Regulators of gene expression in Enteric Neural Crest Cells are putative Hirschsprung disease genes

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1. Introduction

The enteric nervous system (ENS) is required for peristalsis of the gut and is derived from Enteric Neural Crest Cells (ENCCs). During ENS development, the RET receptor tyrosine kinase plays a critical role in the proliferation and survival of ENCCs, their migration along the developing gut, and differentiation into enteric neurons. Mutations in RET and its ligand GDNF cause Hirschsprung disease (HSCR), a complex genetic disorder in which ENCCs fail to colonize variable lengths of the distal bowel. To identify key regulators of ENCCs and the pathways underlying RET signaling, gene expression profiles of untreated and GDNF-treated ENCCs from E14.5 mouse embryos were generated. ENCCs express genes that are involved in both early and late neuronal development, whereas GDNF treatment induced neuronal maturation. Predicted regulators of gene expression in ENCCs include the known HSCR genes Ret and Sox10, as well as Bdnf, App and Mapk10. The regulatory overlap and functional interactions between these genes were used to construct a regulatory network that is underlying ENS development and connects to known HSCR genes. In addition, the adenosine receptor A2a (Adora2a) and neuropeptide Y receptor Y2 (Npy2r) were identified as possible regulators of terminal neuronal differentiation in GDNF-treated ENCCs. The human orthologue of Npy2r maps to the HSCR susceptibility locus 4q31.3-q32.3, suggesting a role for NPY2R both in ENS development and in HSCR.

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at E12 and for their survival at E14-E16 in mice (Chalazonitis et al., 1998; Gianino et al., 2003; Taraviras et al., 1999; Uesaka et al., 2008). In order to develop into a mature ENS, post-migratory ENCCs differentiate into various neuronal subtypes and glial cells. GDNF promotes neuronal, but not glial differentiation of ENCCs in vitro, and acts as a chemoattractant to outgrowing neurites (Hearn et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). The mature ENS contains sensory neurons, interneurons and excitatory and inhibitory motor neurons that use a wide range of neurotransmitters, similar to those found in the CNS (Hao and Young, 2009).

Mutations in RET and GDNF in humans contribute to Hirschsprung disease (HSCR), a congenital disorder that is characterized by intestinal aganglionosis in a variable segment of the distal gut. HSCR patients present with tonic contraction of the muscle layers in the affected segment, which consequently leads to dilatation of the proximal bowel. The prevalence of HSCR is 1:5000 live births and it is considered an inherited disease. Based on the families reported, the mode of inheritance can differ. Most non-syndromic familial HSCR cases show a dominant pattern of inheritance, mostly with reduced penetrance, whereas many syndromic HSCR families show a recessive pattern of inheritance. The sporadic cases are considered polygenic or oligogenic. Of the approximately 15 HSCR genes identified so far, the RET gene is by far the most important gene. Coding mutations in RET are found in ~50% of familial and 15–35% of sporadic HSCR patients and represent the great majority of genetic mutations found in HSCR patients (Attié et al., 1995; Hofstra et al., 2000). Mutations in all other HSCR-associated genes, including GDNF, are rare (Alves et al., 2013; Amiel et al., 2008; Brooks et al., 2005a).

The colonization of the intestine by ENCCs, followed by their neuronal and glial differentiation, is a complex process controlled by various signaling pathways. These include Hedgehog, NOTCH, WNT, Retinoic acid and TGF-β/BMP signaling (Goldstein et al., 2005; Ikeya et al., 1997; Okamura and Saga, 2008; Ramalho-Santos et al., 2000; Sato and Heuckeroth, 2008). Several other pathways that are important for ENS development have been identified in gene expression profiling studies. Iwashita et al. (2003) reported that the HSCR genes Ret, Gfra1, Ednrb and Sox10 are highly expressed by ENCCs in mice, thereby explaining how mutations in these genes contribute to aganglionosis. A subsequent expression study by Heanue and Pachnis (2006) showed that at E15.5 in mice, ENCCs express markers of early and late neuronal development and glial differentiation, representing different stages of ENS development. These authors identified Sox2, Cart, Sema, Fgf and Jnk as novel signaling pathways in ENCCs (Heanue and Pachnis, 2006). In addition, Vohra et al. (2006) found that E14 mouse ENCCs highly express genes with synaptic functions and showed that these genes are important for ENCC migration and neurite outgrowth. Some insight in the pathways downstream of RET signaling has come from expression profiling of GDNF-treated ENCCs by Ngan et al. (2008), who analyzed the changes in gene expression in ENCCs after 8 and 16 h of GDNF treatment. These authors showed that the Prokineticin pathway is activated by RET signaling, but found that few other genes were differentially expressed by the short-term GDNF treatment of ENCCs (Ngan et al., 2008).

These gene expression studies have focused on individual genes that are highly expressed by ENCCs and showed that some of these genes are critical for the development of the ENS. However, for many highly expressed genes it is still unknown if and how they contribute to ENS development. In this study, we used an unbiased, systematic pathway analysis to identify the biological processes that are important for ENS development. Moreover, we treated ENCC cultures with GDNF for 14 days to identify the stable, long-term gene expression changes induced by RET signaling. We show that the expression of differentially expressed genes is regulated by known ENS-related genes, and by several genes not previously implicated in ENS development. These regulatory genes present novel candidates genes for HSCR.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice carrying Wnt1-Cre<sup>+</sup>/− were mated with C57BL/6 females that were homozygous for the Rosa26-LoxP-Stop-LoxP-YFP reporter locus. The stage of embryonic development was set to E0.5 on the day the vaginal plug was seen. Embryos carrying both Wnt1-Cre and Rosa26-LoxP-Stop-LoxP-YFP were identified based on YFP expression in the gastrointestinal tract and craniofacial tissues.

2.2. ENCC isolation, culture conditions and RET activation

Full-length guts (foregut to hindgut) were dissected from E14.5 embryos. Single cell suspensions were made using 0.05 mg/ml Collagenase XI/Dispase II (Sigma Aldrich, St. Louis, MO, USA), followed by incubation for 3–5 min at 37 °C and trituration. YFP-positive ENCCs were FACS sorted and directly seeded in Fbronectin-coated plates containing DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 20 ng/ml FGF (Peprotech EC, London, UK), 20 ng/ml EGF (Peprotech EC) and 1% Penicillin-Streptomycin (Invitrogen). YFP-positive ENCCs from one gut were equally divided over two wells; one of which was supplemented with 50 ng/ml GDNF (Peprotech EC). ENCCs were incubated at 37 °C for 14 days in a humidified atmosphere with 5% CO₂. Neurosphere-like bodies appeared after 3–5 days and were kept in culture for 14 days without passaging.

2.3. Immunostaining

Wells with neurosphere-like bodies were fixed with 4% PFA for 15 min at room temperature (RT) and washed with PBS+1% Triton X 100 (PBTr) for 2 × 5 min. They were incubated in blocking solution (PBTr+1%BSA+0.15% glycine) for 1 h at RT followed by primary antibodies diluted in blocking solution overnight (O/N) at 4 °C: rabbit anti-human p75<sup>+</sup> (1:500, Promega, UK), mouse anti-βIII-Tubulin (1:500, Covance UK). After several washes in PBTr, secondary antibodies were applied: goat anti-rabbit Alexa Fluor 568 and goat anti-mouse Alexa Fluor 568 (1:500, Invitrogen) O/N. DAPI was added to secondary antibodies (1:1000). Antibody was washed off (3 × 15 min with PBTr). Slides were mounted using Vectashield (Vector labs, UK) and visualized using confocal microscopy imaging on Zeiss LSM 710 (Zeiss, Cambridge, UK).

2.4. RNA isolation and gene expression quantification

RNA was isolated from untreated and GDNF-treated ENCC cultures, as well as from uncultured E14.5 embryonic guts using the RNeasy Mini Kit (Qiagen, Crawel, UK). RNA yield, purity and integrity were determined using the Agilent 2100 BioAnalyzer with 6000 Nano Chips (Agilent Technologies, Amsterdam, The Netherlands). DNA microarray analyses and RNA quality controls were performed by ServiceXS (Leiden, The Netherlands) using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). For the ENCC vs gut experiment, 3 ENCC samples and 8 mouse gut samples entered the analysis. For the ENCC+GDNF vs ENCC experiment, 7 pairs of untreated and GDNF-treated ENCC cultures were analyzed.
2.5. Data normalization, statistical analysis and functional analysis

Quantile normalization and background correction were applied to the raw intensity values of all samples using Partek version 6.4 (Partek Incorporated, St. Louis, MO, USA) via GC Robust Multichip Analysis. For the ENCC vs gut analysis, 2 sample T test statistics were applied in Partek to calculate the fold changes with associated p-values. Paired T test statistics were applied to calculate the fold changes with associated p-values for the ENCC+GDNF vs ENCC analysis. The fold change and p-value data were uploaded into Ingenuity Pathway Analysis (IPA) (Ingenuity/Qiagen, Redwood City, CA, USA). The probe level data was mapped

![Diagram](image)

**Fig. 1.** Isolation and characterization of ENCCs. A) Schematic overview of the Wnt1-Cre:Rosa26-YFP mouse model. At embryonic day 14.5, transgenic mouse embryos expressed YFP in neural crest-derived cells, including craniofacial tissue and ENCCs. B) YFP-expressing ENCCs were FACS-sorted from dissected guts and were cultured in the presence or absence of GDNF for 14 days. RNA was isolated from ENCCs as well as whole gut tissue and relative RNA expression levels were quantified by microarray analysis. C) ENCCs expressed the neural crest marker p75/NGFR at both 24 h and 14 days after isolation. Pronounced βIII-Tubulin-positive connections between NLBs were observed in ENCC cultures treated with GDNF.
to the gene level and averaged based on the median fold change values. For the differential gene expression analysis, significance thresholds of fold change $>5$ and p-value $<1E-5$ were applied for the ENCC vs gut comparison. For the ENCC + GDNF vs ENCC analysis, significance thresholds were set at fold change $>1.8$ and p-value $<0.05$. Gene Ontology enrichment was performed using DAVID Bioinformatics Resources 6.7 and Upstream Regulator Analysis was performed in IPA. For the Upstream Regulator Analysis the significance thresholds were set to z-score $>1.8$ and p-value $<0.05$. Hierarchical clustering of Upstream Regulators and their downstream target genes was performed using the heatmap functionality in the HIV sequence database (http://www.hiv.lanl.gov).

2.6. Quantitative real-time PCR

Gastrointestinal tracts from E14.5 Wnt1-Cre:Rosa26-YFP mice were dissociated and FACS-sorted as described above. YFP-positive cells were collected in $100 \mu l$ TRIzol reagent (Life Technologies, UK) and stored at $-80^\circ C$ until RNA extraction. Guts from non-YFP embryos from the litter were also dissected out and frozen in TRIzol Reagent. Once thawed, samples were homogenized and RNA was extracted according to manufacturer’s instructions. cDNA synthesis was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). RNA was extracted according to manufacturer’s instructions. cDNA was loaded with primers and cDNA samples to a 96-well Hard-mer-BLAST software. KAPA SYBR FAST qPCR Kit (Kapa Biosystems) was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). The gene expression profiles of pure populations of ENCCs were generated. Mice expressing Cre recombinase under the neural crest-specific Wnt1 promoter were crossed with mice carrying the Rosa26-LoxP-Stop-LoxP-YFP locus. ENCCs were isolated at embryonic day E14.5, since at this stage ENCCs express markers of both early and late ENS development (Heanue and Pachnis, 2006). Double transgenic mouse embryos expressed YFP in neural crest cells in craniofacial and gastrointestinal tissues as expected (Fig. 1a). YFP-expressing cells were isolated from E14.5 gut using Fluorescence activated cell sorting (FACS) (Fig. 1b). In culture, these cells formed neurosphere-like bodies (NLBs) within 3–5 days (Fig. 1c). Notably, NLBs from GDNF-treated ENCCs were larger than NLBs from untreated ENCCs. Compared to untreated cultures, GDNF-treated ENCC cultures showed marked βIII-Tubulin-positive connections between NLBs, indicating that GDNF treatment induced neuronal differentiation of ENCCs (Fig. 1c). Both untreated and GDNF-treated ENCC cultures expressed the neural crest marker p75/NGRF at both 24 h and 14 days in vitro.

3.2. E14.5 ENCCs were enriched for markers of neuronal development and neuronal signaling

The gene expression profile of cultured ENCCs (harvested from E14.5 embryos) was compared to E14.5 embryonic gut to identify genes that are highly expressed by ENCCs. Since whole gut samples contain a variety of cell types, such as endothelial cells, smooth muscle, connective tissue, serosa, enterochromaffin cells and approximately 5% ENCCs, the expression profiles of ENCCs and whole gut tissue are expected to be very different. Therefore, only genes with an $>5$-fold change in gene expression (975 genes) were included in downstream analyses. Gene Ontology analysis was performed to identify the biological pathways these genes are involved in. ENCC-expressed genes were highly enriched for the Gene Ontology terms transmission of nerve impulse, synaptic signaling and cell-cell signaling, that are associated with neuronal signaling (Fig. 2). In addition, genes that are highly expressed in ENCCs play a role in biological-cell adhesion and neuron differentiation. 1187 genes were at 5 times less abundantly expressed in ENCCs than in the gut and these genes are involved in cell cycle control, in particular during active cell division (M-phase). Although the culturing period may have affected the expression of cell cycle genes in the ENCCs, this finding is consistent with the high proliferation rate of cells in the developing E14.5 mouse gut.

Interestingly, genes highly expressed by ENCCs (and low in gut tissue) and genes highly expressed in total gut tissue (and low in ENCCs), are both significantly enriched for Gene Ontology terms biological- and cell adhesion and regulation of cell proliferation (Fig. 2). However, these represent sets of genes with different properties and localizations. Adhesion molecules in ENCCs included genes expressed in the ENS, such as Immunoglobulin superfamily cell adhesion molecules (Ncam1, Ncam2, L1cam, Nrcam), (proto-)cadherins (Cdh2, Cdh6, Pcdh15, Pcdh4) and integrins (Igα4) (McKeown et al., 2013). Of note, Cdhl3 was highly expressed by ENCCs and its human orthologue CDH13 maps to the HSCR susceptibility locus 16q23.3 (Carrasquillo et al., 2002). Adhesion molecules that were highly expressed in total gut tissue included Cdh1, which is expressed by Paneth cells and goblet cells in the intestinal epithelium (Schneider et al., 2010), the Claudin family of tight junction molecules (CLDN 2, 3, 4, 5, 6, 7, 8, 15 and 23), as well as genes involved in the apical junction complex (Desmocollin 2, Desmoglein 2, Plakophilin 2 and 3). These gut-expressed junction molecules are most likely expressed by the enterocytes of the intestinal epithelium.

3.3. GDNF treatment of ENCCs induces terminal neuronal differentiation

RET signaling is critical during all stages of ENS development and is important for survival, neuronal differentiation and neurite outgrowth at E14-E16 (Chalazonitis et al., 1998; Hearn et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). To identify the molecular pathways underlying these processes, the changes in gene expression in ENCCs after 14 days of GDNF treatment were analyzed. Compared to untreated ENCCs, the expression of 445 genes was at least 1.8 times higher in GDNF-treated ENCCs. The neuronal signaling genes that were highly expressed by ENCCs compared to the gut were even further up-regulated upon GDNF treatment (Fig. 2). This finding suggests that GDNF treatment induced terminal neuronal differentiation in E14.5 ENCCs and is consistent with the pronounced βIII-Tubulin-positive connections that were observed between NLBs grown from GDNF-treated ENCCs (Fig. 1c). Additionally, the expression levels of 352 genes was at least 1.8 times reduced by treatment with GDNF, including genes involved in biological- and cell adhesion and neuron differentiation. The adhesion molecules that are down-regulated by GDNF include Cdh13, Col5a1, Fibronectin-1, Reelin, Thrombospondin 1 and 4, and Vcam1. These adhesion molecules are involved in cell migration and cell motility, and thus corroborate the observation that ENCCs stop migrating when they undergo terminal neuronal differentiation (Young et al., 2004, 2005). Consistent with its proliferative role in ENCCs and its inhibitory effect on neuronal differentiation, endothelin receptor type B (Ednarb) expression was down-regulated by GDNF. The down-regulation of genes involved in neuron differentiation and up-regulation of neuronal signaling genes, shows that different genes are involved in early and late neuronal development.
The mature ENS displays a great diversity in neuronal subtypes, but the molecular mechanisms controlling neuronal subtype specification in the ENS are poorly understood. To test whether RET signaling in ENCCs contributes to the specification of neuronal subtypes, the expression of neuronal subtypes markers was determined in untreated and GDNF-treated ENCCs. The expression levels of cholinergic (\textit{Acat2}, \textit{Ache}, \textit{Chrna5}), glutamatergic (\textit{Grik2}, \textit{Gria1}, \textit{Grid2}) and GABAergic (\textit{Gabra1}, \textit{Gabrg2}, \textit{Gabrg3}) markers were increased by GDNF, as well as the expression of CART pre-propeptide (\textit{Cartpt}), NPY receptor Y2 (\textit{Npy2r}), Substance P precursor \textit{Tac1}, Dopamine beta-hydroxylase (\textit{Dbh}) and Vasoactive intestinal peptide (\textit{Vip}). The elevated expression of these subtype markers supports our observation that RET signaling is involved in terminal neuronal differentiation of ENCCs. Several other neuronal subtype markers, such as 5-HT, Calbindin, Calretinin, CGRP, Enkephalin and NOS were not differentially expressed. The wide range of neuronal subtypes markers that are up-regulated by GDNF suggests that RET signaling might be involved in the specification of different, but not all, neuronal subtypes.

3.4. Upstream Regulator Analysis identifies known and new genes important for ENS development

Having identified gene expression patterns in untreated and GDNF-treated ENCCs, we next aimed to identify regulatory genes in ENCCs and in GDNF-induced neuronal maturation. To address this, we used the Upstream Regulator Analysis tool in Ingenuity Pathway Analysis (IPA). Upstream Regulators are predicted to
regulate the expression of differentially expressed genes, taking into account the enrichment for genes that are known to be regulated by a particular Upstream Regulator (p-value of overlap) and the direction of differential gene expression (activation z-score) (Krämer et al., 2014). Many genes that are known to have important regulatory functions in ENS development are highly expressed by ENCCs. Therefore, genes were selected from the Upstream Regulator Analysis that were either highly expressed by ENCCs or up-regulated by GDNF. The Upstream Regulator Analysis of differentially gene expression between ENCCs and the gut yielded nine regulatory genes that are highly expressed by ENCCs themselves (Table 1).

These Upstream Regulators were expressed in ENCCs that were cultured in vitro for 14 days. To confirm that these genes are endogenously expressed in ENCCs, qRT-PCR analysis was performed on RNA from acutely isolated, uncultured ENCCs from E14.5 embryonic gut. The cell cycle-associated genes Elavl1 and Nupr1 were not differentially expressed between uncultured ENCCs and whole gut.

**Table 1**

Upstream Regulators of gene expression in naïve and GDNF-treated ENCCs. Regulatory genes in ENCCs were predicted by IPA Upstream Regulator Analysis. Significant Upstream Regulators were defined as having an activation z-score > 1.8 and p-value of overlap < 0.05. From the Upstream Regulator Analysis, genes were selected that were expressed > 5 times higher in ENCCs than in the gut, or that were > 1.8 times up-regulated by GDNF. The Upstream Regulator Analysis can be linked to ENS development and HSCR (see also Fig. 5).

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Upstream Regulator</th>
<th>Activation z-score</th>
<th>p-Value of overlap</th>
<th>Target genes</th>
<th>Fold change</th>
<th>Link to HSCR</th>
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<tr>
<td>High in ENCCs</td>
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<td>9</td>
<td>19.8</td>
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<td>Inhibitor of MMP2 that is important for migration of ENCCs&lt;sup&gt;c&lt;/sup&gt;</td>
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Fig. 3. Expression of the Upstream Regulators in acutely isolated ENCCs. Except for Cdkn2a, all predicted Upstream Regulators were detected by qRT-PCR analysis on acutely isolated, uncultured ENCCs from E14.5 embryonic gut. The expression levels of Elavl1, Nupr1 and Timp1 were not higher in uncultured ENCCs than in whole gut, suggesting that the high expression of these genes in the microarray data was induced by culturing. The high expression of Sox10, Ret, Bdnf, App and Mapk10 was confirmed in acutely isolated, uncultured cells. Adora2a and Npy2r were near-significant regulators of gene expression in GDNF-treated ENCCs and both genes were expressed in acutely isolated ENCCs. *Benjamini-Hochberg adjusted p-value < 0.05.
Regulators are required for survival of ENCCs and maintenance of their progenitor differentiation, in a broad range of processes, including proliferation, differentiation, inflammation, cellular stress and neuronal apoptosis and is regulated by RET signaling in ENCCs (Ngan et al., 2008).

3.6 Synergistic regulation of gene expression

The Upstream Regulators with a high expression in acutely isolated ENCCs compared to whole gut were Sox10, Ret, Bdnf, App and Mapk10. Of these genes, Sox10 and Ret are well-established ENS development genes. Sox10 is a transcription factor that is required for survival of ENCCs and maintenance of their progenitor state (Bondurand et al., 2006; Kapur, 1999). The identification of Ret and Sox10 as regulatory genes in ENCCs demonstrates the validity of the Upstream Regulator Analysis and suggests that the remaining Upstream Regulators and their respective pathways are key regulators of ENS development. These remaining Upstream Regulators are Bdnf, App and Mapk10.

Brain Derived Neurotrophic Factor (Bdnf) is expressed by ENCCs and the mucosa and its receptors TRKB and p75/NFGR are abundantly expressed in the human and mouse ENS (Hoehner et al., 1996; Lommatzsch et al., 1999). BDNF signaling promotes neuronal survival, growth and differentiation and functions in the mature ENS by controlling peristalsis and colonic emptying (Coulie et al., 2000; Grider et al., 2006).

Amyloid Precursor Protein (App) is the precursor of Amyloid-β plaques in Alzheimer disease. The endogenous function of APP is not completely understood, but APP has been shown to be involved in synapse formation and plasticity and is expressed in the human ENS (Arai et al., 1991; Cabal et al., 1995; Turner et al., 2003; Tyan et al., 2012).

Mitogen-Activated Protein Kinase 10 (Mapk10/Jnk3) is highly involved in processes such as proliferation, differentiation, inflammation, cellular stress and neuronal apoptosis and is regulated by RET signaling in ENCCs (Ngan et al., 2008).

3.5 Adenosine and neuropeptide Y signaling modulate GDNF-induced changes in gene expression

Upstream Regulator Analysis of GDNF-induced changes in gene expression did not result in any genes that have both a significant activation z-score and p-value. There are, however, two Upstream Regulators with near-significant z-scores and p-values; the adenosine receptor A2a (Adora2a) and the neuropeptide Y receptor Y2 (Npy2r). Adora2a and Npy2r were both expressed in acutely isolated ENCCs from E14.5 embryos and Npy2r was more abundantly expressed in ENCCs than in whole gut (Fig. 3). Adenosine regulates intestinal secretion, motility and immune responses and acts through adenosine receptors A1, A2a, A2b and A3. The adenosine A2a receptor is expressed by the myenteric- and submucosal plexuses in both the small and large intestine (Antonioli et al., 2008). ADORA2A modulates intestinal motility by inhibiting motor activity in the duodenum and colon of mice and humans, respectively (Fornai et al., 2009; Zizzo et al., 2011), thereby allowing relaxation of the bowel.

Npy2r encodes the neuropeptide Y receptor Y2, a receptor for neuropeptide Y (NPY) and peptide YY (PYY) (Chen et al., 1997). NPY and PYY induce colonic smooth muscle relaxation and inhibit intestinal contractility and motility in response to feeding, which is exerted through the neuropeptide Y1 and Y2 receptors (Hellström, 1987; Tough et al., 2011).

4. Discussion

In this study we have analyzed gene expression profiles of GDNF-treated and untreated mouse ENCCs (isolation time E14.5) and E14.5 mouse embryonic gut. Unbiased, systematic Gene Ontology analysis showed that, compared to the whole gut, ENCCs highly express genes involved in cell adhesion, neuronal differentiation and synaptic signaling, representing various aspects of ENS development. These data corroborate findings by Heanue and Pachnis (Heanue and Pachnis, 2006). These authors compared gene expression profiles of E15.5 ganglionic (Ret+/−) and aganglionic (Ret−/−) gut and found that ENCCs express markers of early differentiating glia and neurons, terminally differentiated neurons, axonal outgrowth and synaptogenesis. In fact, of the 47 genes that were confirmed to be expressed in the ENS by in situ hybridization in that study, 35 genes (74%) were highly expressed by ENCCs in our study. Likewise, the gene expression study by Vohra et al. confirmed expression in the ENS for 38 genes by in situ hybridization, of which 23 genes (61%) were differentially expressed between ENCCs and the gut in the present study (Vohra et al., 2006). Of the 105 ENCC-expressed genes found by Iwashita et al., 19 genes (18%) overlap with our data (Iwashita et al., 2003). Importantly, this shows that the 14 days in vitro culturing of ENCCs in our study did not lead to major changes in gene expression. Of the 975 genes that were expressed higher in ENCCs than in total gut tissue, 848 genes (87%) were not reported in any of the three expression studies mentioned above. However, part of these 848 genes are already reported to be involved in ENS development (Kuo et al., 2010; Teng et al., 2008). This study therefore represents a more complete catalog of known and newly identified genes that are expressed in the developing ENS.

4.1 Regulators of gene expression in ENCCs are putative HSCR genes

Sox10, Ret, Bdnf, App and Mapk10 were identified as genes that are highly expressed in ENCCs and that regulate the expression of other ENCC-expressed genes. Of these regulators, RET and SOX10 are known HSCR genes. Mutations in RET and SOX10 contribute to HSCR in humans and mice with null mutations for Ret and Sox10 display total intestinal aganglionosis (Edery et al., 1994; Herbach et al., 1998; Pingault et al., 1998; Romeo et al., 1994; Schuchardt et al., 1994; Southard-Smith et al., 1998). Given the known neuronal functions of Bdnf, App, and Mapk10, this raises the hypothesis that these regulatory genes might also be implicated in HSCR and other neuropathies, such as intestinal pseudo-obstruction, incontinence or constipation.

Mapk10 is a highly interesting regulator of gene expression in ENCCs with regard to HSCR. MAPK10 modulates neuronal
differentiation through phosphorylation of APP and STMN2 (Kimberly et al., 2005; Neidhart et al., 2001). STMN2 (SCG10) interacts with Kinesin Binding Protein (KBP, KIAA1279) and mutations in KBP have been linked to the HSCR-associated Goldberg-Shprintzen Syndrome (Alves et al., 2010; Brooks et al., 2005b). Loss of MAPK10 could therefore contribute to HSCR through impaired APP and/or STMN2 signaling in enteric neuron differentiation. Since Mapk10 was reported to be highly expressed in ENCCs, it was included in a copy number variant screen for candidate genes in HSCR (Jiang et al., 2011). The authors reported one deletion in MAPK10 among 18 HSCR patients. Segregation analysis showed that the MAPK10 deletion was neither necessary nor sufficient to cause HSCR in this family and the authors therefore hypothesized that the deletion in MAPK10 acts as a modifier for developing HSCR (Jiang et al., 2011).

ENS development is of interest in relation to Down syndrome (Trisomy 21). The incidence of HSCR in Down syndrome is 2.6%, which is over a hundred times higher than in the general population (Friedmacher and Puri, 2013). This suggests that the duplication of one or more genes on chromosome 21 contributes to the onset of HSCR. APP is located on chromosome 21 in humans and App is an Upstream Regulator of gene expression in the developing murine ENS. It has been shown that overexpression of wild type human APP in mice causes loss of cholinergic neurons through disruption of Nerve Growth Factor transport and impairs neurogenesis in the CNS (Salehi et al., 2006; Trazzi et al., 2013). Moreover, mice overexpressing an Alzheimer’s disease mutant form of APP show reduced neuronal density in the myenteric plexus and altered intestinal motility (Semar et al., 2013). In the latter study it is not clear, however, whether the phenotype was caused by the increased expression level of APP or by the presence of the Alzheimer mutation in the overexpressed gene. APP contains an inhibitor domain that inhibits matrix metalloproteinase 2 (MMP2) (Miyazaki et al., 1993) and pharmacological inhibition of MMP2 impairs the migration of ENCCs along the mouse gut (Anderson, 2010). Inhibition of MMP2 is another mechanism by which overexpression of APP might contribute to the development of HSCR in Down syndrome. These reports, combined with the regulatory function of App in ENCCs, raise the intriguing hypothesis that overexpression of APP might contribute to the high incidence of HSCR and other gastrointestinal abnormalities in Down syndrome patients.

**Fig. 4.** Upstream Regulators regulate gene expression synergistically. The regulation of downstream target genes (right-hand side) by Upstream Regulators (bottom) is shown for all genes that are regulated by two or more Upstream Regulators. Red and blue boxes indicate regulation by an Upstream Regulator in naive and GDNF-treated ENCCs, respectively. Yellow indicates that the gene is not regulated by the specific Upstream Regulator.
In our Upstream Regulator Analysis, App and Bdnf shared several downstream target molecules that are involved in synaptic signaling, including Snap25 and Vamp2. Snap25 and Vamp2 mediate the fusion of synaptic vesicles and the high expression of these genes in ENCCs was reported by Vohra et al., who subsequently showed that Snap25 and Vamp2 are important for ENCC migration and neurite outgrowth (Vohra et al., 2006). Since both genes are regulated by APP and BDNF, aberrant APP and BDNF signaling might result in similar defects in ENS development.

As discussed here, the Upstream Regulators with neuronal functions are either known HSCR genes, or are linked to the molecular pathways underlying HSCR or animal models of aganglionosis. There is a large body of literature suggesting interactions between these genes, allowing us to construct a hypothetical network of regulatory genes in ENCCs (Fig. 5). The transcription factor SOX10 binds to an enhancer of Ret, thereby regulating Ret expression (Lang and Epstein, 2003). In turn, RET activates the MAPK pathway, including MAPK10 (Ngn et al., 2008; Plaza-Menacho et al., 2006). MAPK10 phosphorylates APP, which regulates the expression of synaptic genes Snap25 and Vamp2 together with BDNF, and inhibits MMP2 activity (Kimberly et al., 2005; Miyazaki et al., 1993). MAPK10 also phosphorylates STMN2, leading to cytoskeletal remodeling in conjunction with KBP (Alves et al., 2010; Neidhart et al., 2001).

4.2. Modulators of RET-signaling in ENCCs

Several signaling cascades are activated by RET, including PI3K/AKT, ERK, p38-MAPK and JNK pathways (Plaza-Menacho et al., 2006). Little is known, however, how these pathways contribute to RET-induced proliferation, migration, differentiation and survival of ENCCs. Similar to our study, Ngn et al. analyzed the changes in gene expression induced by GDNF treatment of ENCCs and found that the expression levels of 29 genes were increased by GDNF treatment. Only 4 of these 29 genes were up-regulated by GDNF in our data. These different findings can be explained by two fundamental differences in study design, duration of GDNF stimulation and developmental stage of ENCCs. Ngn et al. investigated the short-term changes (8 and 16 h GDNF treatment) in E11.5 ENCCs, when RET is required for migration and proliferation. In the present study, on the other hand, we have analyzed the long-term changes (14 days GDNF treatment) in ENCCs at E14.5, a stage in which RET is involved in survival and differentiation. The four genes that are up-regulated by GDNF treatment in both studies (Kcnq2, Vip, Olfrm1 and Tagln3), are therefore likely to be critical modulators of RET signaling and are possibly involved in different aspects of ENS development.

Long-term GDNF treatment of ENCCs resulted in reduced expression levels of genes involved in neuronal migration and early neuronal differentiation. Conversely, GDNF increased the expression of genes that are associated with terminal neuronal differentiation and synaptic signaling. Upstream Regulator Analysis showed that the adenosine receptor A2a and the neuropeptide Y receptor Y2 are near-significant modifiers of these cellular processes in GDNF-treated ENCCs. Signaling via NPY2R modulates the neurotrophic effect of GDNF on ENCCs, as a study by Anitha et al. (2006) has shown that GDNF treatment of ENCCs results in increased proliferation and reduced apoptosis of ENCCs in a NPY-dependent manner. This report is in line with our observation that NBs from GDNF-treated ENCCs are larger than NBs from untreated ENCCs. Of interest, the human orthologue of NPY2R maps to the HSCR susceptibility locus 4q31.3-q32.3 and increased NPY levels have been reported in aganglionic bowel segments from HSCR patients (Brooks et al., 2006; Hamada et al., 1987).

5. Concluding remarks

In conclusion, systematic analysis of E14.5 ENCCs gene expression profiles showed that they express markers of neural migration, axonal outgrowth and neuronal signaling. Upstream Regulator Analysis predicted genes that serve critical regulatory functions during ENS development and we propose a mechanistic network of action for these regulatory genes. Additionally, we found that GDNF treatment of ENCCs caused terminal neuronal differentiation into a variety of neuronal subtypes. These regulatory genes in ENS development give insight into the molecular pathways regulating ENS development and provide novel candidate genes for HSCR.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.004.

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