QUANTITATIVE CHARACTERISATION AND NEUROCHEMICAL CODING OF THE HUMAN HINDGUT MYENTERIC PLEXUS

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INTRODUCTION

• Normal gut function dependent on integration of complex processes involving motor and sensory function

• Orchestrated by an intricate enteric nervous system (ENS)
INTRODUCTION

• Normal gut function dependent on integration of complex processes involving motor and sensory function

• Orchestrated by an intricate enteric nervous system (ENS)

• ENS frequently referred to as ‘little’ brain of gut
INTRODUCTION

- Recent appreciation of ‘Gastrointestinal Neuromuscular Disorders’
  - presumed secondary to gut neuromuscular dysfunction
  - impaired motor activity leading to abnormal transit +/- visceral dilatation
INTRODUCTION

Neuromuscular disease classification

The London Classification of gastrointestinal neuromuscular pathology: report on behalf of the Gastro 2009 International Working Group

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ABSTRACT
Objective Guidelines on histopathological techniques and reporting for adult and paediatric gastrointestinal neuromuscular pathology have been produced recently by an international working group (IWG). These addressed the important but relatively neglected areas of histopathological practice of the general pathologist, including suction rectal biopsy and full-thickness intestinal tissue. Recommendations were presented for the indications, safe acquisition of tissue, histological techniques, reporting and referral of such histological material.

Design Consensual processes undertaken by the IWG and following established guideline decision group methodologies.

Results and conclusion This report presents a contemporary and structured classification of gastrointestinal neuromuscular pathology based on defined histopathological criteria derived from the existing guidelines. In recognition of its origins and first presentation

Significance of this study
What is already known about this subject?
- Numerous case reports and small case series have associated gastrointestinal neuromuscular diseases with a number of underlying histopathological abnormalities.
- Recently published guidelines delineating techniques and histopathological reporting have offered some diagnostic standardisation in an area in which huge methodological variations had previously confounded the significance and reliability of reporting.
- An internationally agreed unifying classification of gastrointestinal neuromuscular pathology based on diagnostic criteria and related to clinical entities was, however, still lacking.

- Supplementary materials are published online only. To view these files please visit the journal online (http://gut.bmj.com).

For numbered affiliations see end of article.

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INTRODUCTION

Table 2  Diagnostic criteria for histological phenotypes

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>QL/QT</th>
<th>Minimum*</th>
<th>Adjunctive</th>
<th>Findings (brief)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1 Aganglionosis</td>
<td>QL, QT</td>
<td>H&amp;E or EH</td>
<td>EH (AChE)</td>
<td>Complete absence of neurons</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IHC (calretinin)†</td>
<td>Hypertrophic submucosal extrinsic nerves</td>
</tr>
<tr>
<td>1.2.1 Hypoganglionosis</td>
<td>QL</td>
<td>H&amp;E</td>
<td>IHC (PGP9.5, NSE)†</td>
<td>Severe reduction in ganglia and neurons</td>
</tr>
<tr>
<td>1.3.1 Ganglioneuromatosis</td>
<td>QL</td>
<td>H&amp;E</td>
<td>IHC (PGP9.5, NSE, S100)†</td>
<td>Hamartomatous increase in neurons and glia</td>
</tr>
<tr>
<td>1.3.2 IND, type B</td>
<td>QT</td>
<td>EH (LDH)</td>
<td></td>
<td>&gt;8 neurons in &gt;20% of 25 submucosal ganglia</td>
</tr>
<tr>
<td>1.4 Degenerative neuropathy</td>
<td>QL</td>
<td>H&amp;E</td>
<td></td>
<td>Degenerative cytological appearances</td>
</tr>
<tr>
<td>1.5 Inflammatory neuropathies</td>
<td>QL</td>
<td>H&amp;E</td>
<td></td>
<td>Gross infiltrates or eosinophils</td>
</tr>
<tr>
<td></td>
<td>QT</td>
<td>IHC (CD45, CD3)</td>
<td></td>
<td>≥1 intraganglionic and/or &gt;5 periganglionic lymphocytes/ganglion</td>
</tr>
<tr>
<td>1.6. Abnormal content in neurons</td>
<td>QL</td>
<td>H&amp;E</td>
<td>IHC (SUMO1), TEM</td>
<td>Intraneuronal nuclear inclusion bodies</td>
</tr>
<tr>
<td>1.7 Abnormal neurochemical coding</td>
<td>QL, QT</td>
<td>IHC†</td>
<td>IHC†</td>
<td>Increased immunostaining vs controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IHC (PGP9.5, NSE)†</td>
<td>Reduced defined subset of neurons</td>
</tr>
<tr>
<td>1.8 Neuronal immaturity</td>
<td>QL</td>
<td>H&amp;E</td>
<td>EH (LDH, SDH)</td>
<td>Morphologically immature neurons</td>
</tr>
<tr>
<td>1.9 Abnormal enteric glia</td>
<td>QL</td>
<td>H&amp;E</td>
<td>IHC (S100, GFAP)</td>
<td>Marked increase</td>
</tr>
<tr>
<td>2.1 Muscularis propria malformations</td>
<td>QL, QT</td>
<td>H&amp;E</td>
<td></td>
<td>Any departure from 2 muscle layers</td>
</tr>
<tr>
<td>2.2.1 Degenerative leiomyopathy</td>
<td>QL</td>
<td>H&amp;E</td>
<td>Tinctorial¶, IHC (SMA)</td>
<td>Myocyte damage and loss, fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TEM</td>
<td></td>
</tr>
<tr>
<td>2.2.2 Inflammatory leiomyopathy</td>
<td>QL</td>
<td>H&amp;E</td>
<td></td>
<td>Inflammatory cell infiltrate</td>
</tr>
<tr>
<td>2.3.1 Muscularis mucosae hyperplasia</td>
<td>QL</td>
<td>H&amp;E</td>
<td></td>
<td>Increased thickness muscularis mucosae</td>
</tr>
<tr>
<td>2.4.1 Filament protein abnormalities</td>
<td>QL</td>
<td>IHC (SMA)</td>
<td></td>
<td>Absent SMA in circular muscle**</td>
</tr>
<tr>
<td>2.4.2 Inclusion bodies</td>
<td>QL, QT</td>
<td>H&amp;E</td>
<td>Tinctorial (PAS)</td>
<td>Smooth muscle amphilophilic 'M' bodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TEM</td>
<td>Smooth muscle polyglucosan bodies</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Megamitochondria in myocytes</td>
</tr>
<tr>
<td>2.5.1 Atrophic desmosis</td>
<td>QL</td>
<td>Tinctorial¶</td>
<td></td>
<td>Total or focal lack of connective tissue scaffold</td>
</tr>
<tr>
<td>3.1 Abnormal ICC networks</td>
<td>QT</td>
<td>IHC (CD117) IHC (Ano1)</td>
<td></td>
<td>&gt;50% reduced ICC in comparison with control sections</td>
</tr>
</tbody>
</table>

**Criteria listed in table 2 (and Supplementary material I). Table 3 shows the provisional relationships currently ascribed by the international working group guidelines for gastrointestinal neurogastrointestinal encephalomyopathy (GINMP). The diagnosis of GINMP requires adequate morphological study of the different components of the enteric neuromusculature and the placement of degenerative and inflammatory changes in distinction. The criteria listed in table 2 have been designed to assist in diagnosis, but are not a recommendation of the international working group guidelines for GINMP. The criteria are provisional and may be modified, added or deleted with appropriate evidence. In certain instances, the presence of a finding should be confirmed by co-expression with another finding, for example AChE, cholinergic staining; H&E, hematoxylin and eosin; IHC, immunohistochemistry; IND, intestinal neuronal dysplasia; QL, quantitative light; QT, quantitative tone; TEM, transmission electron microscopy; Tinctorial, Tinctorial light microscopy; S100, S100 protein; GFAP, glial fibrillary acidic protein; SMA, smooth muscle alpha-actin; PAS, periodic acid-Schiff; EH, enzyme histochemistry; ICC, interstitial cells of Cajal; CD117 synis synonymous with c-kit; Ano1 is synonymous with DOG1.
EDITORIAL

Counting neurons is not as easy as ‘one-two, three’

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Abstract
An accurate determination of the number of neurons in a segment of bowel is fundamental to establish population norms and identify neurodegenerative conditions, including age-related loss of myenteric ganglion cells. Although the latter phenomenon has been observed by several laboratories in various mammals, in this issue of Neurogastroenterology and Motility, Gamage et al. present evidence that colonic myenteric ganglion cells are maintained in aged mice. These discordant findings prompt a thoughtful consideration, the range of variables affecting the diagnosis of neuronal losses or gains associated with pathology or aging.

To better comprehend some potential reasons for the discordant results in this field, it is useful to consider some of the many factors that influence estimates of the size of the myenteric ganglion cell population:

Location and size of tissue samples
The density of myenteric neurons differs significantly in different parts of the intestinal tract, in general being lower in the small intestine than the large intestine.
INTRODUCTION

Current challenges in human tissue processing:

• Variability in tissue obtained
  – Intra-operative tissue handling
  – Unpaired data

• Heterogenous processing of tissue
  – Sectioning vs. wholemounts
  – Stains:
    • H/E staining
    • Histochemistry
    • Immunohistochemistry
INTRODUCTION
INTRODUCTION

Nerve fibres in circular muscle

Distribution of nerve fibres

Using antibodies to NOS or SP, labelled nerve fibres and cells were present in the myenteric ganglia, submucosal ganglia, longitudinal and circular muscle layers (Fig. 5A and B). SP-IR nerve fibres were also present in the mucosa. Labelling was performed on mid-colon from 7·2-week animals, 13·6-month animals, and 26·2-year guinea-pigs. Omission of primary antibody resulted in no detectable labelling.

Immunoreactive nerve fibre percentage area

The percentage area of NOS or SP-labelled nerve fibres in circular muscle in cross-section was measured (Fig. 5C and E). NOS-IR fibres comprised the highest percentage

Figure 4

Density of nerve cells in myenteric ganglia in 2-week, 6-month, and 2-year-old guinea-pigs. (A–C) Whole-mounts of myenteric ganglia immunolabelled with anti-Hu antibody showing nerve cell bodies in (A) 2 weeks, (B) 6 months, and (C) 2-year-old guinea-pigs. (D) Numbers of neurons per mm$^2$ at each age, (E) Numbers of neurons per unit box at each age with adjustments for the change in dimensions (circumference·length) occurring with colonic growth, (F) Neuron cell body area ($l_m^2$) in the Myenteric Ganglia at each age, (G) Numbers of neurons per ganglionic area (mm$^2$), (H) ratio of Ganglionic area (mm$^2$)/total area (mm$^2$) and (I) Ganglionic area mm$^2$ per unit box with corrections for the change in dimensions occurring with colonic growth. Number in brackets is n for each population; mean and SEM shown. P values from Tukey/C213 post hoc test following ANOVA. Scale bar = 300 µm.

C. J. Peck et al. Neurogastroenterology and Motility 2009 Blackwell Publishing Ltd

• **Wholemounts** remain ‘gold standard’ for quantitative neuronal analyses
INTRODUCTION

• Regional variation likely adds sophistication to ENS

• Different regions of colorectum serve varying function(s), raising possibility of concomitant variation in ENS.

• This may have clinical implications in understanding functional bowel disorders and post-surgical motility changes.
AIMS

• To quantitatively assess and neurochemically code the myenteric plexus of the human colon and rectum

• To assess for regional variation within the hindgut ENS
METHODS

- Specimen procurement / preparation
- Tissue Fixation / Clearing
- Tissue dissection
- Antibody incubation
- Microscopy
Specimen Procurement

- Paired samples human colon & rectum
- Fresh *anterior resection* specimens
- Full-thickness sections (1 - 2 cm) obtained from resection margins.
- Transported to lab in PBS with nicardipine (3 μM).
Specimen preparation

- Mesentery removed
- Gut tube opened \textit{longitudinally} along anti-mesenteric border
Fixation

- Tissue stretched maximally in all directions, and pinned mucosa side down in Sylgard-lined petri-dish.
- Tissue fixed with modified Zamboni’s fixative (2% paraformaldehyde / picric acid) – two nights, 4°C
- Tissue cleared with DMSO and PBS washes
Tissue dissection

- Sample of tissue (1.5 cm x 1.5 cm) obtained
Tissue dissection

- Mucosa dissected off
- Circular muscle carefully dissected off, leaving only longitudinal muscle and myenteric plexus
Antibody incubation

Pre-incubation:
- Tissue pre-incubated with BSA, donkey serum, triton (2hrs, RT)

Primary antibodies:
- Anti-Hu (1:500) (mouse)
- Anti-NOS [nitric oxide synthase] (1:1,000) (rabbit)
- Anti-ChAT [choline acetyltransferase] (1:200) (goat)

Secondary antibodies:
- Hu – AF488 (1:200)
- NOS – Cy3 (1:400)
- ChAT – AF 647 (1:100)
Microscopy

- Preparations viewed with P.A.L.M. DuoFlex Combi System fluorescence microscope (Carl Zeiss) equipped with motorised stage and filters to distinguish fluorophores (10X objective).

- Images acquired using MosaiX software:
  - Tile stitching
  - Allocation of focus points and ‘Z-positions’
Image analysis

- **100mm²** wholemount images acquired

- Images analysed using MetaMorph software:
  - Ganglia identified and ROI drawn
  - Background flattening
  - Threshold intensity
  - Automated cell body counting

- **Manual validation of counts** - random 10% of ganglia
Outcome Measures

- **Ganglionic density** (stretch uncorrected / corrected)
  - number of ganglia per 100mm$^2$
Outcome Measures

• Ganglionic area density
  – area of ganglia (mm$^2$) per 100mm$^2$
Outcome Measures

• **Neuronal density** (stretch uncorrected / corrected):
  – number of neurons per mm$^2$

• **Neurochemical coding:**
  – NOS+ or ChAT+
RESULTS

• 15 paired colon / rectum samples
• 8 males, median 63 yrs [51 – 86]
• All resections performed for cancer
Wholemount image acquisition
Myenteric ganglia

- Ganglia (10X objective)
Ganglionic density – stretch corrected

Ganglionic density (ganglia per 100mm²)

- **Ganglionic density – stretched uncorrected (median, range):**
  - Rectum: 162 ganglia per 100mm² (126 – 362)
  - Colon: 184 ganglia per 100mm² (113 – 296)

- **Ganglionic density – stretch corrected (median, range):**
  - Rectum: 585 ganglia per 100mm² (308 – 922)
  - Colon: 510 ganglia per 100mm² (386 – 1170)

\[ P = 0.99 \]
Ganglionic area density

- Ganglionic area density (median, range):
  - Rectum: 9.83 mm² per 100mm² (5.80 – 17.19)
  - Colon: 11.92 mm² per 100mm² (7.53 – 18.64)

P = 0.100
• Neuronal density – stretched uncorrected (median, range):
  – Rectum: 50.6 neurons per mm² stretched tissue (27.8 – 106.3)
  – Colon: 65.5 neurons per mm² stretched tissue (34.6 – 114.1)

• Neuronal density – stretched corrected (median, range):
  – Rectum: 182.0 neurons per mm² relaxed tissue (89.4 – 361.3)
  – Colon: 188.9 neurons per mm² relaxed tissue (116.7 – 387.9)
Neurochemical Coding

50 μm
Neurochemical Coding

Hu+ (pan-neuronal)
Neurochemical Coding

NOS +
‘inhibitory’

50 µm
Neurochemical Coding

ChAT + ‘excitatory’

50 μm
Neurochemical Coding

- NOS + / ChAT +
- NOS + / ChAT −
- NOS − / ChAT +
- NOS − / ChAT −
Neurochemical Coding

![Graph showing neurochemical coding percentages for different groups.]

- **Rectum**
  - NOS+/ChAT+
  - NOS+/ChAT-
  - NOS-/ChAT+
  - NOS-/ChAT-

- **Colon**
  - NOS+/ChAT+
  - NOS+/ChAT-
  - NOS-/ChAT+
  - NOS-/ChAT-

**P > 0.10**
DISCUSSION

• Accurate quantitation and neurochemical profiling of the human hindgut myenteric neurons / ganglia

• No difference in quantitation nor neurochemical coding of intrinsic innervation of human colon and rectum.

• Limitations:
  – Inability to clinically correlate neuro-histological findings
CONCLUSIONS

• Successful staining of the human hindgut myenteric plexus using wholemount techniques, permitting the development of a robust ‘normative’ dataset

• May advance current understanding and diagnostic acuity of enteric neuropathies

• Remarkable similarity in the intrinsic innervation of human colon and rectum,
  ✴ potential insight into pathoetiologia of bowel dysfunction following rectal surgery – ‘anterior resection syndrome’
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