Supporting Online Material for

Expression and Function of Junctional Adhesion Molecule–C in Myelinated Peripheral Nerves


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## Supporting Online Material

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Materials and methods

**Animals**

JAM-C KO mice (on a 129Sv x C57BL/6 background), and mice over-expressing JAM-C in their endothelial cells under the control of the Tie-2 promoter (on a C57BL/6 background), were generated and bred as previously detailed ([S1], [S2]). As appropriate, control mice were either heterozygous litter mates of the JAM-C KO mice or commercially purchased C57BL/6 mice (Harlan-Ölac, UK). Unless stated otherwise, all studies employed adult mice (~8-16 weeks old). Whilst JAM-C KO mice can exhibit numerous health defects (e.g. susceptibility to lung infections) during early phases of their lives ([S3]), all mice used in the present studies were of generally normal health status, e.g. exhibited normal breathing, and had normal appearance of fur.

**Antibodies**

The following primary antibodies were used: Rabbit anti-mouse JAM-C polyclonal antibody (clone 322501), rat anti-mouse JAM-C monoclonal antibody (mAb) (clone H36), rabbit anti-human JAM-C polyclonal antibody (clone 714) and rabbit anti-mouse claudin-19 polyclonal antibody were generated as previously detailed ([S4]-[S6]). Rat anti-mouse PECAM-1/CD31 mAb (clone Mec13.3; BD-Pharmingen, Oxford, UK), mouse anti-mouse neurofilament mAb (clone RMdO20), mouse anti-mouse pan sodium channels mAb (clone K58/35; both from Sigma-Aldrich, Poole, UK), rat anti-mouse E-cadherin mAb (clone ECCD-2), rabbit anti-mouse connexin 32 polyclonal antibody, rabbit anti-human neurofilament mAb (clone 2F11; Dako, Ely, UK) were obtained commercially. Rat anti-mouse laminin γ1 mAb (clone 3E10) was a gift from Dr. L. M. Sorokin (University of Muenster, Germany) ([S7]), rat anti-mouse JAM-A mAb (clone BV-11) was a gift from Dr. E. Dejana (IFOM, Milan, Italy) ([S8]), mouse anti-mouse MOG mAb (clone Z12) was a gift from Dr. S. J. Piddlesden (Burnet Institute at Austin, Australia) ([S9]) and rabbit anti-mouse P0 polyclonal antibody was a gift from Dr. D. R. Colman (Montreal Neurological Institute, Canada) ([S10]). Appropriate secondary antibodies conjugated to Alexa Fluor dyes were purchased from Molecular Probes (Invitrogen, Paisley, UK). Alkaline phosphatase (APAAP) (code D0651), mouse monoclonal and polyclonal goat anti-mouse immunoglobulins (code Z0420) were obtained commercially (Dako, Ely, UK).

**Immunofluorescence microscopy**

Analysis of cremaster muscles was performed as previously detailed ([S11]). Briefly, cremaster muscle tissues were dissected away from mice, fixed in methanol or 4 % paraformaldehyde (PFA), blocked and permeabilised in phosphate buffered saline (PBS) supplemented with 20 % fetal calf serum (FCS) and 0.5 % Triton X-100 for 2 h, and incubated with primary antibodies at room temperature (RT) overnight. Tissues were then
washed thoroughly in PBS and incubated with the appropriate secondary Abs conjugated to Alexa Fluor dyes 488 or 568 for 2 h. For whole-mount staining of sciatic nerves, the isolated nerves were fixed in 4 % PFA, teased on polylysine-coated glass slides and air dried. Teased fibers were postfixed/permeabilised in ice cold methanol for 20 min, rinsed in PBS, blocked for 30 min in PBS containing 10 % normal goat serum (NGS) and 0.1 % Triton X-100 and stained with primary antibodies and secondary mAbs as described above. In some experiments nuclei were post-stained with Draq5 (Invitrogen, Paisley, UK). The immunostained samples were then mounted on glass slides and observed at RT using a Zeiss LSM 5 PASCAL confocal laser-scanning microscope (Zeiss Ltd, Welwyn Garden City, UK) equipped with Argon (excitation wavelength: 488nm) and HeNe (excitation wavelengths: 543 and 633 nm) lasers. Multiple optical sections of tissue samples were captured with the software’s automatic scanning mode. Z-stack images were obtained for 3D-reconstruction of whole vessels and nerves using the LSM 5 Pascal software (version 3.2). For PNS/CNS comparison, perfusion fixed (4 % PFA) mouse spinal cords with the ventral roots were post-fixed for a minimum of 4 h in 4 % PFA, cryoprotected in 30 % sucrose/PBS and cryosectioned at 8 μm. Longitudinal frozen sections were mounted on glass slides, air dried and fixed in ice cold methanol for 10 min. Sections were rinsed in PBS and processed as described above for nerves. Images were obtained with a Nikon E1000M epifluorescence microscope linked to a digital camera and analyzed using Image ProPlus 5.1 (Media Cybernetics, UK). In all studies isotype-matched control Abs were employed in parallel with the specific primary antibodies.

**Immunohistochemistry**

All human samples were obtained after full informed consent of patients and with the approval of the Local Research Ethics Committee. For staining of human tissues, control sural nerves (n = 6, F:M, 1:5, mean age [range] 34 y [16-58 y]) were obtained during nerve repair surgery (i.e. normal nerves, harvested for grafting). Specimens of demyelinating sural nerves (n = 5, F:M, 1:4, mean age [range] 65 y [50-77 y]) were obtained from patients with a clinical, electrophysiological and histopathological diagnosis of typical chronic inflammatory demyelinating polyneuropathy (CIDP), which is an acquired immune mediated condition of idiopathic etiology. Specimens of post-traumatic neuromas (n = 10, F:M, 1:9, mean age [range] 30 y [20-38 y]) were obtained during nerve repair, and avulsed dorsal root ganglia (DRG, n = 2, F:M, 0:2, mean age [range] 26 y [25-28 y]) were obtained during surgery for brachial plexus repair. All tissues were snap frozen in liquid nitrogen and stored at -80°C until use. Frozen sections (15 μm) were collected onto poly-L-lysine-coated glass slides and fixed in 4 % PFA in PBS for 30 minutes. After washing in PBS, endogenous peroxidase was blocked by incubation with 0.3 % hydrogen peroxide in industrial methylated spirits (IMS) for a further 30 minutes. For immunohistochemical co-localisation studies, serial sections of human tissue were double-labelled by incubation with a mixture of primary polyclonal antibody to recombinant human JAM-C (dilution 1:10,000) and a monoclonal antibody to neurofilament. Immunoreaction for neurofilament was revealed using alkaline phosphatase to give a red product, whilst recombinant human JAM-C immunoreactivity was revealed using the nickel-enhanced ABC peroxidase method to give a grey/black product. Double-labelled preparations were air dried and mounted in Ultramount medium (Vector Labs, UK). Specificity was confirmed in controls which
included omission or serial dilutions of primary antibodies. For analysis of the JAM-C paranodal staining, the total number of paranodes was counted per section using a calibrated eye piece, and results were expressed as mean number of JAM-C positive paranodes/mm².

**Methylene blue staining**

Transverse semithin (1.5 μm) Epon 812-embedded sections of sciatic nerves were mounted on a microscope slide and stained with a solution containing 0.5 % Methylene blue, 0.5 % Azur II and 0.5 % Borax (Merck, Darmstadt, Germany). The slide was immediately rinsed with distilled water and mounted with Eukitt (O. Kindler, Freiburg, Germany).

**Electron microscopy**

Sciatic nerves from WT and JAM-C KO mice were either fixed by immersion in a 2 % solution of glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 mol/L sodium cacodylate buffer, containing 0.1 % tannic acid, at room temperature or subjected to perfusion fixation (S12). Briefly, the aorta of anesthetized mice was cannulated with a Butterfly-25G (Venisystems) that was inserted in the blood direction, and was connected to a perfusion pump. Through this system, the lower part of the mice was infused with a solution of 1 % (v/v) heparin and 1 % xylocaine (v/v) in 0.9% NaCl, at the rate of 1 ml/min. As soon as the perfusion started, the inferior vena cava was sectioned to avoid increase in vascular pressure. After 5 min of infusion of this rinsing solution, the animals were infused by the same route with a 2.5 % solution of glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 mol/L sodium cacodylate buffer. 5 min later, the sciatic nerves were dissected and immersed in the same fixative until further processing. In both series, samples were then post-fixed in 2 % osmium tetroxyde, stained en bloc in uranyl maleate for 1 h, dehydrated, and embedded in Epon 812 (Fluka Chemie, Buchs, Switzerland). Thin sections were cut and stained with uranyl and lead citrate and examined by a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

**Immunoelectron microscopy**

Sciatic nerves dissected from WT and JAM-C KO mice were fixed for 5 min at RT in a mixture of 4 % PFA and 0.1 % glutaraldehyde and then 60 min in 4 % PFA (all fixatives diluted in 0.1 M PBS, pH 7.4) or fixed by perfusion as indicated above. The nerves were washed three times in 0.1 M phosphate buffer, cut in small segments, embedded in 12 % gelatin and cooled on ice. Blocs of gelatin-embedded nerves were infused with 2.3 M sucrose, frozen in liquid nitrogen, and sectioned with an EMFCS ultracryomicrotome (Leica, Wetzlar, Germany). Ultrathin sections were mounted on Parlodion-coated copper grids. The sections were processed as per previously described protocols (S13, S14), which, in these experiments, included a 1 h exposure at RT to the anti-JAM-C antibodies mentioned above, diluted 1: 100, and a 20 min exposure at room temperature to Protein A-coated gold particles of 15 nm diameter, diluted 1:150. Cryosections were screened and
photographed with a CM10 electron microscope (Philips, Eindhoven, The Netherlands). Negative controls were run by exposing the sections to only the Protein A-coated particles.

**Electrophysiology**
Sciatic nerves were dissected with their surrounding connective and muscle tissues from JAM-C KO and control mice (heterozygous littermates) and stored in oxygenated Krebs solution at RT for up to 2 h. Tissues were transferred to an interface recording chamber (33 ºC), and the sciatic nerve was stimulated at a voltage empirically determined to elicit maximal compound action potential (CAP) amplitudes (0.02 ms pulses, 0.5 Hz) via a bipolar tungsten-in-glass recording electrode (tip separation ~100 μm). Recordings were made in a blind fashion from the nerve via a glass microelectrode (~1 M-Ohm) filled with Krebs solution and connected to an Axoprobe 2A amplifier. Recordings were digitized at 50 kHz via a CED1401 interface and Spike2 software and stored on a computer for off line analysis of conduction velocities. Stimulation voltage was increased and appearance and latency of A-fiber compound action potential waves was noted.

**Behavioral tests**
Experiments were performed according to the guidelines of animal care of the University of Geneva. Motor coordination was examined in a blind fashion in a footprint and grip strength assay as previously described (S15). For the footprint assay, the hind feet of JAM-C KO mice and littermate controls were coated with nontoxic ink and mice were allowed to walk through a tunnel (100 cm long, 10 cm wide) with paper lining the floor. Mice were given one practice run after which measurements of the footprint tracings were analyzed and the stride lengths averaged. Muscular strength was measured by a blinded investigator using a grip strength meter (Bioseb, Chaville, France) to measure the forepaws’ strength. Mice were allowed to grip on to a wire while the experimenter gently pulled them by the tail in the opposite direction. Mice performance was measured by a digital scale reflecting their muscular force at the point preceding the loss of grip. Results are presented as forelimb grip strength according to the manufacturer’s unit of force (gr). Each session consisted of three consecutive trials with a 30 s recovery time that allowed for calculating a mean value.

**Statistics**
All results are expressed as mean ± SEM. Statistical significance was assessed by unpaired Students t-test or Mann-Whitney test (for the human data). Differences were accepted to be statistically significant at P < 0.05*.
Figure S1. JAM-C is expressed in peripheral nerves.
Cremaster muscles were dissected away from mice and immunostained for structures within blood vessels (v) and nerves (n) before being analyzed by confocal microscopy. (A) Confocal images of tissues obtained from control heterozygous mice and JAM-C KO mice stained with antibodies against laminin γ1 (red) recognizing laminin 2 and laminin 8, which are expressed in the basement membrane of nerves and nerves/endothelial cells, respectively (S11, S16, S17), and JAM-C (green). These images confirm the antibody’s specificity for JAM-C and its detection in nerves and blood vessels. (B) Confocal images of cremaster muscles obtained from transgenic mice over-expressing endothelial JAM-C, under the control of the endothelial cell-specific promoter Tie2 (S2) stained with antibodies against PECAM-1 (red) and JAM-C (green). As expected, vascular JAM-C expression was higher than that detected in WT animals (see also Fig. 1A) whereas the neural expression of JAM-C remained unchanged. Scale bars: 15 μm.
Figure S2. Neural expression of JAM-C is restricted to junctional regions of Schwann cells of the PNS.

(A) Schematic diagrams of Schwann cells enwrapping an axon illustrating the locations of junctional sites of non-compact myelin that collectively seal the glial cell borders and maintain the integrity of the myelin sheath. (B) Paranodal regions and Schmidt-Lanterman incisures of teased sciatic nerve fibers double stained with antibodies against JAM-C (green) and connexin 32 (Cx32), voltage gated sodium channels (Na$_v$), myelin associated glycoprotein (MAG) or E-cadherin (E-Cad) (all in red) in WT (upper panels) and JAM-C KO mice (lower panels). All molecules show normal distribution in JAM-C deficient mice. Scale bar: 10 μm.
Figure S3. Expression of JAM-C during postnatal development.
Teased sciatic nerves from WT mice at postnatal days P2, P5 and P16 stained with antibodies against JAM-C (green) and laminin γ1 (red) (upper panels), and P0 (green) and laminin γ1 (red) (lower panels) with nuclei stained with Draq5 in blue. JAM-C concentrated at developing paranodes at P5 and showed mature expression at P16 around nodes of Ranvier (arrow heads). P0 was highly expressed in compact myelin at P2 and was excluded from the paranodal region (also indicated by strong laminin γ1 deposition) at later time points. Scale bar: 10 μm.
Figure S4. Junctional localization of JAM-C at paranodal regions of Schwann cells.

(A) Schematic diagram of a node of Ranvier illustrating its organization on either side by two Schwann cells, whose cytoplasm increases at paranodal regions (non-compact myelin) to form terminal loops that closely interact with the axon (at paranodal junctions, PNJ) and lateral myelin lamellae (tight junctions, TJ). (B) Immunoelectron microscopy of longitudinal sections of sciatic nerves of WT mice revealed expression of JAM-C in the non-compact, paranodal myelin, but not in the axon (ax) and the compact myelin (cm) regions. (C) No JAM-C labelling was detected in sciatic nerves of JAM-C KO mice confirming the specificity of the procedure. (D) Ultrastructural analysis of longitudinal sections of sciatic nerves from WT and JAM-C KO mice (16 weeks old) prepared for conventional electron microscopy indicated that a proportion of KO terminal loops exhibited a disrupted sequential organization, with an apparent looser adhesion to adjacent loops and axon. Scale bars: (B) and (C) 300 nm, (D) 240 nm. Of relevance, additional
analysis of KO nerves showed no evidence of axonal damage as indicated by staining for amyeloid precursor protein (APP), a marker for axonal injury (S18).

Figure S5. JAM-C is expressed in human peripheral nerves.
Double staining of JAM-C (black, indicated with arrows) and neurofilament (red) in human nerves. In neuroma from a patient with nerve injury (A) the number of JAM-C positive paranodes is reduced in accordance with the demyelinated state of the fibers. (B) In sections from dorsal root ganglia obtained during surgery for brachial plexus repair, JAM-C was observed in nerve fiber paranodes (arrows) but not in neuronal cell body (arrow heads) confirming its non-neuronal origin. (C) Quantification of the number of JAM-C positive paranodes/mm² in the different groups including normal nerves harvested for grafting during nerve repair surgery, neuropathic nerves from patients with a clinical and histopathological diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP), and injured nerves (see also Fig. 4), n = 6 control nerves, and n = 5 and n = 10 for neuropathic and injured nerves, respectively, ** p<0.01 and *** p < 0.001. Scale bar: 50µm.
References