Changes in the enteric nervous system and surrounding cells during ageing

Conference or Workshop Item

The 3rd International Symposium on the Development of the Enteric Nervous System: Cells, Signals and Genes

Poster Abstracts

Posters

All poster presenters with odd-numbered posters must attend their posters on Monday, 26th March, from 12:45-14:00.

All poster presenters with even-numbered posters must attend their posters on Wednesday, 28th March, from 13:15-15:00.

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Neural Crest Cells

A01

Retinoblastoma protein mutations cause selective ENS defects and early death

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Background and objectives: Retinoblastoma (pRb1) is a nuclear phosphoprotein that arrests cells in G1 by repressing transcription of genes required for G1 to S transition via E2F binding. pRb1 deficient cells exit the cell cycle inefficiently and have genomic stability causing aneuploidy. pRb1 closely associates with lamin A, a type V intermediate filament protein that is an important component of the nuclear lamina. Lamin protein mutations ("laminopathies") cause a wide array of human disease including muscular dystrophy, lipodystrophy, neuropathy, and Hutchinson-Gilford progeria syndrome. One striking feature of cells in individuals with progeria is abnormal nuclear morphology with large irregular nuclei, but the mechanism underlying this problem is poorly understood. Recent data suggest that loss of pRb1 may be important for this phenotype. The role of pRb1 in the enteric nervous system (ENS) is not yet known and our goal was to determine how pRb1 loss affects ENS structure and function.

Methods and Results: We used tyrosinase promoter driven Cre expression to delete pRb1 in the ENS (called pRb1ENSKO). Mutant mice gain weight slowly and die of intestinal obstruction typically at around postnatal day 50 with distended distal small bowel and collapsed colon, but they do not have distal bowel aganglionosis or abnormal ENS precursor migration. Instead they have poorly organized enteric ganglia seen as early as P0 using whole mount staining. EdU injection at P8 demonstrated increased myenteric neuron precursor proliferation consistent with failure to exit the cell cycle. Because some myenteric neurons had unusually large nuclei, we stained with antibodies to lamin A/C (found in all cells) and lamin B2 (abundant in enteric neurons and less abundant in glia). Lamin antibody staining demonstrated many enteric neurons in pRb1ENSKO mice have remarkably large and irregularly shaped nuclei with blebbing reminiscent of cells seen in people with progeria. Nuclear size abnormalities are worse in older mice and many very large neurons had markers of apoptosis (cleaved caspase-3 antibody staining). For reasons that are unclear, myenteric neurons with giant nuclei are primarily NADPH diaphorase expressing cells and this neuronal population is reduced in older pRB1ENSKO mice. Staining with TuJ1 and synaptotagmin antibodies demonstrated disorganized neurites and fewer intraganglionic synapses in mutant animals.

Conclusions: pRb1 is essential for normal ENS morphogenesis and function. The nuclear abnormalities in pRb1ENSKO mice suggest that pRb1 loss is sufficient to induce the nuclear changes seen in people with lamin A mutations that cause progeria.

A02

Hoxb5 trans-activates *Sox9* and regulates trunk neural crest cell development

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Background and objectives: We have previously shown that *Hoxb5* regulates the development of vagal neural crest cells (NCC) through *Ret*. In the mutant *b3-IIIa-Cre/enb5*, in which the engrailed-Hoxb5 (enb5) protein was specifically expressed in vagal NCC under Cre induction, retarded migration of vagal NCC and defective enteric nervous system (ENS) development ranging from aganglionosis to hypoganglionosis were observed. The enb5 protein is a chimeric protein consisting of the *Drosophila* engrailed repressor domain and the mouse Hoxb5 DNA binding domain. Hence, enb5 protein functions as a dominant repressor of Hoxb5 by competing for the same binding site and normal Hoxb5 signaling would then be perturbed. *Hoxb5* is also strongly expressed in NCC originated from trunk region of the neural tube, suggesting *Hoxb5* may also regulate trunk NCC development. This study aims to investigate the function of *Hoxb5* in trunk NCC.

Methods: We crossed *enb5* mice with *Wnt1-Cre* mice to induce enb5 expression in NCC along the entire AP levels of the neural tube, investigated the NCC developmental abnormalities in *Wnt1-Cre/enb5* mice and compared with those of *Wnt1-Cre/Sox9*$^{flox/flox}$ mice. Expression of *Sox9* in *Wnt1-Cre/enb5* mice was evaluated by *in situ* hybridization and immuno-histochemistry. *In silico* analysis, electro-mobility shift assay (EMSA), luciferase reporter assay and chromatin immuno-precipitation (ChIP) assay were performed to investigate the binding and trans-activation of Hoxb5 from *SOX9* promoter.

Results: *Wnt1-Cre/enb5* mice displayed neurocristopathies of hypopigmentation, hypoplastic dorsal root ganglion and ENS defects, which were similar to the neurocristopathies in *Wnt1-Cre/Sox9*$^{flox/flox}$ mice. In *Wnt1-Cre/enb5* embryos, expression of *Sox9* in NCC was down-regulated, and apoptosis of NCC was observed. Hox binding sites were predicted in *SOX9* promoter by bioinformatics software, suggesting that *SOX9* could be a downstream target of Hoxb5. Physical interaction between Hoxb5 proteins with *SOX9* promoter was shown by EMSA. Trans-activation of Hoxb5 from *SOX9* promoter in human neuroblastoma cell line, HTB-11, was confirmed by using luciferase reporter assay. Moreover, binding of Hoxb5 proteins onto the *Sox9* promoter was further consolidated by ChIP assay in the central nervous system of mouse embryos.

Conclusions: *Hoxb5* regulates the development of trunk neural crest cell by trans-activating *Sox9.*

A03

The spatiotemporal role of the cell cycle regulator Geminin in enteric neural crest development

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The Neural Crest (NC) is a transient structure which is located at the dorsal neural tube of the vertebrate embryo and gives rise to the Peripheral Nervous System and cranial structures. The Enteric Nervous System (ENS) is predominantly derived from the vagal NC that corresponds to somites 1-7. Enteric Neural Crest Cells (ENCCs) of vagal origin, invade the foregut mesenchyme and colonise a rapidly expanding gastrointestinal tract by coordinating fundamental cellular processes such as self-renewal, proliferation and differentiation. Geminin (Gem) is a small nuclear protein that is expressed in cycling cells and that has been shown to play a role in the coordination of self-renewal and differentiation during embryogenesis. Deletion of *Gem* in premigratory NCCs of mouse embryos leads to severe malformations of Cranial Neural Crest (CNC)-derived structures as well as total intestinal aganglionosis. Gem-deficient NCCs exhibit increased apoptotic cell death and reduced expression of commitment factors.

Although these studies establish the requirement of Gem in NC formation, the spatiotemporal requirement of this factor during mouse NC development is unknown. We have been addressing this question by deleting *Gem* in NC lineages at different stages of embryogenesis.

Our findings suggest that the requirement of Gem for the development of NC derivatives is temporally controlled and depends on the axial level of NC origin. We are currently exploring the molecular and genetic mechanisms that underlie the spatiotemporal requirement of Gem in NCCs.

A04

Patterning and distribution of enteric cell lineages in ganglionic postnatal intestine of *Sox10*^{Dom} Hirschsprung mouse mutants

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Hirschprung disease (HSCR) is a congenital disorder that affects approximately 1 in 5000 people and occurs due to aberrant development of the enteric nervous system. The disorder is

defined clinically by a variable length of aganglionosis in the distal gastrointestinal tract and is in part due to the abnormal migration of neural crest-derived progenitors of the enteric nervous system. Mild cases manifest as constipation while severe cases lead to toxic megacolon and death. The first line of treatment for pediatric HSCR patients is surgical resection of the aganglionic portion of their bowel. However, many patients continue to suffer from intestinal dysfunction, including enterocolitis and gut dysmotility, despite the presence of ganglia in the intact proximal bowel. The basis for these chronic symptoms is unknown and suggests that abnormal neural crest migration is not the only factor contributing to gastrointestinal deficits in HSCR patients. We hypothesize that aberrant lineage segregation and differentiation of neural crest cells in proximal regions of the intestine contribute to the chronic postoperative complications suffered by HSCR patients despite the presence of ganglia in the intact, proximal intestine post-surgery. To test this hypothesis, we are evaluating the distribution of distinct neural crest-derived lineages in proximal and distal postnatal bowel of the *Sox10^{Dom}* HSCR mouse model. Cre-LoxP fate mapping approaches have been applied to comprehensively map architecture and distribution of enteric neuronal and glial lineages in the postnatal intestine of these mutants. Mean neuronal and glial density as well as neuron to glia ratios in *Sox10^{Dom}* mice and WT littermates are being quantified by co-localization with general cell type markers (Phox2b, Hu, S100b) in discrete subregions of postnatal intestine. Specific neuronal subtypes identified by labeling with specific neuronal markers (e.g. TH, SERT, NADPH) are also being examined. Our analysis will determine whether deficiencies of enteric ganglia composition in proximal intestine among HSCR mouse models occurs and may lead to the implementation of alternate pathology methods to identify HSCR cases most likely to suffer from post-surgical complications.

A05

Duplication and divergence of SoxE function during chordate evolution

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During chordate evolution, there were two genome-wide duplications that are thought to have facilitated evolution of vertebrate traits, like the neural crest. This uniquely vertebrate cell type gives rise to many of the defining characters of vertebrates, including a well-defined head, jaw and peripheral nervous system. The SoxE gene family, comprised of Sox8, Sox9, and Sox10, plays important roles ranging from initiating migration to specification of diverse neural crest lineages. For example, Sox9 is essential for the formation of neural crest-derived cartilage, by directly regulating expression of Col2a1. Sox10 on the other hand plays an important role in formation of nearly every other neural crest lineage, ranging from melanocytes, to peripheral neurons and glia. The varied functions of Sox10 appear to be conferred by differential recruitment other co-regulators, cooperating with Mitf to specify pigment cells, Pax3 to specify neurons. Whereas vertebrates have several SoxE paralogues that have assumed diverse functions, the basal chordate, amphioxus, has only a single SoxE gene. Given the two genome-wide duplications occurring during chordate evolution, we speculated that the varied functions of SoxE proteins in vertebrates may have resulted from duplication and divergence of an ancestral gene. Acquisition of new functions in turn may have facilitated elaboration of the diverse neural crest lineages. To test this hypothesis, we have compared the functional properties of a amphioxus SoxE protein to those of vertebrate SoxE genes, Sox9 and Sox10 using the well established vertebrate systems (In ovo neural tube electroporation and mouse transgenesis). Our results reveal important functions conserved in all SoxE family members at the early neural crest development as well as critical examples of divergent activities unique to vertebrate SoxE paralogues in specifying neural crest lineages. The results suggest that duplication and divergence of SoxE genes played an important role in elaboration of neural crest traits.

A06

Sox10 is essential for neural crest invasion in cochleo-vestibular ganglion

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Background and objectives: *Sox10* is a HMG domain transcription factor required for survival of neural crest cells (NCCs) and for gliogenesis in the peripheral nervous system. *SOX10* mutations are associated with sensorineural deafness in Waardenburg-Shah syndrome (WS4) patients. In the developing inner ear, Sox10 is expressed in the otic vesicle and the cochleo-vestibular ganglion (CVG). The aim of this study is to investigate the role of *Sox10* during CVG development, in order to understand the underlying basis for the hearing defect in WS4.

Methods: To investigate the relationship between NCCs and the developing CVG, we used *Wnt1-cre/ZEG* and *Pax2-cre/ZEG* mice to trace the neural crest and otic placode derived cells in CVG respectively. To further investigate the function of *Sox10* in the glial lineage, we analysed a mouse mutant $Sox10^{NGFP}$ in which the *Sox10* N-terminal domain was fused to EGFP.

Results: Sox10-positive NCCs were first detected in the peripheral region of the geniculate ganglion at early E9.5 in *Pax2-cre/ZEG* embryos. From 30-somite stage, NCCs colonized the CVG in a discrete pattern, these NCCs then acquired a glial identity, indicating that NCCs invade the CVG and contribute to glial cells. Using the $Sox10^{NGFP}$ mutant, we found that GFP expression persisted in the otic epithelium and glial cells in the spiral ganglion, recapitulating the expression of *Sox10* in the inner ear. $Sox10^{NGFP/NGFP}$ mutants lacked NCCs in CVG from E10 onward, but neuronal specification was unaffected, indicating that *Sox10* is important for early invasion of NCCs into the CVG. In the absence of glia, spiral ganglion neural fibers displayed disorganized alignment and fasciculation in $Sox10^{NGFP/NGFP}$ mutants at E13.5. Our results indicate that *Sox10* expressing glia were essential for radial growth of spiral ganglion neurons.

Conclusions: Taken together, our data demonstrates that *Sox10* is required for both early neural crest invasion in the CVG and gliogenesis during inner ear gangliogenesis, and for the normal maintenance of spiral ganglion neurons.

Genetic Models of ENS Development

B08

Decreases of enteric neurons and enteric glia in the ileum of a mouse model of Alzheimer's disease

Chin Wai Hui, Chi Kong Yeung,
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Background and objectives: Alzheimer's disease (AD) is associated with a deposition of amyloid plaques and a loss of cholinergic neurons in the central nervous system. This can be modelled to a certain extent using transgenic mice (Tg2576), which express the human amyloid precursor protein (APP). Studying gastrointestinal (GI) tract functions using a valid AD model may provide new perspectives on the treatment of degenerative diseases. In the present study, we compare the morphology of the antrum and ileum of Tg2576 mice and their wild type controls using immunohistochemistry.

Methods: Six-month old Tg2576 mice (n=6) and their wild type controls (n=7) were killed and sections of antral and ileal tissues were dissected and fixed with 4% paraformaldehyde. Cells in the myenteric plexus were stained with primary antibodies against PGP to label enteric neurons, anti-CD117 to label interstitial cells of Cajal (ICC), and anti-S100 and anti-GFAP to label glial cells prior to area quantified using confocal microscopy and ImageJ software (National Institutes of Health). Statistical comparisons were made using Student's t-test (Prism, version 5, GraphPad Software Inc., U.S.A.). Wax paraffin sections of the ileum were prepared and subjected to antigen retrieval using 80% formic acid. Sections were then incubated

with antibodies against amyloid beta protein. The presence of amyloid plaques was detected using Zeiss Axioskop Microscope.

Results: The areas (mm2) of neurons, GFAP positive-glial cell and ICC in the antrum of wild type controls were 0.063±0.005, 0.054±0.005, and 0.093±0.009, respectively; and the areas observed in Tg2576 tissue were not significantly different (P>0.05). In the ileum of wild type controls, the areas of neurons, GFAP-positive glia, S100-positive glia and ICC were 0.068±0.003, 0.036±0.002, 0.053±0.007 and 0.099±0.008, respectively. Neuronal, GFAP-positive glial and S100-positive glial areas in Tg2576 tissues were 53.6 (P<0.001), 63.7 (P<0.001) and 36.4% (P<0.05) lower, respectively, but there were no differences for ICC (P>0.05). There was no statistical difference between the ratio of GFAP-positive glia and S100-positive glia (Tg2576: 0.43±0.07, n=6 and wild type controls: 0.47±0.10, n=7, P>0.05). Immunohistochemistry for ileal wax paraffin sections showed the presence of amyloid plaques in Tg2576 mice but not in wild type controls.

Conclusions: In conclusion, the areas of neuronal and glial cells of Tg2576 mice were lower in ileum compared with their wild type controls. It is not known if these changes were the result of amyloid plaque deposition. The studies provide a foundation for the investigation of degenerative diseases by studying GI tract morphology.

B09

Characterization of the role of RET on enteric progenitors using Mosaic Analysis with Double Markers (MADM)

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The assembly of enteric neurons and glia into discrete ganglia and formation of functional neuronal circuits depends upon spatio-temporally regulated mechanisms that control the migration, proliferation and differentiation of ENS progenitors. Both enteric neurons and glia are derived from a pool of Sox10 expressing progenitors that have high neurogenic potential during early stages and a high gliogenic potential during late embryonic and postnatal stages of Enteric Nervous System (ENS) development. A key molecule that controls many aspects of ENS development is the receptor tyrosine kinase RET, which is expressed by undifferentiated multi-lineage progenitors and postmitotic enteric neurons. A series of genetic studies have established that Ret functions in both cell autonomous and non-cell autonomous manner, but the role of Ret at the single cell level has not been addressed.

To examine the effect of Ret deletion on individual ENS progenitors we used a novel strategy called Mosaic Analysis with Double Markers (MADM). Using this strategy we have been able to differentially label daughter cells of single ENS progenitors with two different fluorescent markers – GFP and RFP. Taking advantage of the chromosomal location of the Ret gene on chromosome 6 in mice (distal to Rosa26, which has been targeted to generate the MADM alleles), we have been able to clonally delete Ret, specifically in GFP-expressing progenitors. This single-cell deletion resulted in a decrease in the neuronal population at embryonic (E16) and early postnatal (P5) stages. This was followed by a total absence of Ret-/- neurons at adult (P30) indicating the importance of Ret for enteric neuronal survival. Interestingly, as gangliogenesis is still ongoing, an increase in the glial population was observed at early postnatal stages (P5) indicating an effect of Ret deletion on gliogenesis. Intriguingly, a distinct decrease in the number of encapsulating (Type I) glial cells was observed in the ganglionated plexus and a concurrent increase of Type III glia in the non-ganglionated plexus of the Myenteric Plexus.

Our work establishes a genetic system which can be used to address the role of Ret signalling on ENS development at the single progenitor cell level and more particularly, a novel role for Ret in glial development is implicated in this study.

B10

A new mouse model under-expressing Gfra1 phenocopies Hirschsrung's disease

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Background and objectives: Childhood Hirschsprung's disease (HD) is a congenital malformation characterized by the lack of enteric ganglia in the distal gut, constipation and distention of the gut. Constipation gradually worsens after birth and condition is lethal without surgical correction. The most common genetic causes for HD are mutations in GDNF's co-receptor ret comprising about 50 % of the cases. A reduced level of Gfra1, part of the same receptor complex, has been described in a subpopulation of HD patients. However, it has remained unclear whether reduction in Gfra1 expression level is sufficient to cause HD. Mouse models with conditional Gfra1 deficiencies have been described but the postnatal observations are lacking.

Methods: Because of total aganglionosis of the gut starting from duodenum and lack of kidneys Gfra1 knock out animals are unviable. To avoid these severe deficits we have generated a mouse line that under-expresses Gfra1 from the endogenous locus by placing a selectable marker gene with a strong recombinant promoter in an opposite transcriptional direction into the sixth intron of the Gfra1 gene. The mRNA-level of Gfra1 in our new GFRa1 mouse line at E13,5, when the GDNF dependent migration of ENS-neuron precursors should have reached the colon, is five fold down in the gut. Kidneys, despite of the similar reduction of Gfra1 mRNA levels at that age, develop normally. Mice were designated as Gfra1 hypomorphs (Gfra1h).

Results: Homozygous Gfra1h mice develop Hirschsrpung's disease phenotype by P5-P20 with a 100 % penetrance. Both the myenteric and the submucosal ganglia are missing from the distal colon and the constipation and distention of the gut are pronounced. The condition is lethal. Similar to the human HD the distal part of the gut lacks enteric ganglia and is innervated with hypertrophic cholinergic fibers. About 30% Gfra1h mice develop symptoms of enteric colitis, a common complication among the HD patients. Heterozygous Gfra1 mice are asymptomatic. The genotype distribution at the time of birth is close to Mendelian.

Conclusions: Our results demonstrate that reduction in Gfra1-levels is sufficient to cause HD. Gfra1h mice can be used to study the postnatal etiology of HD and as a tool for defining new intervention strategies.

B11

GDNF is a key regulator for the formation of the submucosal plexus

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Background & Objectives: Enteric nervous system (ENS) is composed of the myenteric and submucosal plexuses. ENS precursors initially migrate rostro-caudally within the outer gut mesenchyme and form the myenteric plexus, whereas a population of ENS precursors undergoes radial migration later in development to form the submucosal plexus. Glial cell line-derived neurotrophic factor (GDNF) signals through a receptor complex composed of GDNF family receptor α1 (GFRα1) and RET receptor tyrosine kinase and regulates ENS development. It has been established that GFRα1 and RET are essential for rostro-caudal migration of ENS precursors in the outer mesenchyme of the gut. However, it remains unclear whether GDNF signaling is also required for radial migration of ENS precursors from the myenteric plexus to the submucosal region.

Methods: To understand the physiological roles of GDNF signaling in the formation of submucosal ganglia, we used *Gfrα1* and *Ret* conditional knockout mice. To examine expression pattern of GDNF during submucosal plexus formation, we employed mice with a *LacZ* reporter inserted in the *Gdnf* locus.

Results: Inactivation of *Ret* or *Gfrα1* after colonization of the myenteric region by ENS precursors severely impairs their invasion into the submucosal region. After formation of myenteric plexus, expression pattern of GDNF is shifted from the surrounding mesenchyme to the submucosal side of the circular muscle layers, suggesting that the GDNF gradient triggers invasion of ENS precursors into the submucosal region.

Conclusions: GDNF signaling via GFRα1 and RET is essential for development of not only the myenteric but also the submucosal plexus.

B12

Modelling Hirschsprung's disease using patient-specific induced pluripotent stem cells

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Hirschsprung's (HSCR) disease is a congenital disorder in which the enteric nerve cells are absent in the bowel, causing chronic constipation. The incomplete colonization of bowel with enteric neural crest (NC) cells is the main cause of the disease. *RET* gene encodes for a tyrosine kinase receptor and is highly implicated in the neural crest development. Mutations or genetic variants in *RET* are accounted for most of the HSCR cases. In particular, a single nucleotide polymorphisms (SNP, rs2435362) residing in the intron one of *RET* gene is predominantly found in HSCR, which may cause a reduced *RET* expression in patient, is significantly associated with HSCR susceptibility. In this study, a HSCR patient carrying a risk allele T in rs2435362 of *RET* gene, exhibiting a short segment aganglionosis and atrial/ventricular septal defects (ASD/VSD) was selected to establish a human model for HSCR. The patient's skin fibroblast cells were reprogrammed into iPS cells by ectopic expression of four reprogramming factors. Three patient-specific iPS cell lines were currently obtained. They were ES-like in morphology, expressing the pluripotency markers and showing low DNA methylation levels of CpG sites in the promoter regions of *NANOG* and *OCT3/4*. Importantly, they could generate teratoma comprising all three germ layers when they were injected in SCID mice, further corroborating the cells had acquired pluripotency. Subsequent differentiation experiments revealed that these HSCR iPS cells were able to differentiate into NC cells of a comparable capacity as that of the control iPS cells (IMR90). In addition, these iPS-derived NC cells were multipotent and could commit to both neurogenic and smooth muscle lineages under defined differentiation conditions. Nevertheless, in general, all the HSCR-iPS cells showed a lower competency to form neurons and smooth muscle cells, suggesting that differentiation defects of NC may represent a cause of HSCR and other NC-associated disorders. Taken together, these results substantiate the potential use of our patient-specific model to study the etiology of HSCR and other NC-associated diseases.

ENS and the Surrounding Cell Types

C13

Changes in the enteric stem cell niche of children with Hirschsprung's disease

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Background: The transplantation of neural crest derived stem cells (NCSC) is a potent alternative for the treatment of Hirschsprung's disease (HSCR). Ideally, the cells transplanted are derived from progenitors within the gut. To gain further insight in possible stem cell niches in human colon from infants with Hirschsprung's disease was investigated with a panel of stem cell markers.

Material and Methods: The tissue samples from ganglionic, aganglionic and transient segments were immuonstained either for S100/nestin, S100/p75 or GFAP/nestin. Altogether twenty-five tissue samples from infants with Hirschsprung's disease were investigated along the gut axis.

Results: In all samples investigated, nestin positive ganglia could be found, even in the distal parts were a severe hypo- or aganglionosis was verified. Beside nestin positive cells in all segments, there were also different expression pattern of glial markers within the ganglia, indicating that distinct phenotypes of glia cells could be found.

Conclusion: Neural and glial precursor cells are present in the ganglionic as well as in the hypoganglionic segments of Hirschsprung's colon, suggesting that these cells might be suitable for NCSC generation.

C14

Restoration of pacemaker activity via allotransplantation of interstitial cells of Cajal

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Background and Objectives: Interstitial cells of Cajal (ICC) play a critical role in gastrointestinal motility due to their interactions with both the enteric nervous system (ENS) and smooth muscle cells. Loss or disruption of Kit^{+}-ICC capable of generating pacemaker activity has been implicated in numerous gastrointestinal motility disorders. The ability to restore ICC in tissues where they have been lost could potentially lead to a return of functionality. We sought to develop a model where ICC could be allotransplanted into intestines naturally devoid of these cells.

Methods: Enzymatically dispersed cells from the intestinal *tunica muscularis* of $Kit^{+/copGFP}$ and $Kit^{V558\Delta}/+$ gain-in-function mice were allotransplanted into myenteric plexus regions of W/W^{V} mutants that lack ICC-MY and pacemaker activity. Immunohistochemical analysis fate-mapped the development of ICC-MY networks and intracellular microelectrode recordings provided evidence for the development of functional pacemaker activity in organotypic cultures.

Results: Immunohistochemistry of allotransplanted intestinal tissues revealed Kit^{+}-ICC developed in a time-dependent manner into distinct networks at the level of the myenteric plexus over 28 days in culture. Fate-mapping revealed the presence of isolated Kit^{+}-cells within 7 days. By 28 days clusters of Kit^{+}-cells displayed multipolar projections characteristic of mature ICC.
Intracellular microelectrode recordings of circular muscle cells taken from W/W^{V} tissues transplanted with $Kit^{+/copGFP}$ derived cells revealed the development of pacemaker activity, which increased in both frequency and amplitude over 28 days. Allotransplantation of $Kit^{V558\Delta}/+$ derived cells led to a similar, time-dependent increase in slow wave amplitude between day 10-28 however, slow wave frequency failed to increase within this group.

Conclusions: The present study demonstrates the feasibility of allotransplantation of ICC into the myenteric region of the small intestine and the establishment of functional pacemaker activity into tissues normally devoid of ICC-MY and slow waves, thus providing a possible basis for the therapeutic treatment of patients where ICC networks have been disrupted due to a variety of pathophysiological conditions. Such an allotransplantation model might also provide a mechanism of introducing other cell types, such as ENS precursor cells, into regions lacking these particular cells.

C15

Mesenchymal stem cell transplantation from murine bone marrow differentiated into ICC marker positive cells

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Background: Interstitial cells of Cajal (ICC) play important roles in autonomic gut motility as pacemakers by making "slow wave". It has been reported that the number of ICC is decreased in various conditions such as ischemic-reperfusion injury, mechanical ileus, diabetes mellitus and may be Hirschsprung's disease, however, the origin of ICC is still obscure.

Objectives: To test a possible differentiation of mesenchymal stem cell (MSC) into ICC.

Methods: MSC was obtained from bone marrow of EGFP mice by flowcytometry. 1) Co-culture study: Spheres of MSC were obtained by 3 weeks floating culture and co-cultured with a bowel segment of wild type mice and W/W^v mice for a week. In addition to the immunohistochemistry for AIC as a specific ICC marker, immunoelectron-microscopy was applied. 2) Systemic infusion of MSC: Intra venous infusion of MSC derived from EGFP mice bone marrow was performed in wild type mice and W/W^v mice. Mice were kept for 6 months and their intestines were examined for fluorescent immunostain.

Results: In co-culture study, MSC labeled by GFP were colonized into the gut explant. These cells have spindle shape cell body and many processes and a part of these GFP positive cells also showed AIC positive. These GFP positive cells have increased number of mitochondria and a large amount of intermediate filament in their cytoplasm compatible with ICC. In bone marrow transplantation study, especially with W/W^v recipient, GFP and AIC double positive cells and it's network with native AIC positive cells were observed.

Conclusions: In two different experimental systems, co-culture and bone marrow transplantation, cells derived from MSC spreaded into gut tissues and differentiated into AIC positive cells. MSC is a possible source for regenerative medicine of ICC.

C16

Changes in the enteric nervous system and surrounding cells during ageing

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The enteric nervous system continues to undergo changes throughout life. Changes in both enteric neurons and glial cells in old age have been reported, but our understanding both of the effects of described changes on gastrointestinal function and the causes of age-related changes are poorly understood. We are analysing changes in the enteric nervous system and surrounding cells in the intestine of the C57/Bl6 mouse during ageing, using a multidisciplinary approach. We are focusing on the large intestine and terminal bowel; regions that are implicated in changes that may contribute to the increased incidence of constipation and incontinence in the elderly population. The progression of age-associated changes is studied by analysing tissues from animals at 3, 12, 18 and 24 months of age. Changes in the numbers and phenotypic properties of neurons and nerve fibre density and also changes in Interstitial cells, fibroblast-like cells and enteroendocrine cells are being studied in both tissue sections and whole mount preparations by immunohistochemistry, confocal and electron microscopy. Physiological and pharmacological analysis of tissues is also performed and the combined data from all these types of approach allows us to understand more fully how ageing affects neural and non-neural regulation of gut functions. Our data show that changes in the responses of the distal colon, rectum and internal anal sphincter *in vitro* occur during ageing, and that these changes are complex, involve several different signalling systems and do not simply relate to changes in cell number. In addition, we are investigating the mechanisms that cause age-related changes in both neurons and non-neural cells in the intestine, using a combination of markers and imaging techniques. Our preliminary data indicate that the different cell types involved in the regulation of gastrointestinal motility may be differentially affected during ageing. A detailed understanding of ageing of the ENS and surrounding cells will provide valuable information to increase our understanding of age-associated gastrointestinal dysfunction.

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C17

Endothelial and neural stem cells in the gut - A successful team

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Background and objectives: The enteric nervous systems as well as the blood vessels form an intricate network throughout the gastrointestinal tract. They often run parallel, suggesting interdependence. Beside anatomical similarities there are also equivalent molecular mechanisms

and guidance pathways described. This study investigated the interrelationship between developing nerves and blood vessel in the gut. To examine a possible functional interaction on a cellular level, enteric precursors (ENS-cells) as well as mesenteric vascular cells (MVCs) were used.

Methods: To monitor the migration of ENS-cells and MVCs a spheroid confrontation assay was performed. Briefly, from both cell types spheroids were generated, picked and confronted in a three-dimensional collagen matrix. Further investigation of the mutual interaction was performed analyzing multicellular spheroids consisting of MVCs and ENS-cells. Immunofluorescence staining against neuronal, glial and stem cell markers to determine the nature of migrating cells followed both assays.

The quantification of the stimulatory effect of factors released from ENS-cells on the migration of MVCs resulted from a transmembrane-migration assay. This effect was also investigated performing a scratch assay by comparing the migration distance of MVCs during 6h when cultured in ENS-conditioned medium compared to control medium. The effect of factors released from MVCs on the proliferation and differentiation of ENS-cells was studied by co-cultures using transwell-chambers followed by immunofluorescence staining against neuronal and glial cell markers.
Results: Obviously in the confrontation assay both cell types migrated in a target orientated manner towards each other. In immunofluorescence stainings it could be observed that the outgrowth from the ENS spheroids to MVC spheroids is neuronaly guided and in immunofluorescence staining of multicellular spheroids parallel outgrowth of MVCs and glial cells as well as a nestin positive migration front were detected. The stimulatory effect was confirmed by a two-dimensional transmembrane-migration assay, were the number of migrating MVCs significantly increased when cultured in conditioned medium. The scratch assays demonstrated a significant faster "wound healing" when performed in conditioned medium. Using transwell-chambers co-cultures of ENS-cells with MVCs lead to an increased cell proliferation and neuronal differentiation.

Conclusion: An emerging stimulatory effect of ENS-cells and MVCs towards each other considering migration as well as proliferation and neural differentiation could be examined. This confirms the hypothesis of an interdependence of the vascular and nervous system in the gut.

C18

Morphologic analysis of the components of the enteric nervous system in the normal transverse colon of children

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Background and objectives: Quantitative data on the enteric nervous system (ENS) are lacking at present time. In the search of establish reference values, we performed a histopathological analysis of the ENS of the normal transverse colon in children.

Methods: Full thickness specimens of normal transverse colon were harvested during oesophageal replacement by transverse colic transplant in children suffering from caustic oesophageal stenosis. All children (n=8, mean age 5.6 ± 4.1 years, 7 boys, 1 girl) were free of gastrointestinal symptoms except dysphagia. Specimens were immediately fixed in neutral-buffered formalin and embedded in paraffin. Six µm thick serial sections perpendicular to the axis of specimen were made for standard and immunohistochemical stainings. After Hematoxylin-Phloxin-Safran staining, sections were analysed with light microscopy. We selected the specimens that contained all layers of the bowel wall over 10 mm long. Immunohistochemistry using CD56 as a neuronal marker enabled measurements of size and density of ganglia, number and surface of neurons as well as the count and size of nerve fibers. A ganglion was defined as a cluster of neurons bordered by perineural cells. Submucosal nerve fibers were divided into 3 groups according to their thickness: group I: ≤10 µm, group II: >10µm and <20 µm, and group III: ≥20 µm. We measured nerve fiber thickness, number of cells and their surface. Values were expressed in means ± SD.

Results: In the myenteric plexus, for 10 mm specimen, we found 116.3 ± 29.3 ganglia; their

surface was 4991.9 ± 1560.8 μm^2. The distance between two ganglia was 162.9 ± 64.8 μm. We counted 76.4 ± 26.6 neurons per ganglia and their surface was 109.3 ± 25.9 μm^2. In the submucosal plexus, for 10 mm specimen, in group I, II and III respectively, nerve fiber thickness was 6.6 ± 0.3 μm, 13.8 ± 0.8 μm and 25.5 ± 2 μm, we counted 14.9 ± 30.6, 33.9 ± 15.6 and 40.4 ± 13 cells with a cell surface of 32 ± 9.7 μm^2, 58.8 ± 13.8 μm^2 and 73.8 ± 26 μm^2.

Conclusion: We propose standard values of neuronal components of the enteric nervous system determined in the normal transverse colon of children. These values will help in assessing the ENS of patients with pathological conditions such as aganglionosis or hypoganglionosis, especially during pull-through procedures in patients with Hirschsprung's disease.

Signaling Mechanisms in ENS Development

D19

Protein patterned surfaces as assays for cell migration and axon pathfinding in the enteric nervous system

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Development of the enteric nervous system requires coordinated migration of enteric neural crest-derived cells (ENCCs) down the growing bowel. Cells that have reached their targets and differentiated into one of about 20 different neuronal subtypes found in the enteric nervous system (ENS), must also achieve precise axonal wiring with their synaptic partners to create a functioning ENS. Although much work has been done in understanding these processes, the extrinsic molecular cues regulating the directionality of cellular and axonal movement in the ENS are still not entirely clear. As is common throughout development, these molecular cues are likely to be distributed in precise spatial patterns such as gradients, and may exert their actions locally while attached to cellular membrane or extracellular matrix components (as opposed to acting free in solution). The ability to recreate such spatial patterns in a controlled environment *in vitro* would be extremely valuable in the assessment of potential guidance cues important in ENS development. Here we describe the culture of ENCCs on surfaces with precisely patterned subcellular spots of protein on a background permissive for cell growth and migration. The protein pattern is generated by micro-contact printing with a polydimethylsiloxane (PDMS) stamp coated with hexadecanethiol, a lipophillic molecule that forms stable self-assembling monolayers on gold-plated surfaces and readily adsorbs protein of interest out of solution. The un-stamped background regions of the surface are then backfilled with an azide-modified glycol that permits the use of click-chemistry for the covalent attachment of extracellular matrix molecules such as laminin or fibronectin. The backfill allows cells to adhere, grow and migrate anywhere on the surface. Specific responses to the guidance cue pattern can then be evaluated using live cell imaging with fluorescently labeled cells, or *post hoc* immunohistochemical labeling.

D20

Cell cycle exit of different neurochemical classes of enteric neurons

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Background and objectives: There are many different classes of enteric neurons. The seminal work of Pham and colleagues (J Comp Neurol 314:789-798, 1991) identified the time of cell cycle exit of some types of enteric neurons. In this study, we have extended this work to investigate the peak time of cell cycle exit of myenteric neurons in general, and of classes of myenteric neurons whose time of cell cycle exit has not previously been investigated.

Methods: Time-mated pregnant mice with embryos at E11.5, E12.5, E13.5 and E15.5, as well as P0 and P10 mice were injected with the modified nucleoside, EDU, which is incorporated during DNA synthesis. The mice were then killed at around 4 weeks of age, and preparations of myenteric plexus from the small intestine processed for immunohistochemistry using antibodies to the pan neuronal marker, Hu,

calretinin (to identify excitatory motor neurons and sensory neurons), neurofilament M (NFM, to identify intrinsic sensory neurons), NOS (to identify inhibitory motor neurons and interneurons), 5-HT and TH.

Results: EDU+/Hu+ neurons were observed following injections at all ages, including E11.5, when 2.3% of Hu+ neurons was EDU+. NOS and calretinin neurons exited the cell cycle over a wide range of ages, although none of the NOS neurons was EDU+ following injection at E11.5. Furthermore, following EDU injection at P0, small calretinin+ neurons, which are likely to be excitatory motor neurons, comprised 77% of all EDU+ neurons. NF+ (intrinsic sensory neurons) neurons had all exited the cell cycle by P0. As reported previously (Pham et al., 1991), the peak time of cell cycle exit for 5-HT interneurons was E11.5. The peak time of cell cycle exit for TH neurons was E15.5, when 73% of TH+ neurons was EDU+.
Conclusions: Different neurochemical classes of enteric neurons exit the cell cycle over a wide range of ages.

D21

N-cadherin and β1-integrins cooperate during the development of the enteric nervous system

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Integrins and cadherins are transmembrane receptors that mediate cell-matrix and cell-cell adhesion, respectively, and play important roles during development. We used the Ht-PA-Cre conditional promoter to target β1-integrin and N-cadherin gene disruption specifically in migratory neural crest cells and analysed the crosstalk between these receptors during enteric neural crest cell migration (ENCC) at different stages of enteric nervous system (ENS) development.
Previously, we showed that conditional deletion of β1-integrin (b1-/-) expression in ENCC in mice leads to major alterations in the ENS structure caused by reduced migration and increased aggregation properties of ENCC during gut colonization, which gives rise to a Hirschsprung's disease–like phenotype (Breau et al., 2006, 2009). Here, we report that N-cadherin-null (Ncad-/-) ENCC show delayed colonization in the developing gut at E12.5, although this was to a lesser extent than in β1-/- mutants. This delay of Ncad-/- ENCC migration is recovered at later stages of development. Double mutation of N-cadherin and β1 integrin led to embryonic lethality with severe defects in ENS development. The double Ncad-/-; β1-/- mutant ENCC failed to colonize the distal part of the gut and there was more severe aganglionosis in the proximal hindgut than in the single mutants for N-cadherin and β1-integrin. This was due to an altered speed of locomotion and directionality in the gut wall. The abnormal aggregation defect of ENCC and the disorganized ganglia network in the β1-/- mutant was not observed in the double-mutant. This indicates that N-cadherin enhances the effect of the β1-integrin mutation and demonstrates cooperation between these two adhesion receptors during ENS ontogenesis.

In conclusion, our data reveal that N-cadherin is not essential for ENS development but it does modulate the modes of ENCC migration and acts in concert with β1-integrin to control the proper development of the ENS.

D22

Spatiotemporal ontogenesis of the vasculature and enteric nervous systems in the developing avian gut

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Background and objectives: The vasculature and nervous system share striking similarities in their networked, tree-like architecture and in the way they super-impose upon each other in mature organs. These similarities can be explained by the existence of common developmental guidance cues directing both capillary tip cells and axonal growth cones. These signals, along with paracrine or juxtacrine cues, lead to the layout of adjacent functional networks. These guidance cues include members of the semaphorin, netrin, slits, and ephrin families (Weinstein, 2005, Cell 120:

299-302). A close developmental relationship has been highlighted between the enteric nervous system (ENS) and the intestinal vasculature (Nagy *et al*., 2009, Dev Biol 330: 263-272). During ENS development, it has been suggested that the pre-established intestinal microvasculature serves a critical role by providing signals to enteric neural crest cells (ENCC), promoting and directing their migration along the gut.

The aim of this study was to investigate the inter-relationship between the developing ENS with respect to the formation of the digestive track vasculature.

Methods: We used chick^{GFP}-chick intraspecies grafting to permanently label and fate-map ENCC with GFP, combined with intravascular injection of DiI to stain the nascent blood vessel network. This allowed unequivocal visualization of the formation of both networks along the entire length of the digestive track between embryonic day E4 and E8, time window when ENCC migrate from the rostral midgut to the terminal hindgut.

Results: We found that ENCC migrate along the oesophagus prior to the formation of any observable vasculature. In the lung buds, ENCC first colonize the ventro-proximal region, whereas blood vessels form in the dorso-distal region, without initial developmental overlap. When the ENCC reach the stomach, an extensive vascular network is already established, but this network is located deeper underneath the migrating path of the ENCC. As the ENCC migrate through the midgut, the vascular network is very sparse, with only a few ramifications entering from the mesentery. Here again, ENCC move through regions that are apparently devoid of established vascular networks. The terminal hindgut is the only other region where an extensive vascular system pre-exists in the path of the ENCC. Close ups of the ENCC migration front in this region, using high-resolution confocal microscopy, does not support the idea that migrating ENCC follow a pre-existing vascular scaffold.
Conclusions: These observations suggest that an established vascular tree is not essential for guiding ENCC migration within the developing gut.

D23

Genetic and microbial influences on the development of the zebrafish enteric nervous system

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Background and objectives: Although much is known about early events in enteric nervous system (ENS) development, including how ENS progenitors navigate to the gut, later events, such as the differentiation of ENS precursors into their neuronal and glial progeny, remain poorly understood. The Notch signaling pathway has been suggested to regulate differentiation processes in the ENS. In this study, we analyzed ENS development in Notch signaling pathway mutant zebrafish at larval stages. In addition to a role for Notch signaling, there is increasing evidence that the resident intestinal microbes affect gut and nervous system development and function. However, virtually nothing is known about potential roles of gut microbes in normal ENS development. Thus, we investigated ENS development in germ-free zebrafish larvae.

Methods: We quantified the number of enteric neurons in guts of Notch signaling pathway mutants at larval stages using immunohistochemistry for the pan-neuronal marker Elavl. We compared the number of Elavl-positive enteric neurons between germ-free and conventionally raised zebrafish larvae. We also analyzed the percentage of 5-HT and nNOS positive enteric neurons in guts of Notch signaling pathway mutants and germ-free versus conventionally raised zebrafish larvae.

Results: In zebrafish notch1a mutants, there was no significant difference in the number of enteric neurons or in the abundance of 5-HT and nNOS positive neurons compared to wild types. In deltaD mutants, however, we saw an increase in the number of enteric neurons. In addition, the abundance of nNOS positive neurons was increased in deltaD mutants compared to wild types. However, we found no difference in the abundance of 5-HT neurons from wild types in deltaD mutants. In the absence of gut microbiota, the number of neurons did not differ significantly compared to

conventionally raised zebrafish wild types. Additionally, there was no indication for a shift in neuronal subtype specification, as the percentage of nNOS and 5-HT positive neurons did not change between germ-free and conventionally raised zebrafish.

Conclusions: Our results suggest that in larval zebrafish, the Notch ligand, DeltaD, inhibits neurogenesis in the ENS. Our future experiments will examine which cells in the gut express DeltaD during ENS development and if deltaD mutants display other changes in ENS development. Raising zebrafish germ-free does not seem to affect the number or subtypes of ENS neurons. We are currently examining the effects of micobiota on other aspects of ENS development and are further exploring the effects of manipulating Notch signaling in the ENS.

D24

MicroRNA in enteric nervous system development

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MicroRNA (miRNA) are critical regulators of many aspects of development, but their role in the enteric nervous system (ENS) is not well understood. Recent data suggest that miRNA are essential for normal ENS function. For example, conditional *Dicer* deletion within the ENS leads to enteric neurodegenerative disease. However, although Dicer is critical for the function of all miRNA, only a subset of miRNA are likely to be needed in the ENS, and miRNA may have other roles in ENS development. For this reason, we think it is important to identify miRNA expressed in the developing and mature ENS. We also became interested in this question because we demonstrated that retinoic acid (RA) signaling reduces Pten protein levels in migrating ENCC at the leading edge of the migration wavefront, but have been unable to demonstrate an effect of RA on *Pten* mRNA. One possibility is that Pten undergoes posttranscriptional regulation by miRNA in response to RA. To test this hypothesis, we used a microarray to identify specific miRNAs differentially expressed in undifferentiated ENCC as compared to differentiated ENCC-derivatives. Immunoselected ENCCs were subjected to conditions promoting either differentiation or maintenance of progenitor state in culture. Total RNA was isolated from these cultures for analysis and compared to samples from whole embryos. Preliminary results show differentially expressed miRNAs between these groups. These are currently being validated by qRT-PCR and in-situ hybridization. This will be followed by functional studies of Pten regulation by validated miRNAs. The existence of such mechanisms is promising for our future studies of Pten regulation and importantly, may reveal miRNAs that could be targets for the treatment of ENS disorders, like intestinal pseudo-obstruction syndrome or diabetic gastroparesis.

D25

FGF-16 is present in the myenteric plexus of rats and upregulated during wound healing in small bowel anastomoses

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Purpose: The understanding of intestinal wound healing plays a crucial role for different gut diseases and a variety of bowel surgery procedures like intestinal anastomoses. Two-dimensional difference gel electrophoresis (2D-DIGE) with subsequent mass spectrometry(MS)-analysis was used to investigate proteomic changes during wound healing in the perianastomotic muscle layer including the myenteric plexus and to identify differentially expressed proteins. One of the regulated proteins, FGF-16 was chosen for further immunohistochemical investigation.

Methods: Ileo-ileal anastomoses were performed in 22 SD rats (+ 6 references). Groups were assigned according to sacrifice time and modality of examination (2D-DIGE + MS: day 0,1,2,7,14). The anastomotic samples were resected and processed for proteomic and immunohistological exploration. FITC-FGF16-fluorescence was assessed perianastomotically according to a five-graded scale.

Results: 2D-DIGE analysis showed a continuous decrease in differentially regulated protein spots from day 1 to day 14. Five differentially regulated proteins were identified in the perianastomotic muscle layer at different time points. The change in protein amount was highest for FGF16 (+8.7-fold, day 14). FITC FGF16-fluorescence was concentrated significantly at the myenteric plexus niche ($p<0.05$), higher values were also found in the circular muscle.

Conclusions: This study shows that a proteomic approach coupled with immunhistological methods is feasible for describing anastomotic proteome changes and to localize potential intestinal biomarkers for the healing of injuries. The interaction between smooth muscle and the ENS and the role of FGF 16 among other proteins for healing and development deserves further investigation.

Genetics of Hirschsprung's Disease

E26

Alu-mediated deletion of *SOX10* regulatory elements in Waardenburg-Hirschsprung disease

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Waardenburg-Hirschsprung disease, also called Waardenburg syndrome type 4 (WS4), is a rare neural crest disorder defined by the combination of Hirschsprung disease and Waardenburg syndrome (sensorineural hearing loss and pigmentation defects). Three genes are known to be involved in this syndrome, *i.e*., *EDN3*, *EDNRB*, and *SOX10*. With more than 60 mutations reported up to date, *SOX10* is considered as the major WS4 gene. Heterozygous point mutations or deletions affecting part or the whole gene account for about half of WS4 cases. However, 15–35% remains unexplained at the molecular level, suggesting that other genes are involved and/or that mutations within known genes have escaped previous screenings.

Here we used semi-quantitative fluorescent multiplex PCR (QMF-PCR) strategy to search for deletions within *SOX10* regulatory sequences located upstream (called U1-5) and downstream (D6+7) of this gene, and describe the first characterization of a large de novo heterozygous deletion encompassing 3 of these enhancers (U1, 2 and 3). Analysis of the breakpoint region suggests a complex rearrangement involving three *Alu* sequences localized on chromosome 22, which could be mediated by a FosTes/MMBIR replication mechanism.

The clinical outcome of the patient is in agreement with the known functions of the deleted sequences during enteric nervous system and melanocyte development in mouse and zebrafish as well as in different cell lines. These results strongly suggest that U1 and U3 enhancers are also of major importance in the physiological regulation of *SOX10* expression in humans. Taken together with recent reports, our study demonstrate that the disruption of highly conserved non-coding elements located within or at a long distance from the coding sequences of key genes can result in several neurocristopathies, Hirschsprung disease and Waardenburg syndrome in particular.

E27

Retinoic acid and Estrogen receptor alpha binding sites and the aetiology of Hirschsprung disease

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Introduction: Hirschsprung disease is a complex developmental disorder characterized by the absence of enteric ganglia along variable lengths of the intestine, resulting in intestinal obstruction. HSCR is the most common developmental disorder of the enteric nervous system (ENS) with a prevalence of ~1 in 5000 newborns. A sex-dependent penetrance is observed in S-HSCR with a male:female ratio of 4:1. Linkage and association studies points towards the involvement of the RET gene in almost all cases. RET encodes a receptor tyrosine kinase which is crucial for the normal development of the ENS. RET coding mutations are identified in 50% of familial cases and 15-35% of sporadic cases. Furthermore, a RET enhancer mutation has been implicated in HSCR disease addressing the role of the non-coding genome. Recently, Angrisano et al (2010) investigated epigenetic modifications at the RET locus upon Retinoic acid (RA) stimulation, they identified some regulatory elements involved in RA related RET expression Furthermore, Zachary et. al. (2011) studied both in silico and functionally different Estrogen receptor alpha (ESR1) and retinoic acid receptor alpha (RARE) binding sites at the RET locus. They came up with two promising ESR1 and RARE binding regions, RET +32.8 and RET -49.8. Based on these findings we hypothesised that variations in these two non-coding regions may regulate RET expression and as such play a role in HSCR development.

Methods: We performed direct sequencing on these regions in HSCR patients, without RET mutations. All patients come from the Netherlands and we compared the data with the data generated in 1000 controls from GONL (the Genome of Netherlands).

Results and discussion: The data are currently being analysed. The functional relevance of the identified non-coding variants will be further elucidated using in-vitro and in-vivo strategies. As HSCR has a skewed sex ratio we will also determine whether these SNPs have a sex dependant effect, in other words, whether estrogen related RET expression can protect females from getting HSCR disease. Results will be presented at the meeting. Our data likely will result in a better understanding of HSCR disease pathogenesis.

E28

Genetic study of a family segregating Shah-Waardenburg syndrome

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Background: Type IV Waardenburg syndrome (WS4, MIM_277580), also known as Shah-Waardenburg syndrome or Waardenburg-Hirschsprung disease, is a congenital developmental disorder characterized by pigmentary abnormalities of the skin, eyes and hair, sensorineural deafness and Hirschsprung disease. The WS4 is caused by mutations in any of the following three genes: endothelin-3(*EDN3*), endothelin-B receptor (*EDNRB*), or *SOX10*.

Materials and methods: Exons and exon/intron boundaries of the three candidate genes (*EDN3, EDNRB, SOX10*) were screened for mutations by direct sequencing of PCR products in a three-generation family (14 individuals) whose members were affected with WS4 or only HSCR. The family members were also genotyped for 2.5 million genetic markers (single nucleotide polymorphisms -SNP- and copy number variation probes -CNVs-) using Illumina Human Omni2.5-quad BeadChip. After quality control and pruning of SNPs in high linkage-disequilibrium, the Merlin software was used for parametric and non-parametric linkage. To test the effect of the mutation identified, wild-type and mutated mammalian expression vectors encoding for EDNRB isoforms (with green fluorescent protein -GFP-) were/will (isoform 3) transiently transfected in Human Embryonic Kidney 293 cells.

Results: Direct sequencing revealed a heterozygous novel nonsense mutation in *EDNRB (Chromosome 13).* This mutation corresponds to M1V of the EDNRB isoforms 1 and 2 and M91V of the EDNRB isoform 3. It was present in four affected and two unaffected family members. The PolyPhen and SIFT prediction for M1V (isoforms 1&2) and for M91V (isoform 3) was damaging (abolishes the ATG start site) and benign respectively. Importantly, the 3 *EDNRB* transcripts are expressed in the human new-born gut. HEK 293 cells transfected with vector with wild-type

EDNRB-GFP showed mainly a cell membrane localization, while the M1V mutated EDNRB-GFP revealed a clustered localization in cytoplasm indicating that EDNRB receptor cannot translocate to cell membrane. Analysis of M91V is underway. Our linkage analysis indicated that additional genes may be contributing to the phenotype.

Conclusion: We found and characterized a novel nonsense mutation. The *EDNRB* mutation is deleterious in isoforms 1 and 2 (M1V) but predicted benign in isoform 3 (M91V) (*in vitro is* study underway). The variable manifestation of the disease may depend on the ratio between the EDNRB isoforms expressed in gut or/and additional genetic factors involved in the pathogenesis of the disease.

E29

Intronic *RET* gene variants and somatic mutations in Down syndrome associated Hirschsprungs disease (DS-HSCR) in an African population

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The clinical association between Hirschsprungs disease (HSCR) and Downs syndrome (DS) is well established, but the etiology is unclear. Reported associations between specific *RET* promoter and intron 1 variations [e.g. SNP1 (rs2506004) and SNP2 (rs 2435357)], which have been shown to interfere with RET function, increasing the risk to HSCR pathogenesis.

Aim: This study explores the role of variations of specific *RET* intron 1 single nucleotide polymorphisms (SNPs) [viz: SNP1 (rs2506004) and SNP2 (rs 2435357)] in DS- associated HSCR (DS-HSCR).

Methods: Ethical permission was obtained and DNA extracted from paraffin embedded tissue samples and whole blood in 14 DS patients with histologically proven HSCR. PCR products of *RET* intron 1 were screened for genetic variation by RFLP analysis. These were matched to DS controls and 20 ethnically matched controls from the general population.

Results: DNA derived from blood and /or tissue from 14 DS-HSCR patients [37 samples] were investigated. RET intronic variations *RET* rs2506004 SNP1 variations were detected in all patients, was heterozygous in 9 and homozygous in 5 samples (all aganglionic; 1 total colonic aganglionosis (TCA)) but was present in 3 DS controls (heterozygous). The rs 2435357 SNP2 was absent in 6 patients, heterozygous in 6 and homozygous in 3 patients (2 with TCA). Homozygous intronic SNP *RET* variations were identified in aganglionic tissue as opposed to heterozygous in transitional and normal tissue in 3 same individuals indicating a local somatic mutation

Conclusions: Potential disease-related *RET* intronic and somatic mutations were identified in DS-HSCR patients, suggesting a possible causal relationship.

E30

Novel Zeb2 gene variation in the Mowat Wilson syndrome (MWS) in the first reported case from Africa

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The Mowat-Wilson syndrome (MWS) is uncommon but is associated with typical facial and other phenotypic features, mental retardation with epilepsy and absent corpus callosum in Hirschsprungs disease patients. It has been reported from countries all over the world, but never from Africa. It has been shown to result from haploinsufficiency of the the Zinc finger E-box-binding homeobox 2(ZEB2) gene but is variable in site.

Unreported gene variations outside of usual Zeb2 sites [exons 2, 7 and 8 (T→C416 and T→C263 polymorphisms] are described in a proportion of MWS patients, some of which may be functionally important.

We report the first confirmed case of MWS with Zeb 2 variations in Africa in a child with the typical facial gestaldt, mental retardation, absent corpus callosum, epilepsy and HSCR in addition to other congenital anomalies. Successful PCR

amplifications were subjected to semi-automated bi-directional sequencing analysis. Results were compared to the ZEB2 reference sequence (ENS00000169554).

Two novel Zeb2 variations (-56A/T; -7810G) were detected one of which, (-56A/T) is a potentially functional variant in the promoter region .

Conclusions: Although uncommon, MWS is an important link to the Zeb2 gene and functionality of gene variations should be confirmed on further research.

E31

The RET promoter in Black African patients with Hirschsprungs disease in South Africa

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Introduction: The RET promoter region contains common ancestral RET gene variations which exert cell type-dependent control by promoting suppressor activity to increase the HSCR susceptibility risk. Inter-ethnic variation has been proposed but a paucity of information exists about the nature of HSCR genetics in African populations. This study focuses on specific *RET* mutations in Black African patients with HSCR and the promoter region as compared to other ethnic population groups.

Patients and methods: Following ethical permission DNA was extracted from whole blood samples from similar HSCR within the 3 main South African ethnic groups. Black African HSCR patients (n=10)] were compared with a representative sample of 20 HSCR patients from the other 2 main ethnic groups [viz: Caucasian (10), mixed (Coloured) (10)]. PCR products were screened for genetic variation of the *RET* by direct sequencing analysis.

Results: Extracellular RET variations in Black HSCR patients included homozygous (2) and heterozygous(1) A45 variations in exon 2B; heterozygous A432 variations in exon 7(2) ; two homozygous and 2 hetrozygous S904 in exon 15 and an intronic IVS13-29C/T(he) variation in exon 13.

43% of Black patients had RET promoter variations, situated at -1782A/G (11/30) -1697C/G (3/30) -1479G/A (1/30) -1449G/A (1/30) -1285G/A (1/30) -1239C/T (2/30) and -719T/C (8/30) respectively. Three patients had a combination of different RET promoter variants. Homozygous variations were detected at -1782A/G (5) and -719T/C (2).

Four of the 13 identified variants [viz: -1479G/A ; -1239C/T] were specific to the Black African population. The intronic variant SNP2 (rs 2435357) was present in all Black HSCR patients whereas SNP1 (rs2506004) was present in 45%.

Conclusions: Specific disease-related *RET* variations were identified in the promoter region in Black African HSCR patients and adds to our understanding of HSCR in African patients.

E32

Role of stochasticity in incomplete penetrance of Hirschsprung disease

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Background and objectives: In Hirschsprung disease the distal intestine is aganglionic due to failure to complete proximo-distal colonisation by migrating neural precursor cells. Individuals with identified mutations in known Hirschsprung genes show variable penetrance and even monozygotic twins are often discordant. Incomplete penetrance is challenging to understand, and is problematic for diagnosis. If its determinants are understood, greater diagnostic confidence might be attained. In the course of developing mathematical agent-based models of colonisation of the intestine by neural cells, an unexpected emergent property was the appearance of colonisation failure with stochastic

incomplete penetrance in runs with identical starting conditions.
Methods: A cellular automata model was developed to model neural cell colonisation on a grid which represents the gut, with long axis East-West (E-W). Rules are: neural cells can move to adjacent grid sites, the direction at each round being random (N,S,E,W with frequency ¼). However, a cell cannot move into an occupied grid site. This generates a net impetus for movement into uncolonised regions. Neural cells can divide (at a chosen frequency and random position) to produce two agents, placed in adjacent grid sites. If either grid site is occupied, that division is aborted. This biases neural crest division to less crowded regions. The grid sites at random positions can divide to produce two grid sites which are placed E-W. This represents the longitudinal (E-W) growth of the gut.

Results: In the model, at high neural cell proliferation rate, colonisation is always complete, and at low proliferation rate, colonisation is always incomplete. However, at intermediate reductions of neural cell proliferation rate (the equivalent of a Hirschsprung Disease mutation) while the gut is elongating as normal, there is variable success in completing colonisation. The probability of failure to complete colonisation (and the average length of deficit) rises gradually as the neural proliferation rate is lowered gradually. This probabilistic outcome is the result of the stochasticity of movement and proliferation of each neural cell in the model in conjunction with intestinal growth.

Conclusions: We suggest that, in addition to genetic-deterministic causes of Hirschsprung Disease, part of the incomplete penetrance in Hirschsprung Disease may be the result of normal processes that are governed by stochastic rules of cell behaviour, and that these stochastic elements are uncovered by disease-causing mutations. This may impose prognostic limits even if full genetic information is available.

E33

Identification of novel Hirschsprung genes by exome sequencing

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The genetic dissection of HSCR has led to the identification of 12 genes and four susceptibility loci implicated in the development of HSCR disease. The inheritance of HSCR is considered to be complex. The disease can be transmitted as a dominant trait or as a recessive trait, but in the majority of cases it is probably polygenic with differences in sex ratio, with a male predominance in S-HSCR (4:1), incomplete penetrance and variable expression; associations with a large number of syndromes and congenital malformations have been observed. Together, mutations in these 12 genes account for only about one-fourth of all HSCR cases. It is therefore likely that mutations in other genes can lead to HSCR as well. The heritability of HSCR is reflected by the increased risk of HSCR in siblings of affected individuals, the risk percentage being dependent on the extent of aganglionosis and gender of the HSCR patient. Siblings of females with long-segment HSCR are at highest risk of being affected; 33% and 9% for male and female siblings respectively (Badner et al. (1990) A genetic study of Hirschsprung disease. Am J Hum Genet 46: 568-580). Because of this high heritability, female long-segment HSCR patients were chosen as the subject for mutation screening by exome sequencing. A total of 30 patients (5 patients each from six different countries), who were previously tested negative for RET mutations are currently analyzed by exome sequencing on an Illumina sequencing platform. For some of the patients we were able to include the unaffected parents in exome sequencing, which enables us to assess the pattern of inheritance of mutations we find. Exome sequencing data analysis will be performed using the Genome Analysis Toolkit (GATK) developed by the Broad Institute. From the generated list of single nucleotide polymorphisms (SNPs), SNPs reported in dbSNP 131 and the 1000 Genomes

Project will be filtered out and variants are selected for being missense, nonsense, splice-site mutations and insertions and deletions. After performing these filtering steps, genes in which mutations are found will be checked for overlap with other patients to come up with a list of candidate HSCR-associated genes. Mutations in these candidate genes will be confirmed by Sanger sequencing, since false positive rates are generally high in exome sequencing. Results of this analysis will be presented at the 3rd Symposium on Development of the Enteric Nervous System, Hong Kong, 25th-28th March 2012.

E34

Exome sequencing in a family with Hirschsprung disease

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Hirschsprung disease is a congenital disorder characterized by the absence of enteric ganglia in a variable length of the intestinal tract. A linkage study performed on a large, multi-generational, Dutch family with five family members affected with Hirschsprung disease revealed significant linkage to 4q31.3-q32.3. We have sequenced the exomes of two patients from this family, focusing our search for mutations on this linkage region. We assumed an autosomal dominant model of inheritance with low penetrance, suggesting that the mutation in the linkage region is necessary but not sufficient to cause disease development. Exome sequencing revealed only one possible mutation in the linkage region, a single nucleotide change in exon 20 of the *LRB* gene. This variant was validated in all five affected family members. As this variant is located downstream of *MAB21L2*, a gene which plays a role in enteric neural crest cells (ENCCs) migration during ENS development, we hypothesized that this mutation might regulate *MAB21L2* expression in ENS, thereby disturbing ENS development. However, we identified this variant in four individuals out of 220 screened controls and once in 87 Hirschsprung patients. It is therefore unlikely that it is disease-associated. As this was the only variant we identified in the linkage region, we must have missed the causative mutation, which is quite possible since the coverage of the coding sequences, in general, was only approximately 65%.

E35

RET transcriptional regulation by HOXB5 in Hirschsprung's disease

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Background and Objectives: Hirschsprung's disease (HSCR) is the major enteric nervous system anomaly affecting newborns with the highest incidence in Asians. HSCR is a congenital complex genetic disorder characterised by a lack of enteric ganglia along a variable length of the intestine.

The receptor tyrosine kinase gene (*RET*) is the major HSCR gene and its expression is crucial for ENS development. The human homeobox B5, *HOXB5*, has an important role in the development of enteric neural crest cells, and perturbation of Hoxb5 signaling causes HSCR phenotypes in mice. To investigate the roles of HOXB5 in the regulation of *RET* expression and in the aetiology of HSCR, I sought to (i) elucidate the underlying mechanisms that HOXB5 mediates *RET* expression, and (ii) to examine the interactions between HOXB5 and other transcription factors implicated in *RET* expression and HSCR.

Methods: Luciferase assay was applied to study the trans-activity of HOXB5 from both promoter and intron regions. The *RET* promoter luciferase reporter was constructed as previously reported and the *RET* enhancer mini-gene was constructed by ligating the *RET* promoter region (-1205 to +196 or -177 to +196) to the 5' of luciferase gene and MCS+9.7 to the 3' of luciferase gene. EMSA assay

and ChIP assay were introduced to examine the binding activity of HOXB5 to the putative binding sites, which were predicted by *in silico* analysis. Furthermore, we also utilized IP and Co-IP assay to study the protein-protein interaction between HOXB5 and other transcription factors.

Results: In this study, we demonstrate human HOXB5 binds to the promoter and regulates *RET* expression. HOXB5 and NKX2-1 form a protein complex and mediate *RET* expression in a synergistic manner. In contrast, HOXB5 cooperates in an additive manner with SOX10, PAX3 and PHOX2B in trans-activation of *RET* promoter.

In silico analysis, EMSA and ChIP analysis showed that HOXB5 binds to another HSCR-implicated MCS+9.7 enhancer in Intron 1 of *RET*. Specific binding of HOXB5 was abolished by introducing a deletion of core binding sequence or sequence variant in MCS+9.7 region. Luciferase assay indicated that alternative alleles at several SNPs significantly reduced the trans-activity of HOXB5 from the *RET* mini-gene.

Conclusion: Our data suggest that HOXB5 in coordination with other transcription factors mediates RET expression. Therefore, defects in cis- or trans-regulation of *RET* by HOXB5 could lead to reduction of *RET* expression and contribute to the manifestation of the HSCR phenotype.

Developmental Physiology of the ENS

F36

Proinflammatory cytokines from patients with Crohn's disease affect cultured enteric neurons

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Background: The Enteric Nervous System (ENS) appears to be essential for the maintenance of gut homeostasis and mucosal integrity. Inflammatory diseases of the gut, such as ulcerative colitis and Crohn's disease (CD) also affect the ENS. CD is a chronic spontaneously relapsing disorder of unknown origin. The characteristics of CD include transmural inflammation affecting all layers of the bowel wall, including the ENS.
Material and methods: Serum from patients suffering with Crohns disease in acute and remission phase were collected and analyzed concerning their cytokine content (Multiplex Elisa). Primary myenteric cultures of neurons and glial cells were treated with serum from patients with Crohn's disease, healthy adults and defined media. After 24 hrs immunostainings for neuronal (PGP 9.5) and glial (S100) markers, as well as live dead assays, using calcein and propidium iodide were performed. Total neurite outgrowth of the individual neurons was measured

Results: The sera from the three groups investigated showed significant alterations in their cytokine content. The primary enteric cultures harvested with the patient's serum either in Crohn's disease or healthy revealed a decreased survival of neurons compared to defined media. Only cultures, where the Crohn's patients serum was diluted revealed a stimulation in neuronal survival and total neurite outgrowth compared to healthy serum.

Conclusion: A mild inflammation in Crohn's disease is able to stimulate neuronal outgrowth and survival, which is overcome by increasing inflammation. Serum changes in the cytokines in Crohn's disease might influence the regeneration potential of enteric neurons.

F37

Chasing the RET tyrosine kinase in living cells during ENS development

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Background: Neurotrophic factors influence many aspects of neural development, including neuronal migration, survival and differentiation, by binding and activating their cognate receptors. Accumulating evidences from in vitro studies suggest that neurotrophic receptor trafficking plays a crucial role in various cellular events. However, little is known about how receptor trafficking contributes to organogenesis. Especially, the mechanisms of receptor trafficking of RET tyrosine kinases, a receptor for the GDNF family ligands, remain largely unknown. Although GDNF-RET signaling is known to be critical for migration of

enteric neural crest-derived cells (ENCCs), the precise mechanisms of how GDNF regulates the ENCC migration is not yet resolved, due in part by limited knowledge about the subcellular localization and transport of RET. Understanding the biological significance of RET trafficking in ENS development requires a system that allows feasible detection of RET receptors under physiological conditions.
Methods: To examine RET trafficking under physiological conditions, we generated knockin mice expressing RET51-EGFP chimeric receptor under the Ret promoter. To confirm whether these mice are employable for investigating trafficking of RET receptors, we first examined RET-dependent organogenesis of RET51-EGFP mice. Then we examined distribution patterns of EGFP fluorescence in RET51-EGFP mice at tissue and subcellular levels. Finally, we performed time-lapse imaging using gut explants or ENCCs derived from RET51-EGFP mice.

Results: Mice homozygous for the RET51-EGFP allele survived to adulthood. Development of RET-dependent tissues including the ENS, sensory and motor nerves, and kidneys were largely normal in RET51-EGFP homozygous mice, suggesting that RET51-EGFP chimeric proteins exert physiological functions during organogenesis. In RET51-EGFP mice, EGFP fluorescence was visible in all cells endogenously expressing RET. In addition, RET51-EGFP protein colocalized with Golgi apparatuses and microtubule organizing centers (MTOCs). Time-lapse imaging showed that RET51-EGFP signals accumulated at the frontal side toward the ENCC migratory direction. High magnification live imaging showed dynamic movements of RET51-EGFP particles within ENCC cell bodies, and the anterograde and retrograde movements of these particles were observed at similar rates.

Conclusions: To our knowledge, these results are the first to demonstrate that endogenous RET tyrosine kinases are actively trafficked within migrating ENCCs. They also suggest that polarized localization and trafficking of RET plays an important role in the physiological actions of RET during ENS development. The RET51-EGFP mice thus provide a valuable platform for investigating RET trafficking and their biological significance in ENS development.

F38
NSAIDs inhibit ENS precursor migration

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Hirschsprung's disease (HSCR) is an important developmental disorder of the enteric nervous system with many known genetic factors, but the non-genetic elements that contribute to disease are less well elucidated. As HSCR is a partially penetrant disease with variable expressivity, it is crucial to understand how the environment modulates HSCR susceptibility and severity in affected individuals. To that end, we focus on how factors such as maternal nutrition and medications influence the risk of HSCR. We recently conducted a drug screen to identify commonly used medications that cause distal bowel aganglionosis in zebrafish. The screen demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit ENS precursor migration in zebrafish. Subsequent studies using mice show that therapeutic concentrations of the over-the-counter NSAIDs significantly decrease the distance enteric nerural crest cells (ENCCs) migrate out of E12.5 cultured midgut slice explants. In addition, in slice explants these drugs decrease the percentage of migrating cells with lamellipodia based on the analysis of ENS precursors that had migrated the greatest distance from the explant edge. BRDU labeling experiments show that ENCC proliferation is not significantly altered by NSAIDs at therapeutic concentrations. Together, these data suggest NSAIDs inhibit normal ENCC migration and could increase the risk of HSCR.

F39
Changes in gastric myoelectrical activity in ICR mice and the responsiveness to drug treatment

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Background and objectives: Slow waves originate from the pacemaker network of the interstitial cells of Cajal (ICC) and determine the direction and velocity of propagation of peristaltic activity of the gastrointestinal (GI) tract. The enteric nervous system (ENS) and smooth muscle cells are known to interface with ICCs through excitatory and inhibitory neurotransmitters. Electrogastrography (EGG) can be used to record gastric myoelectrical activity (GMA) and reveals slow wave information, in terms of frequency and power. The aim of the present study, therefore, was to define the characteristics of GMA in mice and to study the in vivo pharmacological effects of nicotine and donepezil.

Methods: Male ICR mice (6 month old, 28-38 g) were anaesthetized and surgically implanted with telemetry devices (PhysioTel® ETA-F20, Data Sciences International, U.S.A.) with recording wires sutured into the serosal surface of the stomach. 7 days later, baseline GMA was recorded for 2 h before injecting saline (2ml/kg, i.p.), nicotine (3mg/kg, i.p.), or donepezil (3 mg/kg, i.p.); recordings then continued for a further 6 h and raw data were analyzed using Spike2 (Cambridge Electronic Design, U.K.). A two-way repeated measures ANOVA was used to compare data from vehicle- and drug- treated mice. A value of $P<0.05$ was considered statistically significant.

Results: Nicotine reduced the DF almost immediately from 6.6 ± 0.4 to 5.9 ± 0.5 cpm ($n=8$; $P<0.001$) and produced a non-significant increase in the % power of bradygastria ($P>0.05$); saline had no effect ($P>0.05$). The effects of nicotine lasted for 2 h before the DF shifted back to pre-nicotine levels (6.6 ± 0.4 cpm). The DF of the baseline of the vehicle and donepezil treatment groups were 7.2 ± 0.3 and 7.3 ± 0.8 cpm, respectively. Donepezil reduced the DF immediately towards the bradygastric range and the effects lasted for an hour. The DF then shifted back to pre-donepezil levels with an increased power; dysrhythmia was also observed in the tachygastric range.

Conclusions: Nicotine, which is known to stimulate ganglia, caused bradygastria, suggesting an action to release inhibitory mediators to affect ICC. Donepezil had a more complex action predominantly through increasing endogenous acetylcholine levels to stimulate both nicotinic and muscarinic receptors.

ENS Stem Cells and Therapies

G40

Neural stem cells from gut and brain are highly similar

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Background: Throughout adult life, the brain possesses a population of neural stem cells (NSCs) that express Nestin, an intermediate filament that in embryogenesis and possibly postnatally, regulates proliferation, survival and differentiation of these cells. We and others have cultured NSCs from rodent and human gut, but the role of Nestin in these enteric NSCs is unknown.

Objectives: To determine whether Nestin-expressing cells in the intestine are stem cells capable of giving rise to neuronal and glial lineages.

Methods: Single cells were dissociated from brain periventricular tissue and from colonic muscularis of adult mice expressing Nestin-GFP. Non-adherent cells were grown in serum-free media with EGF and bFGF. Neurospheres were grown for 7 days, and cells allowed to attach and differentiate upon withdrawal of cytokines. Neuronal and glial differentiation was investigated immunohistochemically. NSCs were transplanted into aganglionic chicken embryonic hindgut to determine cell fate *in vivo*.

Results: 1) Nestin expression was abundant in the periventricular region of the brain and in the myenteric ganglia of the gut. 2) The majority of Nestin+ cells in the ganglia expressed glial markers GFAP or S-100. 3) All brain-derived neurospheres were GFP+, as were all gut-derived neurospheres. 4) When enteric NSCs were allowed to attach and differentiate, the resulting glial and neuronal networks were morphologically identical to those formed by brain NSCs. 5) NSCs from both brain and gut gave rise to neurons *in vivo* when transplanted into aganglionic chicken hindgut. Nestin expression was lost by the neuronal lineage,

but retained by the glial lineage. 6) Addition of glial-derived neurotrophic factor (GDNF) significantly increased the number of neurons in culture.

Conclusions: Brain- and gut-derived NSCs share many similarities. Both arise from Nestin+ cells. As neurospheres, they maintain Nestin expression. Nestin is downregulated as NSC differentiate to neurons, but preserved in newly formed glia. Both can be successfully transplanted into aganglionic chicken, where they give rise to neurons and glial cells.

G41

Intestinal mucosa-derived neural stem cells give rise to neurons and glia

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Background: Neural stem cells (NSCs) derived from the intestinal muscle layers can give rise to neurons, glia, and smooth muscle cells. The enteric nervous system (ENS) extensively innervates the mucosa and regulates many epithelial-related functions, with recent evidence supporting the existence of NSCs in the gut mucosa. Isolating and characterizing these cells is an important goal since the mucosa is readily accessible to endoscopic biopsies, making it a promising source from which to harvest stem cells to be used in cell-based therapies for neurointestinal disease.

Objectives: To isolate and characterize NSCs from the intestinal mucosa.

Methods: Nestin-GFP transgenic mice were used to isolate NSCs from the gut mucosa to generate both adherent organoid-derived cultures and non-adherent spheroids. Mucosal organoids were prepared by dissociating adult small bowel enzymatically and mechanically and then culturing on plastic dishes in defined media with 10% serum. Non-adherent cultures were prepared by growing single cells from the colonic mucosa in the presence of EGF and bFGF in serum-free media. Total mRNA's were extracted from floating spheres and levels of the pluripotency factors cMyc, Sox2 and Oct4 were measured by qPCR with GAPDH used as an internal standard. Neurospheres were grown for 7 days, and cells allowed to attach and differentiate upon withdrawal of cytokines. Neuronal and glial differentiation was investigated immunohistochemically.

Results: 1) Nestin+ cells are abundant in the mucosa and submucosa (submucosal ganglia, pericryptal cells, and within the villus core). 2) In adherent cultures, Nestin+ cells migrate out of the organoids and form extensive networks of elongated and branching cells that stain for neural (PGP9.5, Tuj1) and glial (GFAP, S-100) markers. 3) In the floating colonies derived from seeding single cells, many GFP+ (Nestin+) spheroids were obtained and these expressed pluripotency markers including Oct4, Sox2 and cMyc. 4) When re-plated and allowed to attach and differentiate, networks of elongated and branching cells positive for neural and glial markers were obtained. 5) As GFP+ cells differentiated into neurons, Nestin expression was lost, whereas glial cells retained Nestin positivity.

Conclusions: This study demonstrates that the gut mucosa is a potential source of intestinal NSCs. These cells express Nestin, as spheres express pluripotency markers and are able to give rise to both neurons and glia. Mucosa-derived Nestin+ NSCs may offer an easily accessible source of cells that can be isolated, expanded, differentiated, and ultimately transplanted for the treatment of neurointestinal diseases.

G42

Spatial analysis of the enteric nervous system of GFP-Nestin expressing mice

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Background and Objectives: The capability of the enteric nervous system to self-renew and using it as a neural stem cell source is an exciting matter discussed by several recent studies. We studied the enteric nervous system from different parts of the gastrointestinal tract of adult GFP-Nestin transgenic mice to estimate the neural stem cell potential in the individual segments of the gastrointestinal tract.

Methods: Whole mounts from adult GFP-Nestin transgenic mice were dissected from stomach, duodenum, terminal ileum, cecum and colon. The whole mounts were either used for myenteric plexus isolation or stained with either S100 or PGP 9.5 to be able to assess the nestin ratio
To investigate the ENS in vitro, we have established a method to isolate the myenteric plexus from all parts of the gastrointestinal tract of adult mice without using any cell sorting techniques. The muscular layer was stripped from the submucosar layer, followed by enzymatic digestion with purified collagenase. Subsequently a final mechanical disruption step was performed.
The MP-networks were used to generate neurospheres. The neurospheres were differentiated and the cellular composition was visualized by immunofluorescence staining again.

Results: Nestin positive cells are present in the muscle layer and the submucosal layer as well as the mucosal layer from the stomach to the distal part of the colon.
We identified mainly nestin/S100b double positive glial cells throughout the gastrointestinal tract. Nestin PGP 9.5 double positive neurons occur in the ileum, caecum and colon.

Neurospheres can be generated from all parts of the gastrointestinal tract and the spheres can be differentiated into glial and neuronal lineages. The myenteric plexus from the colon seems to be the most effective part of the GIT, concerning its neural stem cell potential, related to myenteric plexus yield per area.

Conclusions: Our results demonstrate that the ENS is a prospective neural stem cell source and it is possible to isolate the myenteric plexus selectively for transplantation experiments. The isolation of the myenteric plexus in an intact form is also a promising approach to analyze proteomic changes of the myenteric plexus during different intestinal conditions such as the influence of microflora or inflammatory bowl disease.

G43

The appendix, a suitable and autologeous neural stem cell source

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Background: Evidence of neurogenesis in the adult enteric nervous system (ENS) brought new perspective for cell therapy and neural regeneration. Although these cells show a decreased plasticity in the aging gut, they are a potential source for neural stem cells. The appendix might be the appropriate location with a sufficient amount of enteric nervous tissue where these cells could be easily harvested, under the condition that a suitable isolation procedure is provided.
Materials and Methods: Tissue samples from adult appendices were collected and separated for neurosphere generation, further differentiation and transplantation into rat brain slices. On average after seven days freefloating neurospheres were seen in the culture and could be cultivated up to 40 days. Q-PCR, immunohistochemical stainings and transplatation experiments into brain slices from adult rat were performed.

Results: mRNA expression of these spheres demonstrated an increase of nestin, suggesting stemcellness. After dissociation and further differentiation an intricate network with glial cells, neurons and interconnecting fibers developed within two to four days. The immunohistochemistry with a panel of neuronal, gilal and stem cell markers (PGP9.5, ß-Tubulin III, GFAP, S-100, Nestin) revealed different cell types, meaning that the neurospheres generated from human postnatal myenteric plexus keep their plasticity to differentiate in neuronal and glial cells. After transplantation into organotypically cultures with rat brain slices, the cells migrate into the cortex, differentiate and network formation was commenced.

Conclusion: Using the appendix as a potential target opens up a new perspective, which might lead to a minimal invasive harvest of neural stem cells without endangering the patient. This neural stem cell pool, delivering cholinergic and catecholaminergic neurons, could be used for the recruitments of cells for the treatment of neurodegenerative diseases.

G44

Optimisation of the generation of ENS stem cells for therapy

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Background and Objectives: The development of cell replacement therapies for patients with gut motility disorders such as Hirschsprung's disease (HSCR) is a focus of many research teams including ours. Current protocols for the isolation of ENSCs rely on the generation of neurospheres in culture from gut tissue. Such neurospheres comprise a heterogeneous mix of neural crest (NC) and non-NC cells, suggesting more selective protocols for the generation of ENSCs are needed. Ret and $p75^{NTR}$ have been suggested for NC selection. We aim to (i) validate a protocol for the selective generation of ENSCs from mouse gut and (ii) assess the application of the protocol to human tissue in order to optimise the generation of ENSCs from human postnatal gut for therapy.

Method: To validate a selective marker, we utilized the *Wnt1-Cre;R26R-YFP* transgenic mouse where all NC cells express YFP. Dissociated gut cells from these mice were labelled with $p75^{NTR}$ and sorted by Fluorescence Activated Cell Sorting (FACS). The resulting cell populations were then plated and characterised to assess co-labelling with YFP. For human post-natal gut tissue, cultures to generate ENS stem cells were established from either endoscopy (mucosal/submucosal layers) or surgery (full thickness). Cells from dissociated gut tissue were seeded into culture either directly (heterogeneous cells) or following FACS selection using $p75^{NTR}$ and characterised.

Results: FACS of YFP cells resulted in 2 distinct populations (YFP+/NC+ and YFP-/NC-). Specifically, 86% of YFP+ were positive for $p75^{NTR}$. These cells proliferated and gave rise to neurospheres, which contained neurons and glia. No YFP- cells expressed $p75^{NTR}$. All cultures established from $p75^{NTR+}$ cells gave rise to neurospheres unlike $p75^{NTR-}$, which did not.

Immunostaining of human gut biopsy sections showed cells expressing $p75^{NTR}$ and Sox10. Cells expressing these markers were also present within cultures of dissociated tissue generated from both endoscopy and surgery. These cultures gave rise to neurospheres. FACS for $p75^{NTR}$ selected 8% of cells obtained from dissociated human gut, which could then be maintained in culture.

Conclusion: Mouse studies confirmed that $p75^{NTR}$ is a sensitive and selective marker for neural crest cells, and can be used to isolate cells capable of generating neurospheres. In human, preliminary data suggests that $p75^{NTR}$ may also be of practical use to improve protocols for ENSC generation. Our current and future studies are assessing the utilization of selected (versus non-selected) mouse and human cells for *in vivo* transplantation in wild type mice and models of human ENS disease.

G45

Maintenance of "stemcellness" in enteric and central neuronal progenitors - Role of bacterial lipopolysaccharides

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Background and objectives: The enteric nervous system (ENS) responds quickly to inflammatory impulses. This is obvious during inflammatory bowel diseases (IBD). There is a high probability in IBD that the ENS is challenged by translocated microflora due to an epithelial barrier dysfunction. The question still remains whether the inflammation influences the ENS stem cell niche. This study therefore investigated the capacity of the inflammation-induced endotoxin lipopolysaccharide (LPS) to influence the proliferation of cultured neural stem cells from the ENS compared to neural stem cells from the subventricular zone.

Methods: Isolated mouse neural stem cells from the small intestine, colon and subventricular zone were cultured in the presence of increasing LPS concentrations (0.5ng/ml to 50µg/ml). Neurosphere numbers and diameters were assessed. Immunofluorescence staining with neuronal, glial and stem cell marker was performed. The expression level of these markers was investigated using quantitative real-time PCR (qRT-PCR). The change of the amount of cells expressing the LPS receptor toll-like receptor 4 (TLR4) after stimulation was quantified using FACS.

Results: A dose dependent increase of the sphere numbers could be detected from 0.5ng/ml to 5µg/ml LPS. Immunofluorescence analysis

revealed a prolonged proliferation state of the neurospheres during LPS-treatment, a decrease of PGP9.5 positive cells and an increase of nestin positive cells, indicating also a delayed differentiation. This could be confirmed by quantitative real time PCR. With increasing LPS concentration the number of cells expressing TLR4 decreased.

Conclusion: LPS possess the capability to decrease neurogenesis and therefore to prolong the stemcellness. This could be a promising prospect to accumulate neural stem cells in vitro, which could be used for transplantation in aganglionosis. Our data demonstrate that inflammation in general might influence the neural stem cell niches in both ENS and CNS.

G46

Neurogenesis: granulocyte colony-stimulating factor facilitates neural stem cell differentiation

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Background and objectives: The continuous generation of new neuronal cells throughout the life is an indispensable requirement for functions of the brain and gastrointestinal tract (GI). There is a quickly response of the enteric nervous system (ENS) to inflammatory impulses and there is a certain amount of evidence that inflammation influences the ENS stem cell niche. During inflammation granulocyte colony-stimulating factor (G-CSF) is released and the production of granulocytes is induced. G-CSF protects neurons from cell death via selectively activating the neuronal JAK-STAT pathway. In this study the capacity of G-CSF to influence the cell behavior of cultured neural stem cells from the mouse ENS compared the mouse subventricular zone (SVZ) was investigated.

Methods: Neural progenitors generated from small intestine and subventricular zone were harvested and cultivated with increasing G-CSF concentrations (1pg/ml to 10ng/ml). Number and diameter of neurospheres were assessed. Immunofluorescence staining with neuronal and glial cell markers as well as G-CSF receptor antibody was performed. Neurite outgrowth and neurite density after differentiation were quantified.
Results: As G-CSF in general stimulates neural stem cells, we demonstrated a dose dependent increase in cell proliferation. The higher the G-CSF concentration the less was its influence upon cell proliferation of neural stem cells of the SVZ. For neural stem cells of the ENS the proliferation rate increased with a rising G-CSF concentration indicating that higher G-CSF concentration is needed to stimulate neural stem cells from the ENS than from the SVZ. The G-CSF receptor is expressed on cultured enteric stem cells. The differentiation was reinforced after stimulating the neural stem cells with G-CSF, which enhanced the density of outgrowing fibers by 40% in SVZ cultures and the neurite outgrowth more than 70% in enteric stem cells.

Conclusion: G-CSF is a potent stimulator of enteric and SVZ neuron differentiation with the capability to increase neurogenesis by increasing neural survival and neural outgrowth, being a promising compound to expand neural stem cells in vitro prior to be used for transplantation in cases of aganglionosis of the gut.

G47

Differential expression of *Phox2b* marks distinct progenitor cell populations and facilitates analysis of regulatory pathways in enteric ontogeny

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Normal gastrointestinal motility relies upon formation of a balanced complement of cell types from enteric neural crest-derived progenitors (ENPs) that populate the fetal intestine during development. Regulatory processes that control generation of cellular diversity within enteric ganglia have remained elusive in part because tools to capture populations of ENPs during lineage

segregation have not been available. *Phox2b* is an essential transcription factor that is required for normal development of enteric ganglia. Heterogeneous expression of *Phox2b* is present in enteric progenitors from the time these cells first enter the foregut and is maintained into post-natal stages with higher levels in enteric neurons and lower levels in enteric glia. The *Phox2b*-H2BCerulean BAC transgene line (*Phox2b*-CFP) recapitulates this heterogeneous expression in ENPs and facilitates capture of progenitors based on fluorescent CFP reporter expression. We postulated that differential Phox2b expression coincides with distinct lineage potential and defines different populations of cell types during development of enteric ganglia. To examine this hypothesis, we evaluated expression of multiple lineage markers *in situ* relative to *Phox2b*-CFP transgene expression. We found that enteric progenitors expressing high levels of *Phox2b*-CFP (bright) exhibited up-regulation of neuronal markers *in situ* concurrent with down-regulation of glial markers. Enteric progenitors expressing high levels of *Phox2b*-CFP maintained these high levels even over extended live cell imaging in catenary cultures *in vitro* and were observed to undergo cell division. Populations of bright *Phox2b*-CFP progenitors flow-sorted into low density cultures gave rise to discrete colonies that differed in composition by comparison to colonies derived from *Phox2b*-CFP dim progenitors further suggesting that these progenitor pools differ in their developmental potential. Whole transcriptome profiling of the bright and dim *Phox2b*-CFP enteric populations by next-generation sequencing identified increased expression of multiple genes associated with neuronal cell types in the bright pool. Comparative analysis of *Phox2b*-CFP bright and dim RNASeq profiles has identified multiple pathways that are differentially regulated as these two populations diverge. Pathways up-regulated in *Phox2b*-CFP bright may be targeted to stimulate production of enteric neurons to compensate for deficiencies of this lineage in gastrointestinal motility disorders.

G48

The central nervous system as a source of stem cells for treatment of enteric neuropathies

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Background and Objectives: There has been much interest in the potential of neural stem/progenitor cell transplantation to replace absent or defective neurons in enteric neuropathies. Numerous sources of stem/progenitor cells have been proposed, but given the close developmental association of central nervous system (CNS) progenitors and the neural crest cells that ultimately give rise to the enteric nervous system (ENS), it was of interest to determine whether CNS stem cells could give rise to neurons in explants of embryonic gut or in the colon of post-natal mice in vivo.

Methods: CNS stem cells were isolated from embryonic and postnatal cerebral cortex of mice ubiquitously expressing GFP. ENS stem/progenitor cells were isolated from embryonic mice where enteric neural crest cells express fluorescent protein. Both stem cell populations were cultured as neurospheres. These neurospheres were then co-cultured *in vitro* with explants of mouse aneural embryonic hindgut or grafted *in vivo* into the distal colon of postnatal mice. Migration and differentiation of the two stem cell populations was then analysed and compared.

Results: When co-cultured with aneural embryonic hindgut, ENS stem/progenitor cells migrated a significantly greater distance than their CNS counterparts. Both ENS and CNS stem/progenitor cells gave rise to neurons (Tuj1+ cells) within explants of embryonic gut. However, nearest neighbor analysis revealed significant differences in the spatial distribution of neurons derived from the two different sources - neurons derived from CNS stem/progenitors were evenly distributed within the explants whereas ENS stem/progenitors were found in ganglion-like clusters. Preliminary experiments (n = 3) were also performed in which CNS neurospheres were transplanted into the colon of postnatal mice in vivo. After 2-4 weeks some of the CNS-derived stem/progenitor cells had migrated away from the graft site. A sub-population of the CNS graft-derived cells

expressed GFAP, but none expressed the neuronal marker, Hu.

Conclusions: ENS stem/progenitor cells migrated further in embryonic explants of aneural hindgut and gave rise to neurons that clustered closer together than CNS stem cells. These data indicate that ENS stem/progenitor cells are likely to be a more efficacious stem cell source for potential treatment of enteric neuropathies than CNS stem cells.

G49

Human autologous enteric neural crest-derived cells as a potential source for cell therapy for Hirschsprung disease

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Hirschsprung disease (HSCR) is a relatively common and serious developmental disorder of the enteric nervous system (ENS). The failure of colonization of the distal bowel by enteric neural crest (NC) cells leads to an absence of NC-derived ENS ganglia (composed of neurons and glia) in the distal colon. This results in a failure of effective peristalsis in the aganglionic gut segment, resulting in mechanical obstruction. Infants with HSCR often have severe constipation and develop megacolon. Currently, HSCR is treated by surgical resection of the aganglionic bowel segment and adjacent ganglionated region, but intestinal dysfunction may persist after surgery. Cell therapy strategies are emerging, and introduction of autologous NC stem/progenitor cells into the aganglionic bowel could be useful to restore peristaltic function. By immunolabeling, in post-natal human bowel tissue we have identified neural cells which can be distinguished as either undifferentiated NC progenitor cells (p75/HNK1/SOXE positive) or their differentiated progeny; neurons and glia (p75/HNK1/HuCD/Neurofilament/GFAP/S100b positive). We have established primary cell outgrowths from dissociated post-natal HSCR bowel tissue (from both ganglionic and aganglionic regions). Enteric NC cells can be isolated from these explant outgrowths by HNK1 antibody labelling and FACS. These HNK1 positive cells can be grown for extended periods, while the parent (HNK1 unsorted) cell lines reach senescence. A subset of the HNK1 positive cells are SoxE positive and are capable of forming TUJ1 positive neurons at least 1 month after establishment of primary explants outgrowths. The generation of post-mitotic neurons *in vitro* after extended culture periods indicates the existence of a NC progenitor cell source. HNK1 expression is maintained following cryopreservation and infection with a GFP-reporter for live-cell detection. These cells will be used in functional assays of cell engraftment and neuronal differentiation in human aganglionic tissue. Our experiments demonstrate that human NC derived cells can be grown for extended periods in cell culture and may be a source of NC progenitors for autologous cell transplant therapies.