Investigating the interaction of the enteric nervous system with the extrinsic nervous system and the immune system in children and development of cell therapies for Hirschsprung's Disease

HAWAII

Hirschsprung's Advances; Working towards Autologous tIssue therapIes

1. Investigators

Chief Investigator:

Miss Rachel Harwood

Department of Paediatric Surgery
Alder Hey Children's NHS Foundation Trust
East Prescot Road
Liverpool

Co-investigators:

Professor Simon Kenny

Miss Sarah Almond

Dr Bettina Wilm

Dr Megan Smith

2. Background

The gastrointestinal (GI) tract is responsible for many functions essential to life such as digestion, absorption and excretion, all of which are under the control of its own intrinsic nervous system, the enteric nervous system (ENS) (1). The ENS is part of the autonomic peripheral nervous system and provides the intrinsic innervation of the bowel - the submucosal and myenteric plexuses are the two networks that comprise the ENS and consist of bundles of enteric neurons and glia – the enteric ganglia that are connected by nerve fibres. The ENS is essential for the autonomic regulation of gastric motility and secretion (2).

Hirschsprung's disease (HSCR) is a gut motility disorder, affecting 1 in 5000 people worldwide (3). HSCR is characterised by the absence of ganglia and nerve fibres of the ENS to various extents of the bowel: 80% of cases affect the distal colon and internal anal sphincter, but 10% of cases have total colonic aganglionosis and 1% total intestinal aganglionosis (4). Between the ganglionic and aganglionic bowel there is a transition zone. The transition zone is abnormally innervated due to a reduced number of enteric ganglia (3).

Initial presentation of neonates with HSCR typically includes distal intestinal obstruction, with symptoms including abdominal distention or vomiting and/or failure to pass meconium within the first 48hrs of life. Children with HSCR can also have difficulty feeding, severe constipation, and symptoms of HSCR- associated enterocolitis (HAEC) (5, 6).

Due to its lack of ENS, the aganglionic region of HSCR bowel is tonically contracted (3). This results in constipation and without treatment is fatal. The current gold-standard treatment for HSCR is a surgical pull-through procedure in which the aganglionic region and TZ is resected in an attempt to restore normal function.

Our group has previously demonstrated the presence of enteric nervous system progenitor cells (ENSPCs) in the aganglionic bowel of children with HSCR. ENSPCs have both a multipotent nature and proliferative capacity (7). There has been a significant amount of research into the feasibility of using ENSPCs to reduce or correct bowel dysmotility in mouse and rat models of ENS neuropathies (8-10). ENSPCs have commonly been cultured as neurospheres (9, 11-19). Neurospheres are free-floating cultures of neural stem cells that also contain neurons and glia and in the case of enteric neurospheres, ENSPCs (11, 12, 15-17).

ENSPCs cultured in neurospheres have the potential to be used as an autologous therapy for HSCR which would avoid the risk of immune rejection and can be isolated from embryonic and postnatal mouse, rat and human tissue (9, 11, 15, 17, 20). Enteric neurospheres have been shown to contain cells expressing various progenitor, neuronal and glial markers. In our lab, Almond et al. (11), demonstrated that both embryonic mouse caecum derived neurospheres and postnatal human colonic neurospheres expressed p75, Tuj1, S100, the proliferation marker BrdU and the neuronal subtype markers NOS and VIP showing that neurosphere cells were capable of proliferation and differentiation. This work was further explored in our group by Lindley and Wilkinson (15, 17, 20) and neurospheres were cultured from tissue from the aganglionic bowel of patients with HSCR. This demonstration(17), indicates the potential for autologous therapies using the patients' own aganglionic bowel due to the presence of progenitor cells that were capable of differentiation into different neuronal subtypes *in vitro*.

The transplantation of enteric neurospheres to aganglionic bowel has provided insight into the ability of such cells to rescue a model with a HSCR phenotype (9, 11, 15, 17). Using *ex vivo* models of

aganglionic tissue, multiple groups have demonstrated that transplanted neurospheres can integrate into tissue and differentiate into enteric glia and electrophysiologically active neurons (19).

Recently, we have found that there is an interaction in the transition zone of children with HSCR between the intrinsic ENS and the extrinsic nerves of the aganglionic bowel (21). As the transition zone is also resected during surgery for HSCR, this could provide a source of ENSPCs to use for autologous transplantation. However, transition zone neurospheres have not yet been characterised. We plan to include transition zone neurospheres in our investigation to determine whether these cells may be useful for transplantation. This forms part of our next set of experiments to investigate future therapies for HSCR.

The clinical outcomes of children with Hirschsprung's Disease have been poorly reported. Recently a core outcome set has been developed to help to standardise how children's outcomes are reported and to encourage reporting of patient's experiences as well as clinician's experiences. After pull-through 30% of patients suffering constipation or incontinence, with clear 'constipation' and 'rapid transit' phenotypes being evident (22). This may be caused by abnormal motility of the ganglionic region or by the fact that the aganglionic internal anal sphincter remains after surgery (22). Future therapies for HSCR aim to reduce long term morbidities associated with HSCR (23) but a key part of this includes drawing together children's lived experience with the findings within the laboratory. This group is currently undertaking a study of the long-term outcomes of children and adults with Hirschsprung's Disease (REC: 18/NNW/0608) and this study offers an opportunity to combine clinician and patient reported data with immunohistochemical data.

3. Research question

Can enteric nervous system neural progenitor cells (ENSPCs) isolated from the bowel of children with and without Hirschsprung's disease be modulated in vivo or transplanted as mature neural cells to restore function to the abnormal diseased bowel?

4. Aims

The aims of this study are to answer the following questions:

1. How are the nerve progenitor cells different in the different sections of the bowel of children with Hirschsprung's Disease (ganglionic, aganglionic and transition zone) and do these differ to the nerve progenitor cells of children without Hirschsprung's Disease?

2. Do neurospheres cultured from different parts of the bowel of children with Hirschsprung's Disease have the same composition of cells within them?

3. Can the differentiation potential of the progenitor cells within neurospheres be manipulated via signalling pathways such as NOTCH, serotonin, endothelin 3 and Sonic Hedgehog.

4. Can cultured neurospheres from ganglionic, transition zone and aganglionic bowel integrate and persist in aganglionic bowel?

5. Do cultured neurospheres from ganglionic, transition zone and aganglionic bowel restore function to aganglionic bowel?

6. Are there differences in the immune profile of the ganglionic, transition and aganglionic sections of the bowel of children with Hirschsprung's Disease

7. Does ganglionic bowel of children with Hirschsprung's Disease have different properties which relate to their clinical experience of living with the disease?

5. Rationale and significance

The current gold-standard treatment for HSCR is to surgically remove the affected bowel to restore normal bowel function and enable passage of stool. However, this often has unsatisfactory outcomes: after surgery ~30% of children have constipation, incontinence or recurrent episodes of enterocolitis and 10% will need a permanent stoma (6). These outcomes are associated with psychological difficulties and significantly impact the quality of life of children and their families. Thus, there is an urgent need to improve the treatment of babies and children with HSCR.

The proposed work is a logical continuation of our previous studies (Research Ethics Committee (04/09/RE "Isolation of Human Enteric Nervous System Stem Cells" and 10/69/RE "Enteric nervous system stem cell behaviour in mature muscle models"). We have previously demonstrated that we can isolate and culture human ENSPCs and that these cells can be transplanted into aganglionic bowel to restore contractility (17). However, there are still outstanding questions on how these cells can be optimised for successful transplantation before this work can be translated to clinical trials:

1) What are the characteristics of the ENSPCs that can be transplanted and go on to proliferate and differentiate.
2) Safety: Do all these cells differentiate? Are the stem cells adequately controlled within microenvironmental niches that prevent uncontrolled growth?
3) What is the ideal method of transplantation? This may be whole neurospheres or single cells, with and without pharmacological agents to aid the differentiation of the ENSPCs.
4) Can the progenitor cells be modulated in vivo to avoid transplantation using medication?

This project is designed to begin to answer these questions using established protocols from our lab as well as incorporating new approaches such as single cell transcriptomics. Firstly, we aim to compare the progenitor populations in ganglionic, transition zone and aganglionosis bowel from children with HSCR as well as control ganglionic bowel. We will do this by examining full thickness bowel tissue sections and neurospheres with histology and single cell transcriptomic analysis. While recent publications have used single cell analysis of neuronal and glial cell populations (24, 25), it has not been carried out in progenitor populations or in HSCR samples.

Secondly, we will identify and target signalling pathways such as NOTCH, serotonin and sonic hedgehog to try and improve the potential of progenitor cells in neurospheres to differentiate when transplanted. Finally, we will optimise the method used for transplantation of neurospheres using explants of aganglionic bowel to see if we can use transplanted cells to restore function. This may involve transplanting dissociated or intact neurospheres with and without signalling pathway manipulation.

The lived experience of children with Hirschsprung's Disease is very variable, even when the length of bowel affected by Hirschsprung's Disease is the same(26). This heterogeneity may be due to the effect of differing genetic backgrounds or differences in the development of the ENS in the ganglionic part of bowel children with HSCR. These differences may reflect differences in the progenitor population and could affect the success of transplantation. Investigation of whether there are visible differences is a key step towards understanding why children have different experiences and will help to understand the translational impact of this work.

In this project we build on many years of HSCR research undertaken by our team. Our long-term goal is to use the patients' own neural stem/progenitor cell population to innervate the aganglionic bowel of infants with HSCR, thus avoiding the need for life-changing surgery and improving outcomes. To reach the point of clinical translation, a robust understanding of the developmental origin and the

properties of neural progenitor cells is essential. We have shown that neurospheres derived from bowel cell cultures contain ENSPCs that could be used as therapies (17). Therefore, it is necessary to identify any therapeutic cell population within HSCR bowel and subsequently neurospheres, and to determine the impact of external signalling pathways on the cultured cells. Furthermore, understanding the molecular mechanisms that control proliferation is a prerequisite for safe progenitor cell therapies. Linking these findings with clinical investigations, including whole genome sequencing, and outcomes is a key part in determining the potential of autologous therapies for translation to clinical practice.

6. Research design

This will be a prospective study.

7. Demographics

Infants and children undergoing intestinal resection or stoma formation. Samples will be taken from bowel resected for clinical necessity. No additional tissue will be removed.

8. Research methods

Inclusion criteria

Any child undergoing therapeutic intestinal resection. Specifically, patients in which it is not necessary for all resected tissue to be sent for histopathological analysis. No additional tissue will be removed for research purposes.

Exclusion criteria

None

Handling of human tissue samples

Human tissue samples will be stored at the Institute in the Park, Alder Hey Children's Hospital, Liverpool and Stem Cell laboratory, Department of Molecular Physiology and Cell Signalling, Nuffield Wing, Crown Street, University of Liverpool. Samples will be stored and disposed of according to the regulations set down in the Human Tissue Act (HTA) and under the remit of the University of Liverpool HTA governance officers.

Of the following investigations of the tissue – not every tissue will undergo each evaluation but a selection of investigations will be used depending on the study question being answered.

Isolation of ENSC and culture of neurospheres.

The sample of bowel will be mechanically and chemically disrupted in order to produce a dissociated cell suspension which is then cultured to promote the formation of neurospheres (aggregates of stem cells, neurons and glia) using optimised cell culture and culture media. This will be performed as per our previous studies(11, 15, 17). Neurospheres will be used for subsequent analysis using histology and other techniques described below.

Histology

As previously demonstrated, we will analyse the characteristics of the bowel tissue by looking at key markers from tissue sections and neurosphere sections. Using established techniques tissue and neurospheres will be stained using antibodies for immunofluorescence and immunohistochemistry staining such as Haematoxylin and Eosin (17, 21, 27). Such antibodies used may include those to

identify proliferating cells, neuronal and glial cells and progenitor cells. The modified thymidine analogue EdU and apoptosis assays will be used to assess proliferation and apoptosis within neurospheres in culture. This will allow us to match progenitor status with proliferative behaviour and identify aberrant responses. The stained tissue and neurospheres will be imaged using various microscopy techniques including but not limited to confocal and light microscopy.

Single cell analysis

Following dissociation of tissue to single cells from patient tissue we would prepare for a recovery of 10,000 cells and 20,000 reads per cell. The samples will be run on a NovaSeq 6000 using an S2 flow cell for an estimated output of 3.3 billion reads, at the Centre for Genomic Research (CGR, University of Liverpool). We will use the Chromium X fixed RNA workflow which allows collection and storage of samples, followed by library preparation and sequencing of all collected samples in one day. The single cell whole-transcriptome sequencing data will be analysed using the computing program R and cells will be clustered according to their principle components using cell cluster analysis software/programmes CellRanger and Seurat. Neuronal progenitor cells will be identified and transcription factors will be interrogated for significant differences using Ingenuity Pathway Analysis. Specifically, we will be looking at the molecular profiles of individual cells to discriminate between different types of cells.

qPCR analysis

RNA will be extracted from tissue preparations encompassing the myenteric plexus from patient tissue samples to confirm expression of significantly upregulated markers using quantitative RT-PCR (qRT-PCR). Specimens will be analysed for the presence and quantity of markers such as SOX10, p75, Tuj, GFAP, calretinin and GLUT1 using established primers but also those identified in scRNA-Seq; β -actin will be used as a reference gene and each measurement will be performed in triplicate. This analysis will confirm any novel transcript expression detected by scRNA-Seq and differences in expression levels between bowel samples. To include spatial analysis of significant markers we will perform the *in situ* hybridisation technique RNAscope.

Cell sorting

Tissue preparations encompassing the myenteric plexus will be dissociated described and single cell solutions labelled for flow cytometry using standardised methods established within our laboratory on a FACSCanto II flow cytometers within the University of Liverpool Flow cytometry facility. We will quantify cells expressing neuronal, glial and progenitor cell markers, including those assessed by qRT-PCR as well as novel markers determined by scRNA-Seq. Our analysis will provide quantification of numbers of cell types and relative expression levels by signal intensity.

Stimulation of key signalling pathways in neurospheres

The role of the following key signalling pathways will be assessed for their capacity to modulate progenitor cells within cultured neurospheres:

a. Notch signalling – using chemical inhibition (DAPT) and siRNA knockdown of RBPkJ

b. Serotonin signalling – using pharmacological serotonin receptor agonists and antagonists

c. Sonic hedgehog signalling – pharmacological inhibition of the sonic hedgehog pathway

d. Pathways identified in the scRNA-Seq analysis will be included in this work stream.

Transplantation of cells

Cells will be dissociated from neurospheres and then labelled with a fluorescent protein such as GFP using lentiviral transfection to allow the tracking of the migration of these cells and their progeny post transplantation. Following purification (removal of viral particles) a single cell suspension will be produced and microinjected into bowel explants. The transplanted model will then be cultured and assessment of the degree of colonisation made at set time points. This assessment will be made by various microscopy techniques and immunofluorescent analysis as described above. Single cells as well as intact neurospheres may be transplanted to compare the optimal transplantation technique.

Linking with clinical outcomes

Patients recruited for this study who have Hirschsprung's Disease are also eligible for the ALOHA study (REC: 18/NNW/0608) which is being run by the same study team in the same institution. Linkage of the clinical outcomes data with the laboratory-based data described above is expected to shed novel light onto the findings and is an important step towards clinical translation of this work.

Statistical analysis

All quantitative results will be analysed using appropriate statistical measures. For complex statistics the Biostatistics Department at the University of Liverpool will be consulted.

9. Outcome measures

The milestones for outcome will be:

1) Successful optimisation of a scRNAseq protocol using dissociated human bowel cells.

2) Identification and description of the populations of progenitor cells which are present within the dissociated cells from different regions of children with HSCR and control patient groups.

3) Determination of the mechanisms and signalling pathways responsible for ENSPC proliferation and differentiation in neurospheres.

4) Manipulation of neurospheres using targeted agonists and antagonists for key pathways.

5) Transplantation of cells into explant tissue *ex vivo* and characterising of cell integration.

9. Study end-point

It is anticipated that achieving these study outcomes including refining protocols and gaining sufficient data within each workstream will require 150 Hirschsprung's specimens and 300 control specimens.

11. Dissemination

The results will be widely disseminated by presentations at national and international gastroenterology, paediatric surgical and developmental biology meetings, and by peer-reviewed publication in appropriate gastroenterology or developmental biology journals. The research group hold regular patient and family meetings where research findings are fed back to participating families. The group's close links with Hirschsprung's charities, particularly CHAMPS, means that research finds are also fed back through the group's channels.

11. References

1. Nagy N, Goldstein AM. Enteric nervous system development: A crest cell's journey from neural tube to colon. Seminars in cell & developmental biology. 2017;66:94-106.

2. Furness JB. The enteric nervous system and neurogastroenterology. Nature Reviews Gastroenterology & Hepatology. 2012;9(5):286-94.
3. Szylberg Ł, Marszałek A. Diagnosis of Hirschsprung's disease with particular emphasis on histopathology. A systematic review of current literature. Przegląd Gastroenterologiczny. 2014;9(5):264-9.
4. Friedmacher F, Puri P. Classification and diagnostic criteria of variants of Hirschsprung's disease. Pediatric surgery international. 2013;29(9):855-72.
5. Chhabra S, Harwood R, Kenny SE. Hirschsprung's disease. Surgery (Oxford). 2019;37(11):640-5.
6. Heuckeroth RO. Hirschsprung disease — integrating basic science and clinical medicine to improve outcomes. Nature Reviews Gastroenterology &Amp; Hepatology. 2018;15:152.
7. McCann CJ, Thapar N. Enteric neural stem cell therapies for enteric neuropathies. Neurogastroenterol Motil. 2018;30(10):e13369.
8. McCann CJ, Cooper JE, Natarajan D, Jevans B, Burnett LE, Burns AJ, et al. Transplantation of enteric nervous system stem cells rescues nitric oxide synthase deficient mouse colon. Nature Communications. 2017;8(1):15937.
9. Cooper JE, McCann CJ, Natarajan D, Choudhury S, Boesmans W, Delalande J-M, et al. In Vivo Transplantation of Enteric Neural Crest Cells into Mouse Gut; Engraftment, Functional Integration and Long-Term Safety. PloS one. 2016;11(1):e0147989.
10. Hotta R, Cheng LS, Graham HK, Pan W, Nagy N, Belkind-Gerson J, et al. Isogenic enteric neural progenitor cells can replace missing neurons and glia in mice with Hirschsprung disease. Neurogastroenterol Motil. 2016;28(4):498-512.
11. Almond S, Lindley RM, Kenny SE, Connell MG, Edgar DH. Characterisation and transplantation of enteric nervous system progenitor cells. Gut. 2007;56(4):489-96.
12. Stavely R, Bhave S, Ho WLN, Ahmed M, Pan W, Rahman AA, et al. Enteric mesenchymal cells support the growth of postnatal enteric neural stem cells. Stem Cells. 2021;39(9):1236-52.
13. Binder E, Natarajan D, Cooper J, Kronfli R, Cananzi M, Delalande J-M, et al. Enteric Neurospheres Are Not Specific to Neural Crest Cultures: Implications for Neural Stem Cell Therapies. PloS one. 2015;10(3):e0119467.
14. Metzger M, Bareiss PM, Danker T, Wagner S, Hennenlotter J, Guenther E, et al. Expansion and differentiation of neural progenitors derived from the human adult enteric nervous system. Gastroenterology. 2009;137(6):2063-73.e4.
15. Lindley RM, Hawcutt DB, Connell MG, Almond SL, Vannucchi MG, Faussone-Pellegrini MS, et al. Human and mouse enteric nervous system neurosphere transplants regulate the function of aganglionic embryonic distal colon. Gastroenterology. 2008;135(1):205-16.e6.
16. Rauch U, Hänsgen A, Hagl C, Holland-Cunz S, Schäfer KH. Isolation and cultivation of neuronal precursor cells from the developing human enteric nervous system as a tool for cell therapy in dysganglionosis. Int J Colorectal Dis. 2006;21(6):554-9.
17. Wilkinson DJ, Bethell GS, Shukla R, Kenny SE, Edgar DH. Isolation of Enteric Nervous System Progenitor Cells from the Aganglionic Gut of Patients with Hirschsprung's Disease. PloS one. 2015;10(5):e0125724.
18. Bianco F, Bonora E, Natarajan D, Vargiolu M, Thapar N, Torresan F, et al. Prucalopride exerts neuroprotection in human enteric neurons. Am J Physiol Gastrointest Liver Physiol. 2016;310(10):G768-75.
19. Hotta R, Stamp LA, Foong JP, McConnell SN, Bergner AJ, Anderson RB, et al. Transplanted progenitors generate functional enteric neurons in the postnatal colon. J Clin Invest. 2013;123(3):1182-91.
20. Lindley RM, Hawcutt DB, Connell MG, Edgar DH, Kenny SE. Properties of secondary and tertiary human enteric nervous system neurospheres. J Pediatr Surg. 2009;44(6):1249-55; discussion 55-6.

21. Smith M, Chhabra S, Shukla R, Kenny S, Almond S, Edgar D, et al. The transition zone in Hirschsprung's bowel contains abnormal hybrid ganglia with characteristics of extrinsic nerves. medRxiv. 2022:2022.06.15.22276240.
22. Aubdoollah TH, Li K, Zhang X, Li S, Yang L, Lei HY, et al. Clinical outcomes and ergonomics analysis of three laparoscopic techniques for Hirschsprung's disease. World journal of gastroenterology. 2015;21(29):8903-11.
23. Pan W, Goldstein AM, Hotta R. Opportunities for novel diagnostic and cell-based therapies for Hirschsprung disease. Journal of Pediatric Surgery. 2021.
24. Guyer RA, Stavely RS, Robertson K, Bhave S, Hotta R, Kaltschmidt JA, et al. Single-Cell Multiome Sequencing Clarifies Enteric Glial Cell Diversity and Identifies an Intraganglionic Population Poised for Neurogenesis. bioRxiv. 2022:2021.08.24.457368.
25. Morarach K, Mikhailova A, Knoflach V, Memic F, Kumar R, Li W, et al. Diversification of molecularly defined myenteric neuron classes revealed by single-cell RNA sequencing. Nat Neurosci. 2021;24(1):34-46.
26. Allin BSR, Opondo C, Bradnock TJ, Kenny SE, Kurinczuk JJ, Walker GM, et al. Outcomes at five to eight years of age for children with Hirschsprung's disease. Arch Dis Child. 2020;106(5):484-90.
27. Bethell G, Wilkinson D, Fawkner-Corbett D, Mesa A, Shukla R, Edgar D, et al. Enteric nervous system stem cells associated with thickened extrinsic fibers in short segment aganglionic Hirschsprung's disease gut are absent in the total colonic and intestinal variants of disease. J Pediatr Surg. 2016;51(10):1581-4.