initial studies have focused on α tubulin. Both α and β tubulin contain α helices close to their carboxytermini which are predicted to lie on the surface of the microtubule (Nogales et al. *Nature* **391**, 1998) and may have a role in the interaction of tubulin with kinesin. We have carried out alanine scanning mutagenesis of the charged amino acids within these helices. To analyse the effect of the mutations we have expressed the tubulin as a fusion with the green fluorescent protein which enables visualisation of the microtubules within the living cell. Using confocal fluorescence microscopy we can follow both the dynamic instability of interphase microtubules and the formation and elongation of the mitotic spindle. This latter process depends on the activity of kinesin related proteins and we now plan to screen for mutants which affect this motor dependent process but still permit microtubule assembly.

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

This approach will enable us to identify the parts of the tubulin structure that have a significant role in the interaction of kinesin and tubulin.

24 The N-terminal region of myosin binding protein-C binds to myosin S2: implications for genetic cardiac disease and contraction regulation. By M. GRUEN[1,2] and M. GAUTEL[1]. [1]*European Molecular Biology Laboratory, Heidelberg; and* [2]*Max Planck Institute for Molecular Physiology, Dortmund, Germany.*

The myosin filaments of striated muscle contain a family of enigmatic myosin binding proteins (MyBP), MyBP-C and MyBP-H. These modular proteins of the intracellular immunoglobulin superfamily contain unique domains near their N-termini. The N-terminal domain of cardiac MyBP-C, the MyBP-C motif, contains additional phosphorylation sites and may regulate contraction in a phosphorylation dependent way. In contrast to the C-terminus which binds to the myosin rod the interactions of this domain are unknown. We demonstrate that fragments of MyBP-C containing the MyBP-C motif bind to the A band of the sarcomere both in transfected cardiomyocytes and isolated myofibrils, suggesting a second myosin binding site. We localised this site to the N-terminal 126 residues of the S2 segment of the myosin rod. In this region, several mutations in β myosin are associated with familial hypertrophic cardiomyopathy (FHC); however their molecular implications remained unclear. We show that 2 representative FHC mutations in β myosin S2, R870H and E924K, drastically reduce MyBP-C binding ($K_d = 60$ μM for R870H compared to $K_d = 5$ μM for the wild type) down to undetectable levels (E924K). The interaction of the N-terminal domain of MyBP-C with β myosin reveals an interplay of myosin and MyBP-C relevant for the molecular mechanism of FHC. We suggest that the function of MyBP-C might be in the control of head-tail motility of myosin and that this function could be impaired by FHC-mutations in β myosin S2.

25 Targeting the titin gene in mouse myoblasts in culture. By G. MILLER, C. MOSS, S. CROOK, D. M. WRIGHT and M. PECKHAM. *School of Biomedical Sciences, University of Leeds, UK.*

Titin, the largest protein described, is a myosin binding protein found in skeletal and cardiac muscle. A single titin molecule (1 μm long) stretches from the Z-line to the M-line in the muscle sarcomere. It is a multifunctional protein important in such diverse functions as muscle elasticity, Z-line assembly and regulation of the length of the myosin containing thick filament. Recently the kinase domain of titin, close to the M-line, was shown to phosphorylate telethonin at the Z-line early in muscle differentiation (Mayans et al. *Nature* **395**, 1998), an event potentially important for myofibrillogenesis.

To understand better the function of this kinase we have used gene targeting to interrupt the coding sequence of titin in the middle of the kinase domain. The targeting vector contained a 10 kbp genomic fragment of the mouse titin gene, isogenic to genomic DNA of conditionally immortal ($H2k^b$-tsA58) myogenic cells. The pgkNeo gene was cloned into the middle of an exon encoding the kinase domain to interrupt the coding sequence, prematurely truncate transcription and translation of the gene, and to confer resistance to the antibiotic G418 following transfection into mouse myoblasts. The thymidine kinase gene was cloned into the vector outside the region of homology for negative selection (resistance to gancyclovir, $GANC^r$).

The targeting vector was linearised and electroporated into low passage conditionally immortal mouse myoblasts. We recovered 4 potentially targeted clones by screening approximately 250 $G418^r$, $GANC^r$ clones by PCR. We confirmed that these clones were targeted by Southern analysis.

Preliminary data suggest that the karyotype of the targeted clones appears normal and that we have interrupted the titin message in the kinase domain. However immunofluorescence using antibodies to myosin, titin and α-actinin, appears to show normal staining in differentiated cells. We are investigating further the expression of titin at the RNA and protein levels, and myofibrillogenesis.

26 Single-bead single-motor motility assays. M. MILOVANOVIC and R. M. SIMMONS. *MRC Muscle and Cell Motility Unit, King's College, London, UK.*

There is a growing need in the myosin field for a range of simple routine assays for studies of the many myosin isoforms likely to become available in the near future, and for mutation analyses. A single bead assay for single molecule studies would be particularly desirable. However it is apparent from our own work (Trombetta, Sleep & Simmons, unpublished), and also from single bead studies in the kinesin field, that compliance and bead geometry present difficulties. Detection of events when stiffness changes are small is one such problem and we have been exploring theoretically the optimum strategy using applied oscillations.

27 Single molecule fluorescence and optical tweezers: application to molecular motors. By A. E. KNIGHT[1], J. F. ECCLESTON[2] and J. E. MOLLOY[1]. [1]*Department of Biology, University of York; and* [2]*National Institute for Medical Research, London, UK.*

To investigate the mechanism of mechanochemical coupling in myosin and other motor proteins we have constructed an apparatus to make simultaneous measurements of fluorescence and mechanical properties of single myosin molecules. Using total internal reflection fluorescence (TIRF) microscopy in conjunction with a photon counting system we can monitor the binding and release of single molecules of fluorescent nucleotide (Cy3-ATP) from a myosin head (Ishijima et al. *Cell* **92**, 1998). Our preliminary measurement of single myosin ATPase activity is similar to bulk solution measurements at 0.06 s^{-1}. However we find the rate of ATP binding to be slightly faster than expected from the concentration of nucleotide used.

Using a dual optical trap combined with a nanometre position sensor we measure the force, stiffness and movement produced by each actomyosin interaction. By applying a high frequency oscillation to the trap position we can determine the onset of each interaction with a precision of

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

about 1 ms (Veigel et al. *Biophys J*. **75**, 1998). We aim to combine these techniques to determine the timing of ADP release and the mechanical transitions that occur during each biochemical cycle, and to explore how this depends upon the type of myosin tested. (See http://motility.york.ac.uk:85/ for more information).

Supported by the BBSRC and the Royal Society

28 The myosin one-two. By C. VEIGEL[1], L. M. COLUCCIO[2], J. D. JONTES[3], R. A. MILLIGAN[3], J. C. SPARROW[1] and J. E. MOLLOY[1]. [1]*Department of Biology, University of York, UK;* [2]*Boston Biomedical Research Institute, Boston, USA.; and* [3]*Department of Cell Biology, Scripps Research Institute, USA.*

We have used an optical tweezers transducer to measure the mechanical interactions made by a single myosin head while it is attached to actin. We found that 2 members of the myosin I family (rat liver 130 kDa myosin I, myr-1a, and chicken intestinal brush border myosin, BBM-I) produce movement in 2 distinct steps. The initial motion ($\sim$ 5.5 nm) was produced within 10 ms of actomyosin binding and the second step ($\sim$ 5 nm) occurred after a stochastic delay. The lifetime after the second step was variable and shorter at high ATP concentration. At the highest time resolution currently possible ($\sim$ 1 ms) we were unable to detect this second step with the single headed subfragment (S1) of fast skeletal muscle myosin II. The slow turnover kinetics of the type I myosins have enabled us to observe the time course of a single working stroke produced during a single biochemical cycle.

Supported by the American Cancer Society, NIH, Royal Society and the British Heart Foundation

29 A single myosin head progressively moves along an actin filament with regular steps of 5.5 nm. By T. YANAGIDA. *Yanagida BioMotron Project and Department of Physiology I, Osaka University Medical School, Japan.*

Development of techniques for manipulating a single actin filament by a microneedle and optical traps and nanometry has allowed displacements to be measured directly from single molecules of myosin or its subfragments in vitro. The size of displacements reported, however, has varied considerably from 4–6 nm to 15–20 nm. It is essential to determine the displacement unambiguously in order to elucidate how the conformational changes in the myosin head are involved in force generation, and how mechanical cycles are coupled to ATP hydrolysis. Here we have developed a new assay that directly manipulates a single S1 molecule, which was specifically fluorescently labelled at its regulatory light chain and visualised by TIRFM, and measures the process of the generation of displacements with a scanning probe. The displacements did not take place abruptly but instead, developed in a stepwise fashion. The number of steps in each event spread from 1 to 5, and was 2.5 steps on average. The size of steps was $\sim$ 5.5 nm, independent of the concentration of ATP (0.1 and 1 μM) and the temperature (20 and 27 °C). The dwell time between steps ($\sim$ 4 ms at 20 °C) was constant at the ATP concentrations of 0.1 and 1 μM, but depended on the temperature (Q_{10} = 2.7). These results show that the unitary step size is $\sim$ 5.5 nm, and a myosin head can undergo $\sim$ 5 steps to produce a maximum displacement of $\sim$ 30 nm during one biochemical cycle of ATP hydrolysis.

30 How much do we not know about muscle? By A. F. HUXLEY. *University of Cambridge, UK.*

In the last decade major steps have been taken toward understanding how muscle works, notably recording of single molecule interactions between myosin and actin and determination of the x-ray structures of actin and myosin. Nevertheless, many uncertainties remain.

(1) What fraction of myosin heads contribute at any one time to isometric tension? (Estimates range from 10% to 50%). (2) How much tension does one crossbridge contribute? (2–20 pN). (3) What is stiffness of an attached crossbridge? (2 pN/nm from intact fibres; 0.7 pN/nm from single molecule experiments). (4) Do both heads of one myosin in rigor contribute to stiffness? (5) What is length of the working stroke? (11 nm from intact fibres; 5 nm in single molecule experiments with myosin, HMM or S-1 stuck down on a surface; 10–20 nm with myosin-rod cofilaments according to Yanagida). (6) Is the working stroke a single event? Is it entirely due to tilting of the light chain domain, or are there contributions from tilting of catalytic domain on the thin filament or shortening of S-2? (7) If the working stroke is a single step of about 10 nm, isometric contraction would be unstable and filaments would slide to and fro. (8) Where is the elastic element in a crossbridge? (Lever arm; S-2; actin bending. If in S-2 or actin, stiffness with both heads attached would be same as with one). (9) Do all attached states contribute to the actin layer lines? (10) Which if any of the 3 published explanations of 'repriming' (Lombardi et al.) is correct? (11) Which if any of 3 explanations of the hump in the P-V curve (Edman) is correct? (12) How does affinity of myosin for actin vary along the actin helix? (13) What is the cause of stretch activation and of the oscillatory contraction of asynchronous insect flight muscles? (14) What is the chemical state of myosin when it has been forcibly detached from actin by stretch during contraction? Can myosin be detached in a similar way during shortening? (15) The effects of ATP and ADP binding on myosin properties, and of the mechanical state of myosin on rates of ATPase steps, are not yet explained in terms of interatomic forces.

Questions 1–5 interact strongly with one another and with the maximum efficiency of muscle contraction.

31 Phosphate release and muscle fibre ATPase mechanism. By ZHEN-HE HE and M. A. FERENCZI. *National Institute for Medical Research, London, UK.*

The rate of ATP hydrolysis was measured in permeabilised fibres of skeletal muscle during contraction and shortening by means of a fluorescence technique with millisecond time resolution and micromolar sensitivity. The technique uses the increase in fluorescence observed when inorganic phosphate produced by the actomyosin ATPase binds to the phosphate binding protein MDCC-PBP and avoids diffusion time lags by measuring changes directly in the muscle lattice. Immediately following the photolytic

release of ATP from NPE-caged ATP in the presence of Ca^{2+} we observed a period of high ATPase activity which is only partially explained by shortening of the muscle fibre at the expense of the compliant end regions. During the isometric phase the ATPase rate was approximately 3 times higher than has been reported by means of less direct techniques. During shortening the ATPase rate increased, with up to a 4-fold increase reached at the highest shortening velocities. The ATP hydrolysis and power output show that at the optimal shortening velocity the efficiency of contraction reaches 30%. The ATPase rate constant in rabbit psoas muscle fibres shortening at a velocity of 1.2 muscle length.s^{-1} was 21 s^{-1} at 12 °C. The velocity corresponds to each half sarcomere shortening at $\sim$ 1600 nm.s^{-1} so that the distance travelled per ATP hydrolysed was 76 nm. If the distance over which crossbridges are able to remain attached to the thin filaments was 13 nm, they remained attached to the thin filaments for 17% of the cycle time.

32 ADP release measurements in muscle fibres. By M. R. WEBB, M. BRUNE, S. MILLAR and M. A. FERENCZI. *National Institute for Medical Research, London, UK.*

We have developed and applied methods to measure the kinetics of ligand release (ADP and P_i) in fibres from rabbit psoas muscle during ATP hydrolysis to relate biochemical processes of the ATPase cycle to mechanical events such as tension generation. Prerequisites for these methods are: (1) sufficient sensitivity to measure the release processes in single glycerinated fibres; (2) millisecond time resolution to measure rapid changes in ligand concentration following activation by caged ATP photolysis in the presence of calcium or following rapid length changes; (3) it must be possible to introduce the probes into the muscle fibre. Two different types of probe have been developed based on coumarin fluorescence. In one, a fluorescent protein responds to ADP binding in a way somewhat analogous to the P_i probe MDCC-PBP. Thus ADP on release from myosin binds to a sensor protein and produces a fluorescence signal. The other method uses ADP analogues with a fluorescent group attached to the ribose ring such that there is a fluorescence change on binding or release of the nucleotide from the myosin active site. In this method the fluorescent analogue is preloaded onto the myosin and when ATP is released from caged ATP it displaces the analogue, giving a fluorescence signal. The 2 methods gave similar rates of ADP release when a fibre was activated from rigor by caged ATP photolysis: ADP release was much slower than from actomyosin in free solution.

33 Enzymatic and mechanical characterisation of expressed myosin V fragments. By J. R. SELLERS[1], M. BARTOO[3], F. WANG[1], J. A. HAMMER III[2] and J. E. MOLLOY[3]. *Laboratories of [1]Molecular Cardiology and [2]Cell Biology, NHLBI, National Institutes of Health, Bethesda, U.S.A.; and [3]Department of Biology, York University, UK.*

Myosin V is a 2 headed actin-dependent motor characterised by a conserved head domain, 6 IQ motifs and stretches of coiled-coil sequence in the tail region. In order to better characterise its enzymatic and molecular properties we have expressed an HMM-like fragment of myosin VA heavy chain (HC) corresponding to amino acids 1–1100 with a COOH-terminal FLAG-tag to aid in purification. Sf9 cells were coinfected with recombinant baculoviruses for the myosin V HC, calmodulin (CaM) and essential light chain (ELC). The purified recombinant myosin VA HMM-like fragment was soluble at 200 mM KCl for extended periods of time on ice and contained CaM and ELC. It binds actin in an ATP dependent manner and has an actin activated Mg.ATPase activity. The V_{max} at 80 mM KCl and 37 °C was 7.0 ± 2.6 s^{-1} (n = 9) with a range of 4.2–12.8 s^{-1}. The K_{ATPase} was 1.35 ± 0.82 μM. The rate in the absence of actin was less than 0.046 s^{-1}. In contrast to tissue purified avian myosin VA, no effect of calcium and CaM on the actin activated Mg.ATPase activity was observed. The expressed myosin VA HMM-like fragment translocated actin filaments at a rate of 0.45 μm s^{-3} at 30 °C. The in vitro motility rate was not affected by calcium or tropomyosin. In vitro motility could be measured even at KCl concentrations as high as 300 mM (0.75 μm s^{-1}). ADP markedly inhibited the motility rate. Even when applied to the surface at very low density the expressed HMM moved actin filaments. In some cases actin filaments appeared to be translocated about a single attached point. The step size of myosin V could be measured by optical trapping. Preliminary data indicate a step size of about 16 nm with occasional longer steps of 32 nm. These sometimes occur in a staircase like manner suggesting either that myosin V is capable of processive movements or that multiple myosin V molecules are acting on the actin filament.

34 Arrangement of heads in the myosin molecule and relaxed thick filament. By G. OFFER[1], P. J. KNIGHT[1,2], S. A. BURGESS[1,2], L. ALAMO[3] and R. PADRÓN[3]. *[1]Division of Molecular and Cellular Biology, Department of Clinical Veterinary Science, University of Bristol, UK.; [2]School of Biomedical Sciences, University of Leeds, UK.; and [3]Structural Biology Laboratory, IVIC, Caracas, Venezuela.*

The myosin that causes muscle contraction is a 2 headed species but the atomic structure is known only for the isolated head. Control of contraction by regulation of myosin is widespread and requires the 2 headed molecule for activity to be switched off. To explore the structural links between the heads that underlie this requirement we therefore built an atomic model of this part of the molecule (Offer & Knight, *J. Mol. Biol.* **256**, 1996). We have now extended the work to investigate how the molecules may be arranged to form the lattice of heads seen on the surface of the thick filaments of muscle.

The myosin model comprises the 2 chain coiled coil myosin tail, constructed using the scallop amino acid sequence, onto which is grafted 2 copies of the atomic structure of the regulatory domain of scallop striated muscle myosin. This is the base of the head, containing the essential and regulatory light chains (ELC and RLC) and associated heavy chain. The molecule is completed by adding the atomic structure of the skeletal myosin motor domain in such a way that the interactions it makes with the adjacent regulatory domain are similar to those in the whole skeletal myosin head.

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

The resulting model is compact, with the 2 RLCs interdigitating without steric clash. This is an encouraging result as it potentially provides a route for communication between the heads. This junction point also produces a morphological feature not much smaller than a motor domain. The curvature of the head is found to lie in a plane perpendicular to the axis of the tail, so the molecule looks S-shaped in end-on view, and T-shaped from the side.

Communication between heads is needed to switch off activity, and it is in the switched-off state that the heads adopt a regular lattice on the filament. Therefore we have tested whether this model of myosin, with its explicit interactions between the heads, can provide a reasonable fit to the observed filament structure. Previous modelling studies have used independent single heads unconstrained by the requirement that pairs of heads converge on a single tail. We have used the particularly well ordered filaments of relaxed tarantula striated muscle, for which a detailed 3 dimensional reconstruction is available. This shows zigzag helical tracks of high protein density with an associated J-shaped feature of lower density that is about the size and shape of a myosin head. We built many models to simulate the reconstruction. The helical symmetry plus 4 parameters were used to define the position and orientation of myosin molecules in each model. Some bending and torsion within the heads was allowed at specific sites. The motor and ELC of one head was also allowed to have a lower density to accommodate the possibility of disorder. Each atomic model was low-pass filtered and the resulting 3 dimensional density distribution was aligned with the reconstruction and scored by cross correlation. The model was then refined by simulated annealing.

The best model accounts for all the major features of the reconstruction. The tip of each head interacts with the head-tail junction of a neighbouring molecule along a helical track. One of the heads coincides with the J-shaped feature and has lower density suggesting that this site is occupied only intermittently. The head-tail junction lies at a bend in the zigzag. In this position the coiled coil coincides with a radial connection to the filament backbone. The ability of this model to fit the reconstruction suggests that myosin molecules do indeed adopt this compact structure in the relaxed state.

35 Myosin head organisation in relaxed muscle: implications for the contractile mechanism. By J. M. SQUIRE. *Biophysics Section, Blackett Laboratory, Imperial College, London, UK.*

Using fish muscle with its high degree of 3-D organisation as a structural tool we have been trying to answer the following questions. (1) How are the myosin heads organised in relaxed muscle? (2) How far do the heads need to move to attach to actin? (3) Are the heads initially the right way up to attach directly and stereospecifically to an actin binding site? With answers to these we are also asking: (4) What cycle of movements does a head need to go through when the muscle is first activated and then reaches a steady state at the plateau of a tetanus?

We have tackled the first 2 of these questions using structural refinement of the relaxed myosin filament against low angle x-ray diffraction data (Hudson et al. *J. Mol. Biol.* **273**, 1997; Squire et al. *J. Struct. Biol.* **122**, 1998) and by using electron microscopy of either negatively stained isolated myosin filaments or of fish muscle cryo-sections. We have also used electron microscopy of freeze fracture replicas of rapidly frozen fish muscle to address the third question (Cantino et al. unpublished).

The conclusions so far are that (from x-ray diffraction) the myosin heads are organised in relaxed fish muscle with their actin binding domains quite close to the neighbouring actin filaments and (from electron microscopy) with the heads oriented the correct way up for stereospecific attachment to actin to occur without large scale rotations of the heads around their own long axes prior to attachment.

Further evidence from low-angle x-ray diffraction including time resolved studies (e.g. Harford & Squire, *Biophys. J.* **63**,1992) is allowing estimates to be made of both the distribution of myosin heads on actin in rigor muscle and the changing populations of weak-binding (non force producing) and strong binding (force producing) heads during typical isometric contractions of fish muscle, needed to answer question (4).

36 Visualisation of working myosin heads: 3D electron tomography of cryofixed active insect flight muscle (IFM). By M. K. REEDY[1], M. C. REEDY[1], C. LUCAVECHE[1], R. EDWARDS[1] K. A. TAYLOR[2], H. SCHMITZ[2], H. WINKLER[2] Y. E. GOLDMAN[3], C.FRANZINI-ARMSTRONG[3], H. SASAKI[3] and R. T. TREGEAR[4]. [1]*Duke University, Durham, USA;* [2]*Florida State University, Tallahassee, USA;* [3]*University of Pennsylvania, Philadelphia, USA; and* [4]*MRC Laboratory of Molecular Biology, Cambridge, UK.*

We seek to observe working myosin heads in situ. Slam-freeze/freeze-substitution of skinned IFM fibres activated isometrically by high Ca^{2+} (i.e. not stretch activated) traps and preserves the regular lattice of myosin motors as seen in 25 nm longitudinal sections. In 3D electron tomograms computed from tilt views spanning $\pm 72°$, active myosin heads attach to the helically restricted actin target zones midway between 39 nm periodic troponin densities. This is consistent with x-ray diffraction from active IFM (Tregear et al. *Biophys. J.* **74**, 1998) indicating faithful cryo-preservation of the crossbridge pattern in EMs. In most 39 nm repeats, the single headed crossbridges form an opposed pair in the target zone; in some target zones 1–2 more bridges also bind. The unaveraged tomogram displayed a range of crossbridge angles and forms. Axially averaging the 116 nm repeats along each thin filament revealed several consistent crossbridge forms. Using troponin position to determine actin azimuth, the 3D atomic model of acto-S1 was fitted to averaged crossbridges. Many target crossbridges can be fitted by positioning the actin contact of myosin's motor domain as in rigor (Rayment et al. *Science* **261**, 1993) but the regulatory domain must bend axially by up to 30° from that in rigor. This range of regulatory domain bending would provide ~ 5–6 nm of working stroke. Other crossbridges, at an anti-rigor angle, require both a bend between the motor and regulatory domains and a motor domain that is itself rotated relative to the actin interface. If these anti-rigor angles are of working rather than pre-powerstroke myosin, then the overall working stroke is ~ 10–12 nm.

Supported by the NIH, MDA, MRC.

37 Evidence that the start of the cross bridge power stroke in muscle has variable geometry. By M. WALKER[1], J. TRINICK[1], and H. D. WHITE[2]. [1]*School of Biomedical Sciences, Leeds University, UK.; and* [2]*Department of Biochemistry, Eastern Virginia Medical School, Norfolk, U.S.A.*

The primary force producing event of muscle contraction is thought to be a gross conformational change in the heads of the myosin molecule while attached to actin. However the structural and biochemical steps in this mechanism are not well understood. The tightly bound actomyosin complex that forms in the absence of nucleotides and probably corresponds to the end of the power stroke has been described to ~ 3 nm resolution. In smooth muscle another tightly bound actomyosin complex in ADP has been shown to have a substantially different conformation. However the conformation(s) of the earlier stages in the power stroke are not known. For instance, it is not known whether the start of the power stroke has only one conformation, nor is it known whether the various transient intermediates that can be identified by solution kinetics are associated with particular conformations.

Here we report parallel stopped-flow fluorescence and electron microscopy studies of the kinetic mechanism of S1 binding to actin in the absence of nucleotide. The stopped-flow studies were done using actin to which a pyrene group was covalently attached to Cys-374. This provides a sensitive fluorescent measure of the isomerisation steps of the binding reaction. The data show that a transient intermediate, forms a few milliseconds after mixing and is then converted at a slower rate to the rigor complex. Electron micrographs of the complex at early times after mixing were obtained by spraying S1 onto grids layered with actin immediately before freezing. Disordered binding was observed 5 ms after mixing and was followed by conversion to the ordered binding after 50 ms. The appearance of the disordered intermediate is similar to that observed when S1 binds to actin in the presence of nucleotides (M-ATP and M-ADP-P_i). These results suggest that the transient intermediate that most nearly corresponds to the start of the power stroke does not have a single conformation.

38 Fluorescence temperature jump studies of myosin S1. By W. JAHN[1], C. URBANKE[2] and J. WRAY[1]. [1]*Max-Planck-Institut für medizinische Forschung, Heidelberg; and* [2]*Medizinische Hochschule Hannover, Germany.*

The tryptophan fluorescence of unmodified myosin S1 has provided a valuable signal in kinetic analysis of its ATPase mechanism. A fluorescence rise occurring at ~ 100/s following the binding of ATP is often interpreted as due to the hydrolysis step. This and other steps are strongly temperature dependent, at least for warm-blooded vertebrates. We have therefore examined the response of the fluorescence of S1-nucleotide complexes to rapid (10 μs) temperature jumps of ~ 4 °C induced by Coulomb heating, to explore the relationship of fluorescence to structural states of S1.

Both rabbit and chicken skeletal S1s were used. Under all the conditions studied the fluorescence decreased during the T-jump due to nonspecific quenching. Depending on conditions this decrease was or was not followed by changes reflecting structural events within the protein. These changes were in general fluorescence increases. The fluorescence of the steady state mixture of species in the presence of ATP rose with a time course showing more than one phase; the most prominent had a rate constant of 10–100/s depending on temperature. A subsequent slower phase was possibly related to release of ADP from S1.ADP. For S1 with ADP and beryllium fluoride bound, the fluorescence rise was much faster (rate constant ~ 1000 s^{-1}), and of relatively constant amplitude for a range of starting temperatures: the data suggested that 2 different conformations of S1.ADP.BeF_x were present and that the rate constants for their interconversion varied only slowly over a large range of temperature. Certain other complexes (e.g. S1.AMPPNP) showed smaller but similarly rapid fluorescence increases. In several other cases (e.g. S1.ADP) no rate process was observed, implying either that the same fluorescence state(s) predominate over the entire temperature range studied or that redistribution does not occur on the accessible timescale (0.05 ms–1 s).

These findings complement temperature jump observations on structural events in myosin heads in relaxed skeletal muscle fibres using x-ray diffraction (Rapp et al. *Biophys. J.* **59**, 1991). Both approaches illustrate, for several different complexes of myosin with trinucleotides or their analogues, the existence of a temperature dependent equilibrium between different conformations, independently of the chemical step of hydrolysis.

39 Modelling the mechanics of the myosin molecular motor. By G. P. TYRRELL and L. S. D. CAVES. *Department of Chemistry, University of York, UK.*

The availability of crystal structures of myosin fragments allows key aspects of structure-activity relationships to be investigated by molecular modelling methods. Using an empirical potential energy function to represent the atomic interactions we have addressed the nature and origin of the intrinsic structural compliance in myosin S1 by computing low frequency harmonic deformation modes about the local energy minimum. Significant flexibility of the (~ 12000 atom) multisubunit assembly is found in accord with EM and x-ray scattering data. The role of the light chains in stiffening the heavy chain neck region helix (a putative lever arm) is revealed. Hinge points for large scale motions are identified and related to sequence data. New approaches to modelling such large molecular systems are surveyed. The role of molecular modelling in bridging structural-mechanical relationships in molecular machines is discussed.

40 Computer modelling of the binding of nucleoside triphosphates in the active site of myosin. By G. OFFER[1,2,3], L. YU[1] and H. WHITE[2]. [1]*Laboratory of Physical Biology, NIAMS, National Institutes of Health, Bethesda, USA;* [2]*Department of Biochemistry, East Virginia Medical School, Norfolk, USA; and* [3]*Departments of Clinical Veterinary Sciences and Physiology, University of Bristol, UK.*

The hydrolytic step converting ATP to ADP + P_i in the active site of myosin is coupled to a conformational change priming the myosin for tension generation after attachment

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

to actin. In order to understand this coupling, computer modelling may usefully complement the crystallography. For example, the BeF_3 or vanadate moieties in the ADP.BeF_3 or ADP + vanadate complexes may be replaced with a γ-phosphate group (making the ATP complex) or an inorganic phosphate ion (making the ADP + P_i complex which has not been crystallised). The distance between the Be atom in the ADP.BeF_3 complex and the adjacent oxygen atom of the ADP is 1.6 Å, similar to the length of the O-P bond in ATP. The distance between this O atom and the V atom in the ADP.VO_4 complex is longer (2.1 Å) which is why this is considered to be a transition state complex. After energy minimisation of our ADP.P_i structures the distance between the O atom and the γ-P atom increases to $\sim$ 3.3 Å. Thus on ATP hydrolysis the γ-P atom moves $\sim$ 1.5 Å away from the β-P atom; this movement is likely to be the event triggering the conformational change.

The position of the M.NTP to M.NDP.P_i equilibrium is highly sensitive to the nature of the nucleoside base. Thus at 20 °C the equilibrium constant is $\sim$ 5 for ATP, $\sim$ 20 for CTP and < 0.1 for GTP. Understanding the cause of this sensitivity should illuminate the coupling between hydrolysis and conformation. One hypothesis is that changing the base causes the NTP to shift in position within the active site so that the phosphate chain is positioned differently with respect to the catalytic residues. The alternative hypothesis is that changing the base causes changes in the relative stabilities of the 2 conformations. We find that when ADP is replaced by GDP or CDP followed by energy minimisation, the position of the diphosphate chain is unaltered falsifying the first of these hypotheses. However the guanine ring is tilted by $\sim 15°$ compared with the adenine ring, while the cytosine ring although nearly co-planar with the adenine ring is rotated with respect to it, consistent with the second of these hypotheses.

41 A fibre diffraction and atomic modelling study of the actomyosin complex. By K. J. V. POOLE[1], M. LORENZ[1], K. C. HOLMES[1], P. ELLISON[1], M. FURCH[1], D. J. MANSTEIN[1], C. CREMO[2], A. SZENT-GYORGI[3], G. EVANS[4] and G. ROSENBAUM[4]. [1]*Max Planck Institute for Medical Research, Heidelberg, Germany*; [2]*Department of Biochemistry, Washington State University, Pullman, USA*; [3]*Rosenstiel Center, Brandeis University, Boston, USA*; [4]*Structural Biology Center, Argonne National Laboratory, Chicago, USA*.

The current standard model of the actomyosin complex (Rayment et. al. *Science* **261**, 1993) represents a fit of the Rayment myosin subfragment–1 (S1) crystal structure (*Science* **261**, 1993) and the Holmes et al. atomic model of F-actin (*Nature* **347**, 1990) into a 20–30 Å resolution electron microscopic density map. We are using high angle fibre diffraction data from myosin decorated actin arrays to further refine this model and to probe different attached states. Thin filaments of stretched rabbit striated muscle fibres were decorated with myosin motor fragments and the associated intensity changes in the actin based fibre diffraction patterns measured out to 8 Å and better. So far we have looked at nucleotide free chicken skeletal chymotryptic and papain S1 fragments, chicken smooth muscle S1, scallop fast adductor S1 and *Dictyostelium* catalytic domain (761). It is interesting to note that all S1 fragments looked at so far show characteristic x-ray decoration patterns indicating that there are different types of bound head structures in rigor. x-ray patterns from all but the striated muscle chymotryptic S1 decorated fibres show reversible changes on ADP binding, the extent of which was related to the strength of binding of the nucleotide. The effect was most striking in the smooth S1 with a half maximum change at $\sim$ 2 μM ADP, small in the scallop protein (half maximum effect $\sim$ 250 μM) and very slight in the skeletal papain-S1. A refinement of the atomic model of actomyosin against the smooth S1 data showed that the ADP effect results primarily from a $\sim$ 30–35 Å axial displacement of the lever arm, which is remarkably consistent with the electron microscopic data (Whittaker et al. *Nature* **378**, 1995). Model structures were achieved by first using a Monte Carlo, solid body search for the preferred binding region of S1 on actin (5 Å grid used in axial, azimuthal and radial directions) which proved to be clearly in the vicinity of the Rayment et al. (1993) fit. This region on subdomain-1 was then probed more finely in a least squares fitting procedure in which a head had axial, azimuthal and radial freedom to adjust angle and position on actin, rotational freedom around its own axis and freedom to move the light chain binding region around a hinge residue between the 2 essential cysteines. The structural effect of ADP binding to this motor protein is dominated by the swing of the lever arm and we have not yet looked at the nucleotide effect on the catalytic domain alone. However we have measured ADP induced changes in the diffraction pattern from actin decorated with this fragment from *Dictyostelium* myosin which indicates that nucleotide binding causes a significant structural rearrangement in this domain which may be translated to the lever arm in a full motor. A modelling study is in progress.

42 A comparative approach to understanding the fine tuning of molecular motors. By V. MOHAN-RAM, L. GAUVRY and G. GOLDSPINK. *Department of Anatomy and Developmental Biology, Royal Free Campus, Royal Free & University College Medical School, London, UK*.

The molecular motors (myosin S1) that generate the force for muscular contraction are coded for by a family of individual myosin heavy chain (HC) genes, the expression of which is a major determinant of the contractile properties of muscle fibre type. Throughout the animal kingdom there have been evolutionary pressures for economy as well as speed of movement. With regard to the latter we have shown that the myofibrillar ATPase of antarctic fish is considerably higher than that of tropical fish muscle and that some species of fish are able to rebuild their contractile apparatus for cold temperature swimming. In mammalian muscles different isoforms of myosin HC are expressed in different muscle fibre types within the same anatomical muscles, some of which are designed for rapid powerful movements and others adapted for slow repetitive movements and/or postural activity. In order to understand structure and function we have used a molecular cloning and computer graphics strategy to study the differences

between different fish and mammalian myosins. These data showed that the muscle myosin HC genes are highly conserved except for regions that encode 2 flexible surface loops. One of these is loop 1 which projects above the ATPase site and has an overall positive charge compared with the adjacent part of the S1. We postulate that loop 1 acts as an electrostatic latch which regulates the rate of release of the products of ATP hydrolysis during each crossbridge cycle. Comparing sequences it is seen that there is an electrostatic charge motif associated with muscle loop 1 s but the length of the loop differs and so does the distribution of this charge. However myosin HCs with similar contractile velocities have similar loop structures, e.g. the red muscle loop 1 in the antarctic fish is very similar to that of the white muscle in tropical fish which explains how the molecular motors have been fine tuned for living at different temperatures. In the mammal, the embryonic has a very similar loop to the adult slow

Supported by the Wellcome Trust and NERC.

43 Mouse melanocytes transiently transfected with antisense DNA oligonucleotides to myosin Va show reduced dendrite formation. By J. P. BENNETT and A. J. EDGAR. *Division of Biomedical Sciences, Imperial College School of Medicine, London, UK.*

In humans with Griscelli disease and mice with the *dilute* mutation a disruption in the myosin Va gene causes a defect in melanocyte function. There is conflicting evidence about whether the normal role of myosin Va in these cells includes dendrite outgrowth or is restricted to melanosome transport along actin filaments. We transiently transfected antisense oligonucleotides to inhibit its expression in a mouse melanocyte cell line, melan-a. Transient transfection of antisense phosphorothioate oligodeoxynucleotides targeted against 2 different sequences within myosin Va mRNA each reduced expression of myosin Va protein in cultured melanocytes (as determined by Western blotting) by over 70% 20 h after transfection whereas a control (shuffled sequence) oligonucleotide did not. After the transfected cells had been trypsinised and replated they were incubated for 20 h and then fixed and stained for examination by light microscopy. Cells transfected with the specific antisense oligonucleotides appeared different from the untransfected controls in that they had a flattened 'fried egg' appearance and possessed fewer dendritic processes which were shorter than those of the control cells. Image analysis was used to determine a roundness factor $R = P^2/4\,\pi A$ (where A is cell area and P is cell perimeter) which measures the deviation from a perfect circle with $R = 1.0$. The cells transfected with antisense oligonucleotide were much more round in profile ($R = 2.8 \pm 0.4$, mean $\pm$ S.E.M., n = 20, $P < 0.01$) compared with untransfected control cells ($R = 6.4 \pm 0.6$) while the cells transfected with the control oligonucleotide were not significantly different ($R = 7.5 \pm 0.6$). In contrast, when cells were not trypsinised and replated following transfection so that previously existing dendrites could persist, the normal dendritic morphology continued to be observed. We conclude that, in addition to its role in melanosome transport, myosin Va has a role in the extension of new dendrites by melanocytes but not in maintenance of pre-existing dendrites.

Supported by the MRC.

44 N-terminal processing of ACT88F. By S. SCHMITZ[1], H. PRINZ[2], J. D. CLAYTON[1], M. A. GEEVES[2] and J. C. SPARROW[1]. [1]*Department of Biology, University of York, UK.; and* [2]*Max-Planck-Institut für molekulare Physiologie, Dortmund, Germany.*

All actins examined so far show acetylation of the α-amino group of the N-terminal amino acid of the mature protein. Class II actins which include all *Drosophila melanogaster* isoforms and vertebrate skeletal muscle actins usually have a Met-Cys-X sequence where X is an acidic residue. Those actins are expected to be processed to an N-terminal sequence of Ace-X.

Drosophila melanogaster indirect flight muscle specific actin encoded by the gene *Act88F* was purified by 2 methods. A large scale preparation from whole flies uses anion exchange chromatography to separate the actin from most of the nonflight muscle isoforms. A small scale preparation method purifies the actin from dissected indirect flight muscles. Nano-electrospray mass spectroscopy and Edman sequencing were applied to examine the actin.

It is shown here that the bulk of the *Drosophila melanogaster* indirect flight muscle specific actin is processed to an N-terminal sequence of X. It is therefore the first actin reported not to be acetylated. In addition the *Drosophila melanogaster* mutant mod^- is identified here to cause the gene product of *Act88F* to be processed to an N-terminal sequence of Ace-Cys-X. Together with isoelectric point calculations those results explain the migration pattern of the *Drosophila melanogaster* actins on 2D gels and their anion exchange chromatography elution profiles.

The negative effects of mod^- on flight ability and wingbeat frequency allow speculation on the functional role of this unique processing mechanism. The free α-amino group seems to be required for fine tuning the flight muscle for its specific function which is to power flight. The flight muscles have been shown to exhibit a range of structural and functional differences to other muscles, probably to be able to fulfil their unique function. Some unique features are flight muscle specific proteins such as arthrin and flightin, stretch activation and their high contraction frequency. The free α-amino group of the flight muscle actin is probably another one. This has to be examined by looking at flight muscle actins of other insects as well.

45 The small GTPases Rho, Rac and Cdc42 regulate macrophage migration and chemotaxis. By W. E. ALLEN[1,3], A. J. RIDLEY[2] and G. E. JONES[3]. [1]*Department of Clinical Biochemistry, The Queen's University of Belfast;* [2]*Ludwig Institute for Cancer Research, University College London; and* [3]*The Randall Institute, King's College London, UK.*

Three members of the Rho family of small GTPases, Cdc42, Rac and Rho, have been shown to regulate the organisation of actin based cytoskeletal structures. In Bac1.2F5 cells, a CSF-1 dependent murine macrophage cell line, we have shown using a microinjection approach that RhoA regulates cell contraction and rounding, while Rac1 regulates the formation of actin rich membrane ruffles and lamellipodia and Cdc42 regulates the formation of filopodia. We have also shown that Rac1 activity is required for focal complex

assembly in these macrophages. These observations suggest that Rho, Rac and Cdc42 could be important regulators of cell migration. To test their effects on cell migration the Dunn chamber chemotaxis assay was used. This assay allows individual migrating cells to be directly observed and is therefore ideal for studying the behaviour of microinjected cells. In the absence of CSF-1 Bac1 cells lost their motile behaviour and rounded up. Upon restimulation with CSF-1 cells became highly motile and showed a chemotactic response to this cytokine. When cells injected with activated RhoA (V14RhoA) or C3 transferase, an inhibitor of Rho, were placed in a gradient of CSF-1 they failed to show either increased migration speed or chemotaxis. Similarly cells injected with activated forms of Rac1 and Cdc42 or with the dominant negative Rac mutant N17Rac1 were inhibited in their response to CSF-1. In contrast, the dominant negative Cdc42 mutant N17Cdc42 enhanced the speed of cell migration in the presence of CSF-1, although chemotaxis up the gradient of CSF-1 was inhibited. These results suggest that Cdc42 is involved in sensing and responding to the gradient of CSF-1 but is not required for cell migration, while correct regulation of Rac and Rho activity is essential for cell migration.

46 Association of kettin with actin in the Z-disc. By M. VAN STRAATEN, D GOULDING, B. KOLMERER, S. LABEIT, J. CLAYTON, K. LEONARD and B. BULLARD. *European Molecular Biology Laboratory*, *Heidelberg*, *Germany*.

In striated muscle actin filaments from neighbouring sarcomeres are anchored in the Z-disc by α-actinin. The Z-discs of insect muscle also contain kettin, a modular protein of 500–700 kDa. The *Drosophila* protein is made up of a chain of 30 immunoglobulin (Ig) domains separated by linker sequences. Isolated kettin from *Lethocerus* (waterbug) muscle is an elongated molecule 180 nm long which binds to F-actin with high affinity (K_d 1.2 nM) and a stoichiometry of one Ig domain per actin monomer. The linker sequence between Ig domains is necessary for actin binding. Tropomyosin and myosin S1 compete with kettin for binding to actin, and tropomyosin is absent from the region of the sarcomere containing kettin. In contrast, kettin and α-actinin bind simultaneously to actin. The position of kettin in the sarcomere was determined by immunoelectron microscopy. Actin filaments interdigitate in antiparallel fashion in the Z-disc, with the N-terminus of kettin within the Z-disc and the C-terminus some way outside. In *Drosophila* flight muscle the 30 modules of kettin extend 93 nm along the thin filament, which is consistent with an arrangement in which the molecule follows the genetic helix of actin and Ig domains separated by linker sequences bind to each actin monomer. In vitro kettin promotes the antiparallel association of actin filaments and a similar process may occur in the developing sarcomere. The expression of kettin in *Drosophila* embryos was followed by in situ hybridisation with a riboprobe. Kettin mRNA appeared in mesoderm at stage 11 (7 h) and by stage 13 (10.5 h) had a segmental pattern. The function of kettin is probably to align actin in the Z-disc and to act as a template for formation of α-actinin crosslinks in the Z-disc.

47 Fibroblast growth factor-2 has different effects on different stages of oligodendrocyte development in the rat CNS in vivo. By D. R. GODDARD, M. BERRY and A. M. BUTT. *School of Biomedical Sciences*, *St Thomas' Hospital Campus*, *King's College London*, *UK*.

Fibroblast growth factor 2 (FGF-2) is known to affect oligodendrocyte development in vitro. We have previously presented to the Society evidence that IGF-I promotes myelinogenesis in the anterior medullary velum (AMV) following intraventricular injection. In the present study, the in vivo effects of FGF-2 on oligodendrocytes and CNS myelination were determined in the postnatal rat AMV following injection of the cytokine into the cerebrospinal fluid (CSF). FGF-2 was administered via the lateral ventricle twice daily commencing at postnatal day P6 to give approximate concentrations in the CSF of 0.5 μg/ml (low FGF-2) or 10 $\mu g/ml^{-1}$ (high FGF-2). At P9 AMV were immunolabelled with the Rip antibody to enable analysis of the numbers of myelin sheaths and of immature promyelinating oligodendrocytes and mature myelinating oligodendrocytes, or treated for Western blot analysis of changes in expression of the myelin proteins CNP and MOG. Low FGF-2 induced an increase in the number of promyelinating oligodendrocytes but had no effect on the number of oligodendrocytes or sheaths, whereas high FGF-2 caused decreased numbers of promyelinating oligodendrocytes, oligodendrocytes and sheaths. There was decreased expression of CNP and MOG at both doses of FGF-2. Thus FGF-2 exhibited stage and dose dependent actions on oligodendrocytes whereby the timely differentiation of immature oligodendrocytes was arrested, whilst in mature oligodendrocytes it induced dedifferentiation and down-regulation of myelin gene products. The results support a role for FGF-2 in the normal development of oligodendrocytes in vivo, but indicate that high titres such as might occur in CNS injury may damage oligodendrocytes. These divergent effects of FGF may be related to changes in FGF receptor (R) expression in oligodendrocytes during development, whereby FGFR-1 expression increases with oligodendrocyte development, FGFR-2 is expressed mainly in mature oligodendrocytes, and FGFR-3 is expressed mainly in oligodendrocyte progenitors (Bansal et al. 1996).

Supported by the Anatomical Society of Great Britain & Ireland and the Wellcome Trust.

48 Molecular cloning of Tropic 1808 protein, a novel factor induced by nerve injury. By XAIOSONG GU[1], P. K. THOMAS[2], XIANGLING TAN[1], R. H. M. KING[2], DING FEI[1], GAO ZHENG[1], FAN MING[1], J. M. COOPER[2] and A. H. V. SCHAPIRA[2]. [1]*Department of Anatomy and Neuroscience*, *Nantong Medical College*, *P. R. China; and* [2]*University Department of Clinical Neurosciences*, *Royal Free and University College Medical School*, *London*, *UK*.

We have previously shown that an 18 kDa protein produced by the distal stump of transected rat sciatic nerves possesses a strong chemotropic influence on outgrowing neurites from explanted neonatal dorsal root ganglia in tissue culture (Gu et al. *J. Anat.* **186**, 1995). In order to identify its gene and establish its expression a cDNA library of the distal segment of peripheral nerve after transection was constructed and

molecular cloning related to the chemotropic substance was undertaken. A monoclonal antibody termed Tropic 1808 McAb was established through the hybridoma technique by immunizing with the electroeluate protein from 18 kDa band strips from native PAGE. A λgt11 cDNA library was constructed from poly(A)+ RNA derived from the distal segment of transected rat sciatic nerve at 7 and 14 d using the bacterial host strains Y1090 and LE392 (Promega). Immunoscreening was performed with Tropic 1808 McAb by the ABC method. Eight clones from 600 000 recombinant plaques were initially selected. The cDNA inserts were selected from the bacteriophage DNA by digestion with *Eco*R1 restriction endonuclease and subcloned into the *Eco*R1 site of a pUC18 vector. This showed that 5 clones were in the 2.2–2.7 kb size. The nucleotide sequences of the 5 clones were analysed using an automatic sequencer. This revealed that one cDNA was 2651 bp in length with an open reading frame of 276 amino acids that predicted a 33 kDa protein which we have named Tropic 1808 gene (GeneBank AF078811). The Tropic 1808 sequence was used to search nonredundant nucleotide and amino acid sequence databases but no homology to other cDNA or proteins was detected. By means of immunoaffinity chromatography an eluate fraction from a Tropic 1808 McAb column was detected by native PAGE. The protein band appeared in the 18 kDa position, but it was situated at the site of a 32 kDa band on SDS-PAGE. Northern blot analysis showed that the size of the mRNA encoding Tropic 1808 was ~ 3.8 kb. In situ hybridisation demonstrated that Tropic 1808 mRNA was specifically expressed in Schwann cells in the distal stump of the sciatic nerve after transfection. RT-PCR results indicated that Tropic 1808 mRNA levels were high in embryonic rat brain and injured nerve but were not detected in normal nerves of adult rats.

Supported by the National Science Foundation of China, grant 39425006.

49 RT-PCR techniques complement Nomarski and electron microscopy in the detection of somatic crossover mutations between vasopressin and oxytocin precursor genes. BY M. J. EPTON, T. BUDD and J. F. MORRIS. *Department of Human Anatomy and Genetics, University of Oxford, UK.*

Magnocellular neurons with large aggregates of peptide in their rough endoplasmic reticulum (rER) can easily be detected by Nomarski microscopy in the supraoptic and paraventricular nuclei of homozygous Brattleboro rats. These rats suffer from hypothalamic diabetes insipidus due to a single base deletion and frameshift mutation within the vasopressin precursor gene. Electron microscopic immunogold cytochemistry using antibodies for vasopressin, oxytocin, vasopressin neurophysin, and oxytocin neurophysin, shows that the peptide aggregates contain a hybrid molecule consisting of the N-terminal of the vasopressin precursor fused to the C-terminal of the oxytocin precursor. The hybrid peptide is synthesised as a result of a crossover mutation between homologous regions of the vasopressin and oxytocin precursor genes and the appropriate hybrid mRNAs are detectable. The number of neurons containing hybrid peptide aggregates increases with age; the mutation is therefore somatic in type.

Neurons containing similar aggregates of hybrid peptide are also detected in di^+/di^+ mice, which suffer from nephrogenic diabetes insipidus (which stimulates vasopressin expression), but only if they have been treated with estradiol for 10 d to stimulate oxytocin precursor gene expression. In contrast to the Brattleboro rat, such neurons are scarce and their small size makes them difficult to find. However, RT-PCR using combinations of forward and reverse primers specific for mouse vasopressin and oxytocin precursor transcripts permits the easy and rapid detection of hybrid mRNA, and hence the crossover mutation, in both oestradiol treated and untreated di^+/di^+ mice.

Humans are long lived, and their neurons are therefore particularly susceptible to somatic mutations. Nomarski microscopy reveals human magnocellular neurons that appear to contain peptide aggregates, but the delay postmortem to tissue fixation makes structural analysis and peptide immunophenotyping difficult. We can however extract and amplify vasopressin and oxytocin precursor mRNA from human postmortem hypothalamic tissue. A crossover mutation similar to that occurring in rodents would produce hybrid mRNA, although at a smaller copy number compared to wild type vasopressin and oxytocin precursor. As mRNA is only transiently stable, levels will steadily decrease post mortem. Even so RT-PCR should make possible the detection of hybrid mRNA and thereby allow us to study a somatic mutation in an important human neuropeptide.

Matthew Epton is supported by an Anatomical Society Studentship

POSTERS

P1 Achieving neuronal network architectures in vitro by microcontact printing. By M. SCHOLL[1,3], M. C. DENYER[1], C. SPROESSLER[1], K. NAKAJIMA[1], A. OFFENHAEUSSER[2], W. KNOLL[1,2], A. MAELICKE[3], A. NAKAO[1], Y. IWAKI[1], H. SIGRIST[4], D. LÉONARD[5], Y. CHEVOLOT[5] and H. J. MATHIEU[5]. [1]*The Institute of Physical and Chemical Research (RIKEN), Japan;* [2]*Max-Planck-Institute for Polymer Research, Mainz;* [3]*Johannes Gutenberg University, Institute for Physiological Chemistry, Mainz, Germany;* [4]*Centre Suisse d' Electronique et de Microtéchnique, Neuchâtel; and* [5]*Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.*

Receptor mediated recognition of substrate molecules is one of the key guidance cues which nerve cells use in order to find their target structures in vivo. Precise targeting is the prerequisite for the formation of correct neuronal connections and networks. In order to study these mechanisms under simplified in vitro conditions we have applied cultured embryonic hippocampal neurons onto a geometrical grid pattern of a particular substrate, PA22–2, the grid consisting of 3, 5, 8 and 10 μm lines crossing at 50 μm in one and at 100 μm in the other direction. PA22–2 is a synthetic peptide matching the corresponding sequence in the A chain of the extracellular matrix protein laminin, except that a cysteine residue was added at the N-terminal. The peptide was covalently immobilised via cross-linker chemistry on silicon oxide based substrates using microcontact printing (μCP). Successful surface chemistry and pattern formation was confirmed by several means including

immunofluorescence, atomic force microscopy, time of flight secondary ion mass spectroscopy, x-ray photoelectron spectroscopy and time lapse video recording. We demonstrate that the surface modification performed resulted in patterned biofunctionalisation of the surface which induced the hippocampal neurons to form a neuronal network of the same geometry. Based on these and other findings the dimensions of the adhesive pattern are a critical factor in setting directional motility, axon targeting and the formation of neuronal networks. Most of the cell bodies settled within 24 h after plating on the cross points of the pattern. Migration of cells plated between 2 cross points in the direction of the nodes was observed for patterns of 5, 8 and 10 μm diameter, whereas on patterns of 3 μm diameter, the cells remained immobile.

Immunocytochemistry and patch clamp recordings revealed that the patterned neurons were fully functional. Prompted by these encouraging results, we have begun to culture patterned neurons onto an array of field effect transistors, in order to perform extracellular recordings from this network. Such model systems may help to unravel the molecular principles underlying the formation and maintenance of functional networks of neurons.

P2 Cell culture study of the biological compatibility between polyglycolic acid and nerve tissue. By XIAOSONG GU, ZHANG PEIYUN and WANG XAIODONG (introduced by P. K. THOMAS). *Department of Anatomy and Neuroscience, Nantong Medical College, P. R. China.*

In order to investigate the biological compatibility of peripheral nerve tissue with the artificial material polyglycolic acid, dorsal root ganglia from neonatal (1–3 d) Sprague-Dawley rats were cultured on polyglycolic acid filaments for 3 wk. Schwann cells were stained immunocytochemically for S100 protein and observed by inverted phase contrast light microscopy and scanning electron microscopy. Neurites from dorsal root ganglia accompanied by Schwann cells grew along the polyglycolic acid filaments, sometimes surrounding the filaments like bunches of grapes. This experimental study has demonstrated good biocompatibility between the biodegradable artificial material and peripheral nerve tissue in the rat.

Supported by the National Science Foundation of China, grant 39425006.

P3 Development of peripheral nerve abnormalities in PMP22 mutant mice. By A. M. ROBERTSON[1], C. HUXLEY[2], R. H. M. KING[1] and P. K. THOMAS[1]. [1]*Royal Free and University College Medical School and* [2]*Imperial College School of Medicine, London, UK.*

Mutations in the gene for peripheral myelin protein 22 (PMP22) are associated with peripheral neuropathy in mice and humans. PMP22 is strongly expressed in peripheral nerves and localises largely to the myelin sheath but a dual role has been suggested on the basis that 2 tissue specific promotors have been found. In this study we compared the initial stages of postnatal development in transgenic mouse models with 7 (C22) and 4 (C61) copies of the human PMP22 gene and homozygous and heterozygous Trembler-J mice, which have a point mutation in the PMP22 gene. Homozygous and heterozygous Tr^J and C22 mice could be morphologically distinguished from controls by postnatal day P4. They had fewer myelinated and more promyelinated fibres. The number of axons that were singly ensheathed by Schwann cells was the same in all groups indicating that PMP22 does not function in the initial ensheathment and separation of axons. At both P4 and P12 all the mutants had an increased proportion of fibres that were incompletely surrounded by Schwann cell cytoplasm. The 2 most affected strains could be distinguished by differences in the Schwann cell morphometry at the initiation of myelination. In homozygous Tr^J animals the Schwann cell cytoplasm had failed to make a full turn around the axon whereas in the C22 strain most fibres had formed a mesaxon. We have concluded that PMP22 functions in the initiation of myelination and most likely involves the movement of the Schwann cell around and along the axon. Abnormalities may result from a failure of differentiation but more likely from defective interactions between the axon and the Schwann cell.

Supported by Action Research.

P4 The effects of cell shape changes on electrophysiological signals recorded from cardiac myocytes cultured over microfabricated extracellular recording devices. By M. C. DENYER[1], M. RIEHLE[2], M. SCHOLL[1,5], C. SPROESSLER[1], S. BRITLAND[3], M. HARA[1], A. OFFENHAEUSSER[1,4] and W. KNOLL[1,4]. [1]*Laboratory for Exotic Nano-Materials, RIKEN, Saitama, Japan;* [2]*Centre for Cell Engineering, University of Glasgow, UK.;* [3]*School of Pharmacy, University of Bradford, UK.;* [4]*MPI Polymerchemie, Mainz, Germany; and* [5]*Institute for Physiological Chemistry, School of Medicine, Johannes Gutenberg University, Mainz, Germany.*

Cardiac myocytes cultured in high density over arrays of extracellular recording devices can be used to assay bioactive compounds. Electrophysiological signals recorded from these devices vary in amplitude with time. Theoretically, changes in signal amplitude may arise from myocyte movement, induced by fibroblast/myocyte interactions. To test this, we cultured cardiac myocytes from 1–5 d old SD rats for 36.5 h at high densities of 3×10^5 cells/cm^2 and low densities of 3×10^4 cells/cm^2 on fibronectin coated glass. After 36.5 h myocytes were identified by their rhythmic contractions and time lapse video recorded for a further 3.5 h. Length, width and angle of orientation was then determined every 30 min for 5 cells in low density and high density culture. Cells in low density had mean lengths of 65.3 μm ($n = 40$, S.D. = 11.0) and widths of 35.1 μm ($n = 40$, S.D. = 14.5). Length, width and angle of orientation of these cells changed by 4.1% ($n = 35$, S.D. = 2.8), 11.8% ($n = 35$, S.D. = 13.3) and 2.7 degrees ($n = 35$, S.D. = 2.3) every half hour. In comparison cells in high densities had significantly greater mean lengths of 74.2 μm ($n = 40$, S.D. = 15.4, $P = 0.05$) and significantly lower mean widths of 24.3 μm ($n = 40$, S.D. = 15.4, $P = 0.05$). The lengths, widths and angles of orientation of cells in high density changed by 6.4% ($n = 35$, S.D. = 4.1), 10.0% ($n = 35$, S.D. = 10.6) and 4.6 degrees ($n = 35$, S.D. = 7.3). We found no evidence of myocyte/fibroblast interactions influencing cell position or shape in low density culture, but in high density, interactions between fibroblasts and myocytes were evident. We con-

clude that changes in cell shape are largely responsible for the changes in signal amplitude recorded from cardiac myocytes cultured on microfabricated extracellular recording devices. However, there is some evidence that myocyte/fibroblast interactions may augment this process in high density culture. We also propose that cells in low density, because of their morphology, may cover more effectively and so couple more efficiently with 20 μm wide recording sites than cells in high density culture.

P5 Near field optical observations of cultured cardiac myocytes. By R. MICHELETTO[1], M. C. DENYER[1], M. SCHOLL[1], K. NAKAJIMA[1], A. OFFENHAEUSSER[2], M. HARA[1] and W. KNOLL[1,2]. [1]*Frontier Research Program*, *The Institute of Physical and Chemical Research* (*RIKEN*), *Saitama*, *Japan; and* [2]*Max-Planck Institute for Polymer Research*, *Mainz*, *Germany*.

Real time observations of cell behaviour have been performed using optical phase contrast or DIC microscopes. Resolution of these systems is limited by the wavelength of light. More detailed submicrometre studies of cell ultrastructure have been performed using scanning and transmission electron microscope (SEM and TEM) techniques. However live cells cannot be examined in real time using these methods because SEM and TEM systems require cell fixation and observation under high vacuum. More recently atomic force microscope (AFM) systems have been used to examine the morphology of live and fixed cells. These systems have high resolutions but may, because of the direct contact between the cantilever and sample, deform soft tissue. In this study we report on the first observation of live cells using noncontact scanning near field optical microscopy (SNOM). We used the SNOM to examine rhythmically beating cardiac myocytes in culture. By scanning over the surface of cells it was possible to obtain 3 dimensional SNOM images. While scanning the scanning probe could be stopped at any point to record the contractile activity of submicrometre areas of individual cells. We found that the contraction profiles could change dramatically within adjacent 800 nm areas. This suggests that the SNOM system is capable of detecting submicrometre features that directly influence the recorded contraction profiles. There is evidence from acoustic microscopy that submembrane shortening actin filaments can act as detectable scatterers in contractile cells. There is also evidence that near field optical systems can resolve submembrane cytoskeletal elements in fixed cells. Therefore, we propose that the spatially dependent amplitudes of contractions reported in this study may arise because of the SNOMs ability to resolve the behaviour of individual submembrane actin bundles. At present, the spatial resolution of this system is of the order of 800–1000 nm. However once scanning tip fabrication and positioning has been optimis

P6 The effect of maternal nutrition on muscle development in the ovine fetus. By B. DEMIRTAS and N. C. STICKLAND. *Department of Veterinary Basic Sciences*, *Royal Veterinary College*, *London*, *UK*.

Muscle fibre number is a major determinant of muscle mass. Muscle fibre hyperplasia occurs during the fetal period and is completed by birth in many agricultural animals including sheep. Muscle fibre number has been shown to be affected by prenatal conditions in utero.

Prenatally muscle fibres develop as 2 distinct populations in sheep. Fibres which form during the initial stages are primary fibres which provide a structural framework for subsequent formation of secondary fibres. Secondary fibre number has been shown to be affected by maternal nutrition in pigs and guinea pigs. Primary fibre number was unaffected by undernutrition in utero in these species. The purpose of this study was to investigate the effect of maternal nutrition especially in early development of the ovine fetus.

Maternal undernutrition (85% of normal food intake) was imposed from 2 wk before gestation and up to 70 d of gestation (full term = 147 d). Controls were maintained on unrestricted diets throughout. Ewes were killed at 127 d or 128 d gestation by an intravenous injection of pentobarbitone. Fetuses were removed and m. semitendinosus was dissected from each. Complete midbelly slices were frozen rapidly in liquid nitrogen and 10 μm sections were cut on a cryostat. The sections were stained for alkali-stable ATPase with preincubation at pH 10.4. The number of primaries and total number of fibres were counted across the muscle to take account of the deep and superficial part of the muscle. A Seescan image analysis machine was used for the analysis. Nutritional groups were compared by the Student *t* test.

There was a significant 16.7% decrease in total fibre number in the restricted group due to an apparent decrease in both primary and secondary fibre number. We speculate that the apparent decrease in primary fibre number (from 28485 ± 1036, mean $\pm$ S.E.M., n = 5, to 23220 ± 1207, n = 4; $P < 0.05$) is due to the very early restriction period which encompasses conception and primary fibre formation. Alternatively the identification of primary fibres using ATPase staining may be inappppropriate for this species.

P7 The Swiss albino MF1 mouse, a new animal model of streptozotocin induced insulin dependent diabetes mellitus (IDDM) for the study of diabetic teratogenesis. By T. I. CHINNAH and M. E. ATKINSON. *Department of Biomedical Science*, *University of Sheffield*, *UK*.

An increased risk of congenital malformations in offspring of mothers with diabetes (IDDM) is well established. The commonest specific structural defects include caudal dysgenesis complex, congenital cardiac lesions and neural tube defects. IDDM has been induced in different species of animals with the diabetogenic drug streptozotocin (STZ). Susceptibility to both the toxic and diabetogenic doses of STZ vary widely between species and strain of animals. Furthermore the entire spectrum of human malformations associated with IDDM is never observed in a particular species or strain of animal model. The study of a number of animal model systems increases the chance that the models studied will represent the entire spectrum of the heterogeneous set of human malformations associated with IDDM. This study aims to develop a new animal model of STZ-induced IDDM using the outbred Swiss albino female MF1 mouse strain for studying the pathogenesis and mechanisms of diabetic teratogenesis. The female MF1 mice were injected subcutaneously with doses of STZ (75 mg/kg, 100 mg/kg, 75 mg/kg × 2, 150 mg/kg, 200 mg/kg and

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

250 mg/kg). Control animals were injected with vehicle (sodium citrate buffer pH 4.5). Urine glucose levels were determined before and 1–2 wk after treatment. Confirmed diabetic and control females were mated overnight with nondiabetic males. Pregnant diabetic and control animals were killed by cervical dislocation on gestation d 9.5/10. Blood samples were collected to measure the serum glucose levels. Embryos were examined for external malformations. The results suggest that the MF1 mouse strain is susceptible to the diabetogenic effects of STZ. Serum glucose level increased with the dose of STZ administered. A bolus dose of 150 mg/kg body weight of STZ significantly ($P < 0.05$) induced permanent IDDM (serum glucose level 18.4 ± 0.6 mmol/l) in the mice with moderate severity and minimal mortality compared to the other doses administered. Above this dose, a very high mortality rate resulted. The major malformations induced by the diabetic state were cranial neural tube defects observed in 34.9% of the embryos against 8.9% in the controls. The defects were similar to those seen in human and other animal models. This study demonstrates that the Swiss albino MF1 mouse strain can be used as animal model of pregnancy complicated by IDDM for studying the pathogenesis and mechanisms of diabetes induced teratogenesis.

T. I. Chinnah is supported by a Commonwealth Scholarship.

P8 Congenital malformations in a new animal model of streptozotocin induced insulin dependent diabetes mellitus (IDDM). By T. I. CHINNAH and M. E. ATKINSON, *Department of Biomedical Science*, *University of Sheffield*, *UK*.

Maternal diabetes (IDDM) is associated with an increased risk of congenital malformations. The commonest include caudal dysgenesis complex, congenital heart and neural tube defects. Malformations similar to those described in humans have been reported in animal studies in which IDDM has been induced by streptozotocin (STZ) and in culture of conceptuses under diabetic conditions. The entire spectrum of human malformations associated with IDDM is never observed in a particular species or strain of animal model and the cellular and molecular mechanisms of the congenital malformation remain unknown. These studies however have supported the notion that the altered nutrient mixture is responsible for the teratogenicity of diabetic pregnancy. This study examines the congenital malformations in the offspring of mothers of a new animal model of STZ-induced IDDM and the histological appearance of the affected tissues. Female Swiss albino MF1 mice were injected subcutaneously with a bolus dose of 150 mg/kg body weight of STZ. Control animals were injected with the vehicle (sodium citrate buffer pH 4.5). Urine glucose levels were determined before and 1–2 wk after treatment. Confirmed diabetic and control females were mated overnight with nondiabetic males. Pregnant diabetic and control animals were killed by cervical dislocation on gestation d 9, 10, 11 or 12. Blood samples were collected to measure the serum glucose levels. Embryos were staged, examined for external malformations, weighed, crown rump lengths measured and fixed in either 4% paraformaldehyde or methacarn (modified Carnoy's fixative) for histological examinations. The major external malformations observed were cranial neural tube defects with or without haemorrhagic effusion and growth retardation. The highest incidence of the malformations occurred at gestation d 11–11.5 in 47.8% of embryos from diabetic mothers against 10.9% in the controls. Histological examination revealed unfused cranial neural tube, distorted neuroepithelia.

T. I. Chinnah is supported by a Commonwealth Scholarship.

P9 Laterality of jugular foramen external opening size and carotid canal position in Anatolian skulls. By M. A. KURT[1,2] and I. ARI[1]. [1]*Department of Anatomy*, *Uludag University Medical School*, *Turkey; and* [2]*Department of Anatomy and Developmental Biology*, *St George's Hospital Medical School*, *London*, *UK*.

A knowledge of the variation in the positions of the foramina and canals of the cranial base is important since increasingly refined imaging and skull base surgical techniques have been developed during the past decade. An anatomical investigation of the external aspect of the skull base was therefore carried out on 40 adult male Anatolian skulls. The jugular foramen (JF) and carotid canal (CC) were chosen to study because of their critical location, the important structures they contain and their close association with important pathologies including aneurysms, schwannomas and clival tumours. The long and short diameters of the external opening of the JF and CC were measured on both sides of the cranial base. In addition, the distance from the medial border of the external opening of the CC to the midline and the distances from the lateral border of the external opening of the CC to the midline, supramastoid crest and external acoustic opening were also measured following the method of Lang & Schreiber (*HNO* **31**, 1983). The mean (± S.E.M.) long (right 14.66 ± 0.43 mm; left 12.83 ± 0.32 mm) and short diameters (right 7.63 ± 0.37 mm; left 5.98 ± 0.30 mm) of the external apertures of the jugular foramina were significantly longer on the right ($P = 0.001$ and $P = 0.002$, respectively) compared to the left side of the cranial base. However, there was no significant evidence of laterality between the mean long (right 6.23 ± 0.21 mm; left 5.92 ± 0.14 mm) and short (right 3.96 ± 0.07 mm; left 3.94 ± 0.10 mm) diameters of the external apertures of the CCs. The mean distance from the lateral border of external aperture of the CC to the midline was significantly greater ($P = 0.024$) on the right (31.52 ± 0.46 mm) compared to the left ($30.57 \pm 0.4.9$ mm) side of the cranial base. In contrast, the mean distances from lateral border of the external aperture of the CC to the supramastoid crest (right 32.15 ± 0.59 mm; left 34.92 ± 0.68 mm) and external acoustic opening (right 15.91 ± 0.45 mm; left 16.50 ± 0.43 mm) were significantly greater on the left compared to the right side of the cranial base ($P = 0.001$ and $P = 0.031$, respectively). Thus, the external aperture of the JF is larger on the right than on the left and the external aperture of CC is closer to midline on the left compared with the right side. Although our results for the JF were in accord with those of Lang and Schreiber, the closer spatial relationship between the external opening of the left CC and the midline compared to the right external opening of CC and the midline is a novel finding. These

results suggest that possible differences between populations should be taken into account in investigations of the foramina and canals of othe cranial base.

P10 Integrins and focal adhesion kinase localisation within the periodontal ligament of the rat dentition. By J. M. PYCROFT and B. J. MOXHAM. *Anatomy Unit, School of Biosciences, University of Wales Cardiff, UK.*

We have shown that in the aged rat the cells of the periodontal ligament show marked apoptosis (Pycroft et al. in press). Integrin receptors play an important role in the signalling of apoptosis, and the mechanism of protein tyrosine phosphorylation is a major event in the organisation of points of close apposition (termed focal adhesions) between the cell membrane and the extracellular matrix. There is also activation of a large number of membrane associated proteins, one of which is termed focal adhesion kinase (FAK). To date the types and location of integrins and FAK in the periodontal connective tissues supporting the tooth have not been adequately studied. Using a panel of antibodies to integrins and FAK and standard immunofluorescence procedures, 24 male Wistar rats provided material for the periodontal ligaments of the non-continuously erupting mandibular molars and for the continuously erupting mandibular incisor at various ages (1, 2, 3, 4, 8, 104 wk: n = 4). All tissues were fixed in 95% alcohol for 2 h and then decalcified in 10% EDTA. Labelling for FAK was found in the periodontal ligaments of both the rat incisor and molar at 3 wk, with a row of immunopositive cells extending from the base of the tooth to the gingival connective tissues. The pattern of integrin subunit labelling at this age was detected markedly along the incisor. At 4 wk, when the molar teeth had erupted, no labelling for FAK was present in this tooth type. For the incisor, the integrin labelling pattern persisted until 104 wk (in the aged animal) when there appeared to be a marked downregulation in both integrin and FAK. Apoptosis was markedly also seen in the aged tissues of both tooth types. That FAK was seen in the cells lining the tooth surface during eruption suggests a relationship between these cells (putative cementoblasts) and eruptive behaviour. However, in the aged incisor eruption rates are considerably increased and yet FAK labelling is markedly diminished.

P11 Uptake of microparticles by the small intestine in pregnancy. By S. H. SMYTH, M. DOYLE-McCULLOUGH and K. E. CARR. *The Queen's University of Belfast, UK.*

Previous research has shown that the uptake of microparticles in adult rat small intestine varies from animal to animal, limiting the significant differences seen in uptake as parameters are varied. However the importance of the phenomenon in toxicology and drug delivery ensures that studies continue despite these difficulties. This study aims to investigate if particle uptake changes during pregnancy, a state which may have an effect on gastric emptying and intestinal transit.

The model used was an established protocol, with 8–10 wk old Sprague–Dawley female rats given free access to water and standard laboratory chow. Three pregnant and 3 nonpregnant rats were fed 0.25 ml of latex microparticles by gavage, with each group receiving a standard volume of latex. Three different sizes of particles (2, 6 and 10 μm) were used. The pregnant females were exposed to the particles on d 17 of pregnancy. Animals were killed by carbon monoxide asphyxiation, 30 min after gavage.

The small intestine was cut into 9 equal segments. The number of microparticles in several sites was counted using epifluorescence microscopy of cryosections. Particles were taken up in substantial numbers, with villous uptake more marked than at Peyer's patch tissue. The number of particles taken up is generally inversely proportional to particle size, but this difference diminishes when volume instead of particle numbers is analysed by ANOVA and Mann Whitney U tests. There was also substantial variation from animal to animal in uptake of particles of all 3 sizes. There was therefore no significant difference in total uptake across the groups. However the uptake in pregnant rats was greater than that in nonpregnant females, with most of this increase appearing proximally in villous epithelium and lamina propria. There are also substantial differences in the way in which the 2 female groups respond to particles of different sizes. The total uptake is also approximately twice the level of that seen in previously collected data for male rats. Until the explanation has been found for the inter-animal variation, it would therefore be premature to conclude that pregnancy has no effect on microparticle uptake, leaving residual concern about the impact on such individuals of accidental exposure to such large particles.

P12 The influence of total parenteral nutrition on the passage of microparticles across the rat gastrointestinal tract. By R. A. HAZZARD[1], K. E. CARR[1], O. O'SHEA[1], N. MANDIR[2], M. JORDINSON[2] and R. GOODLAD[2]. [1]*The Queen's University of Belfast; and* [2]*Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK.*

Previous studies indicated that total parenteral nutrition (TPN) can alter intestinal permeability, increasing bacterial and macromolecular translocation. The current study examines the effect of TPN on the uptake of 2 μm latex particles across the small intestine, using an established rat model. Twelve male Sprague–Dawley rats, aged 6 to 8 wk, were divided at random into TPN and control groups (n = 6 per group). The TPN group received a continuous infusion of a standard TPN diet (250 kcal/kg i.e. 60 ml per rat at almost 1 cal per ml) via the right jugular vein for 6 d. The control group had free access to standard food and tap water. On d 7, all animals received 0.25 ml latex suspension (containing fluorescent 2 μm microparticles): the TPN group received particles via a stomach cannula (inserted on d 0), while the control group received particles by gavage. Animals were killed by intravenous injection of 0.4 ml Sagatal 30 min following particle administration and the small intestine was divided into 9 equal segments. A 1 cm specimen containing a Peyer's patch from each segment was cryosectioned. Particle numbers at each site were counted using epifluorescence microscopy. Log transformed data were analysed using 1-way ANOVA. Particles were found in most sites, including epithelial and underlying tissues. Control particle uptake was maximal proximally, decreased

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press

steadily until the sixth segment and was negligible thereafter. Uptake in the TPN group was more variable with peaks at segments 1, 4 and 9. The results suggest that TPN may have increased total uptake and enhanced particle distribution along the intestinal length. Both effects may be due to a decrease in mucus production which could increase the possibility of particle contact with the mucosa or the rate at which particles move along the intestine. Another possible relevant factor is atrophy of the TPN gut. This could affect particle uptake either due to the presence of less tissue to take up the particles or, once taken up, having less tissue to pass through on their way to the deeper organs of the body.

P13 Immunohistochemical staining of matrix proteins in renal glomeruli of Lewis and Dwarf rats subjected to subtotal nephrectomy and growth hormone administration. By G. H. COPE and M. SOLEIMANI MEHRANJANI. *Department of Biomedical Science, University of Sheffield, UK; and Department of Biology, Arak University, Islamic Republic of Iran.*

The aim was to determine the effect of exogenous growth hormone on the sclerosis of remnant glomeruli which occurs following subtotal nephrectomy. This was studied immunohistochemically using male Lewis rats and age matched growth hormone deficient (dwarf) Lewis rats. These were subjected to subtotal (5/6th) nephrectomy (SNX) at 16 wk of age, received subcutaneous injections of human recombinant growth hormone (rhGH, 1.6 I.U. per day) between 30 and 60 d later and were killed after 120 d. Control animals received a sham operation, SNX alone or rhGH injections alone. The kidneys were perfused with saline, fixed in buffered formaldehyde solution and embedded in paraffin wax. Sections were incubated with commercially available affinity purified polyclonal antibodies against Type I, III and IV, collagen, laminin and fibronectin, and a monoclonal antibody against alpha smooth muscle actin (αSMA).

Neither Type I nor Type III collagen was detected within the glomerulus of sham operated or injection alone rats but Type III collagen was present in the Bowman's capsule of these animals. Staining intensified following SNX and was greatest in the SNX + rhGH groups. Type IV collagen was detected both within the glomerular tuft and in the Bowman's capsule but staining was strongest after SNX + rhGH administration when some glomeruli were completely hyalinised. Laminin was present in the glomerulus of normal rats but was not seen in the Bowman's capsule. The intensity of staining was, again, heaviest after SNX + rhGH administration and was slightly stronger and more widespread in the dwarf rats. Fibronectin appeared within the tuft matrix of all rats but was greatly increased after SNX + rhGH treatment. αSMA was not observed in the glomeruli or in the Bowman's capsule of sham operated or injection alone rats but slight staining occurred following SNX and SNX + rhGH administration. The heaviest staining was seen however in the Bowman's capsule in the SNX and SNX + rhGH groups and was slightly heavier in the Lewis rats.

We conclude that SNX results in the deposition of substantial amounts of extracellular matrix proteins and that the deposition is exacerbated by rhGH administration. However, little evidence was found to suggest that dwarf Lewis rats respond differently to normal Lewis rats even though they have lower ($< 10\%$) levels of circulating growth hormone.

P14 Demonstration of velocity compartmentalisation within the living cell body. By P. VESELY (introduced by A. BOYDE). *Institute of Molecular Genetics, Prague, Czech Republic.*

Overall 3 dimensional cellular motility is the result of the integration of all types and mechanisms of intracellular movement and net displacement. Until recently it was only possible to observe some of its component parts, such as the displacement of the whole cell and/or its surface motility using phase contrast or differential interference contrast (DIC) optics and cell-to-substratum contacts using reflection interference contrast. Except for the limited applicability of DIC, the interior of the intact thick living cell had been almost excluded from the imaging potential of wide field optical microscopes. The advent of optical sectioning via confocal imaging brought the chance to view intracellular activity within living cells, but this could not be exploited until confocal reflection laser scanning achieved real time speed with a matching video rate recording speed. This technique has enabled the study of the distribution of velocities of entities moving within the cell and between cells at high optical and temporal resolution. Such an examination of the living cell in a well controlled in vitro environment shows that the mutual assessment of the dynamics of cell-to-substratum contacts, the detailed activity around the cell periphery and the integrated intracellular dynamics stratified within the depth of the cell body, can provide information about velocity compartmentalisation. Taken all together, they indicate the current status of the cell and can be used to characterise fast cellular reactions to chemical and physical challenges. The exploitation of this approach in cell biology and medicine may serve to advance our intuitive appreciation of speed and time in cell life.

Supported by an Anatomical Society Senior Visiting Fellowship.

P15 A mutation of myosin VI and its effect on the hair cells of the mouse mutant, *Snell's waltzer*. By T. J. SELF[1], T. SOBE[2], K. AVRAHAM[2] and K. P. STEEL[1]. [1]*MRC Institute of Hearing Research, Nottingham, UK; and* [2]*Sackler School of Medicine, Tel Aviv University, Israel.*

The deaf mouse mutant *Snell's waltzer* (*sv*) has an autosomal recessive mutation on chromosome 9 and is characterised by hyperactivity, circling and head tossing. The *sv* gene has been identified and encodes an unconventional myosin VI.

In our study we examined the morphology of the organ of Corti from mutant mice and their littermate controls. Using scanning electron microscopy we looked at mutants and controls at the stages: newborn, 3, 7 and 20 d after birth (DAB). Using transmission electron microscopy we looked at mutant and control hair cells at newborn and 20 DAB. The stereocilia bundles at the top of the sensory hair cells of the controls were normal. In contrast, the early development

of the stereocilia bundles of the mutants was abnormal with aberrant bundle arrangement, fusion and bulging of the apical surface. The stereocilia continued to fuse and elongate to produce giant stereocilia by 20 DAB. In the latter stages of development there was also evidence of outer hair cell death and scar formation. One possible explanation for our observations is that myosin VI may be involved in anchoring the apical membrane to the upper surface of the hair cell, and if the membrane between the stereocilia is not anchored properly the stereocilia may tend to fuse.

https://doi.org/10.1046/j.1469-7580.1999.19440589.x Published online by Cambridge University Press