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Neurogenesis in the enteric nervous system

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ABSTRACT

The enteric nervous system (ENS) represents the highly organized intrinsic innervation of the gastrointestinal tract and plays a critical role for all stages of postnatal life. Severe disturbances of ENS function can significantly influence life quality or, in severe cases, can have acute life-threatening effects. Recent in vitro and in vivo studies demonstrated the persistence of neural stem cells in postnatal gut and there seem to be many interesting parallels to the more extensively studied neural stem cells in the brain. Enteric stem cells have been proposed as an appropriate cell source to provide an alternative therapeutic option for a number of neurogastrointestinal diseases, however a better understanding of these cells would be crucial for the translation of cell-based therapies into clinic. This review tries to highlight the recent findings in the field of enteric neurogenesis and additionally gives a brief overview about the development, structure and function of the ENS and about the developmental or age-related disturbances affecting the ENS.

Key words

Enteric nervous system (ENS) • Neural crest-derived stem cells (NCSCs) • Hirschsprung's disease (HSCR) • Acquired ENS disorders • Neurogenesis

Development, structure and function of the enteric nervous system (ENS)

The enteric nervous system (ENS) is the largest and probably the most complex division of the autonomic nervous system (ANS) and represents the intrinsic innervation of the gastrointestinal (GI) tract formed by a population of multipotent stem cells derived from the neural crest, a transient component of the ectoderm during embryonic neurulation (Le Douarin and Teillet, 1973). During and shortly after neural tube closure neural crest cells (NCCs) delaminate from the epithelium following induction of epithelial-mesenchymal transition and emigrate from the dorsal neural tube. These processes are mainly controlled by molecular gradients of bone morphogenic proteins (BMPs), Notch, fibroblast growth factors (FGF), and Wnt pathways. NCCs then follow defined, region-specific routes in the embryonic environment to give rise to a number of different epithelial and mesenchymal cell derivatives including neural cells of the ANS, mesenchymal cells of the head, chromaffin cells of the adrenal gland, and melanocytes in the skin (LeDouarin, 1980). To colonize the gut, NCCs first migrate ventrolaterally and then ventromedially along the dorsal aorta towards the proximal foregut. Using quail-chick interspecies grafting techniques the vagal neural crest region was mainly identified as the precise axial origin of most ENS stem cells and, to a lesser extent, sacral levels of the neural tube (Burns and LeDouarin, 1998) and is driven by a complex program of different genes (Newgreen and Young, 2002). The exact molecular mechanisms which guide NCCs within the gut are not yet well understood, however the Ret/GFRa1/GDNF signaling pathway plays a crucial role in driving the migration in a rostro-caudal pattern, a process highly conserved in all investigated verte-

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brates. Other important pathways are endothelin-3 (EDN3)/endothelin receptor B (EDNRB) signaling, BMPs, the hedgehog family, various growth factors and neurotrophins, guidance molecules (e.g. semaphorins, repulsive guidance molecule/RGM) and a number of further molecules such as transcription factors e.g. Sox10, Phox2b, Pax3, Mash1 and others (Heanue and Pachnis, 2007). Recent experimental studies indicated that a minimal number of NCCs is required for the normal rostro-caudal colonization of the complete gut (Barlow et al., 2008). Interestingly, NCCs often migrate in chain-like formations and interact with the tissue environment and neighboring NCCs via extensions and retractions of filopodia.

Gut colonization by vagal NCCs is completed by ~E15 in mice and after ~7 weeks gestation in humans. Shortly after vagal cells have reached the hindgut sacral cells, which have already accumulated in close proximity to the distal end, also start to migrate into the gut wall and thus contribute to ENS formation in the distal midgut and hindgut region. After complete colonization vagal and sacral neural crest-derived ENS cells generate a complex network of differentiated neuronal and glial cells arranged in interconnecting ganglia within the longitudinal and circular smooth muscle layers (myenteric or Auerbach's plexus) as well as the connective tissue that lies beneath the mucosa (submucosal or Meissner's plexus) of the gut. It has been shown that the myenteric plexus is formed first and the submucosal plexus develops later after a secondary centripetal migration wave mediated by the netrin/DCC guidance molecules (Jiang et al., 2003). Evidences exist in pig and human gut that a third ganglionated plexus of the ENS may exists within the intestinal mucosa (Fang et al., 1993; Balemba et al., 2002; Metzger et al., 2009). The size and number of ganglia is variable between the gut region and different species, but typically myenteric ganglia are larger within the same region. Taken together all ENS ganglia are estimated to contain more neurons than the spinal cord (i.e. up to 10^{10} cells in humans) and, interestingly, many of the neural subtypes and neurotransmitters can also be found in the central nervous system (CNS). About 20 different neuronal cell types were identified by their neurochemical coding so far (also variable between species) and provide a complex local neuronal circuitry consisting mainly of intrinsic primary afferent neurons (IPANs; monitor gut lumen and gut wall alterations), ascending/ descending interneurons (connect neurons and ganglia with each other), and inhibitory/excitatory motor neurons (target smooth muscle cells). Although several distinct phenotypic classes of neurons have been identified there are basically two major morphologic forms termed as Dogiel type I neurons, having many short processes and a single long process, and Dogiel type II neurons which are multipolar with many long, smooth processes. Another classification, based on their electrophysiological potential, was introduced in the 1970s and termed neurons exhibiting brief action potential followed by short duration after-hyperpolarizing potentials (AHP) as S neurons (S historically stood for synaptic) and neurons having large action potentials followed by two separate and very prominent phases of hyperpolarization as AH neurons (AH stood for after-hyperpolarizing). In the guinea-pig intestine, where most of these studies have been done, all S type neurons have a Dogiel type I morphology whereas AH neurons have a Dogiel type II phenotype (Furness, 2000). In addition to neurons, enteric glial cells are an integral and important component of the neuronal circuitry and they largely exceed neurons in cell number. Enteric glial cells resemble the astrocytes of the CNS and are different from the Schwann cells of the peripheral nervous system (Gershon and Rothman, 1991). They form a widespread network at all levels of the gastrointestinal tract with multidirectional cellular interactions to neurons, epithelial cells, mesenchymal cells and immune cells. In comparison to enteric neurons their physiological role has not that extensively studied but there is accumulating data indicating an active role of enteric glial cells in enteric neurotransmission, maintaining the integrity of mucosal barrier and serving as link between the ENS and immune system (Ruhl, 2005). The fully mature ENS circuitry develops in the first days after birth and, even after complete extrinsic denervation, allows a largely independent control of gut peristalsis and secretory activity. Because of its size, autonomy, complexity, and structural analogies to the CNS, the ENS has been entitled as "Little Brain" in the gut (Wood and Grundy, 1998). However, despite its great autonomy the ENS is well connected to the CNS via motor and sensory pathways of the sympathetic and the parasympathetic nervous system which is necessary

for fine tuning of the diverse functions of the ENS. Noteworthy, coordinated gastrointestinal motility also requires functional interaction with gut smooth muscle and interstitial cells of Cajal (ICCs or gut 'pacemaker' cells) which are intimately related functionally and developmentally (Burns, 2005, 2007). In particular, ICCs generate electrical slow waves that regulate the phasic contractile activity of the gut and mediate neurotransmission from enteric motor neurons to smooth muscle cells. Beside control of peristalsis and secretion activity there is evidence that enteric cells are also involved in blood flow control, the function of the mucosal immune system and influence the epithelial stem cell compartment of intestinal mucosa (Bjerknes and Cheng, 2001; Toumi et al., 2003).

Because of its important role in various gut functions the ENS is absolutely essential for all stages of postnatal life and severe disturbances of ENS function therefore can significantly influence life quality or, in severe cases, can have acute life-threatening effects.

Developmental disturbances and acquired disorders affecting ENS integrity

Congenital or developmental disturbances of the ENS

In the last two decades, enormous progress has been made in understanding the genetics and developmental disturbances of the ENS. This is best illustrated in Hirschsprung's disease (HSCR) which is the most common identifiable congenital disorder of ENS with an incidence of 1:4500 live births (Newgreen and Young, 2002). Other defects such as intestinal neuronal dysplasia type B (INDB) are less clinically defined and therefore a precise characterization was much more challenging in the past. HSCR is caused by disturbances during formation of the ENS leading to characteristic absence of enteric ganglia (aganglionosis) in variable lengths of terminal gut. This leads to peristaltic misregulation and tonic contraction within the aganglionic segment causing intestinal obstruction and massive distension of the proximal bowel. HSCR has been described as heterogeneous genetic disorder that can be either familial or sporadic. Although 10 different genes have been identified to be involved in HSCR so far, the Ret tyrosine kinase receptor gene (RET) appears to be the principal causative gene with a mutation frequency of about 50% in familial cases and 15-35% in sporadic cases (Hofstra et al., 2000). Furthermore, hypomorphic alleles of RET account for the majority of isolated HSCR, constituting 70% of all HSCR cases (Uesaka and Enomoto, 2010). In particular, extensive enteric progenitor cell death was identified as main mechanism of intestinal aganglionosis in a RET hypomorphic or deficient state, which could be prevented by elevated Bcl-xL (an anti-apoptotic protein of the Bcl-2 family) expression. However, in contrast to rescued hypomorphic mice, Bcl-xL could not prevent disturbances in neuronal differentiation in RET-deficient mice revealing the importance of RET in neuronal differentiation and ENS function (Uesaka and Enomoto, 2010).

Dependent to the extent of aganglionosis HSCR is termed short-segment (S-HSCR) or long-segment HSCR (L-HSCR). The majority of cases are sporadic (> 80%) and S-HSCR while the L-HSCR and total aganglionosis are less common. The inherited form of HSCR is influenced by gender (male:female ratio 4:1), seems multifactorial and has a low penetrance. In most cases, soon after birth HSCR can be diagnosed by signs of obstruction (megacolon), vomiting or enterocolitis. Treatment of HSCR has essentially been limited to surgical intervention but despite tremendous progress in surgical techniques the long-term outcome often remains unsatisfactory and many patients suffer from considerable postoperative morbidity or develop severe functional gut disturbances (Amiel and Lyonnet, 2001; Thapar, 2009). In severe forms of total aganglionosis, the provision of artificial nutrition and small intestinal transplantation currently seems to be the only therapeutic option for these patients.

Acquired or age-related enteric neurodegeneration

Besides intestinal genetic neuropathies that manifest in fetal or early postnatal development, there is increasing scientific and clinical interest in acquired or age-related gastrointestinal dysfunctions which can generally be classified as either secondary to a variety of primary pathological conditions or idiopathic in nature (Firth and Prather, 2002; Wade and Cowen, 2004). Observations in human as well as

rodent gut revealed an age-related, often immune-mediated, reduction of the total numbers of neurons, mainly within the myenteric plexus, or abnormal nerve fiber density and ganglia structure (deSouza et al., 1993; El-Salhy et al., 1999; Phillips and Powley, 2007). Some studies correlated ENS alterations with reduction of distinct neuronal subtypes (e.g. intrinsic primary afferent neurons/IPANs) or neurotransmitter expression (Feher and Penzes, 1987; van Ginneken et al., 2001; Wade et al., 2002). Others report a selective protection of nitrergic and loss of cholinergic myenteric neurons during aging which can vary between animal strains (Wu et al., 2003; Thrasivoulou et al., 2006). The reasons for a specific cell vulnerability are unclear but oxidative stress by reactive oxygen and nitrogen species (RONS), which also play a role in neurodegenerative processes of the CNS, have recently been discussed for ENS degeneration (Thrasivoulou et al., 2006; Wade and Cowen, 2004). In humans, significant ENS alterations can lead to symptoms that, depending on the gut region, include dysphagia, gastro-esophageal reflux, delayed emptying of the stomach, abdominal pain and bloating, diarrhoea, constipation, and faecal incontinence (De Giorgio et al., 2004). Symptoms are manifested in diseases such as achalasia, gastroparesis, congenital hypertrophic pyloric stenosis, chronic intestinal pseudo-obstruction, chronic idiopathic constipation, and probably irritable bowel syndrome (deGiorgio et al., 2004; Di Nardo et al., 2008). In addition to neuroplastic changes in the ENS in physiological states, acquired ENS alterations are also associated secondary to pathological or stress conditions such as gut inflammations, infections or multisystemic diseases e.g. diabetes (Collins, 1996; Mawe et al., 2004; Chandrasekharan and Srinivasan, 2007). Inflammatory bowel disease in particular can lead to ENS remodeling including increased size and/or numbers of nerve bundles (neuronal hypertrophy and hyperplasia), enteric glia activation (i.e. upregulation of glial fibrillary acidic protein/GFAP and glial hyperplasia) within the (sub-)mucosa, and changes in neuropeptides and neurotrophin release (e.g. Substance P, GDNF) induced by cytokine mediators of inflammatory cells (Geboes and Collins, 1998; Cabarrocas et al., 2003; von Boyen, 2004; Margolis and Gershon, 2009). Interestingly, in transgenic mouse models lacking enteric glia an extremely severe form of small bowel inflammation, hemorrhagic necrosis and disruption of mucosal integrity have been observed suggesting these cells as an integral element of the inflammatory process (Bush et al., 1998; Ruhl, 2005). Further, evidences continue to accumulate to indicate that the enteric and central nervous systems may demonstrate parallel pathologic changes in a number of disease processes previously regarded as confined to the central and somatic nervous systems. Thus, GI dysfunction is a frequent and occasionally dominating symptom of Parkinson's disease (PD) manifesting mainly as dysphagia, disorders of gastric emptying and constipation. The most likely causes of these disorders are cerebral degeneration and degeneration mainly of the myenteric plexus (Martignoni et al., 1995; Pfeiffer, 1998; Lebouvier et al., 2009). Taken together, the ENS not only continuously changes during development but also keeps a significant potential of postnatal plasticity adapted to the actual physiological conditions.

Stem and progenitor cells of the enteric nervous system

The presence of migrating enteric neural-crest stem cells (NCSCs) in the embryo has long been established and a number of characteristic genes were considered as markers allowing identification and purification of NCSCs and their progenies (Stemple and Anderson, 1992; Teng and Labosky, 2006). Undifferentiated NCSCs are initially characterized by high expression of Sox10 (SRY-related HMG-box gene) (Paratore et al., 2002; Kim et al., 2003). During the progressing gut colonization an increasing number of NCSCs then start to upregulate a panel of progenitor marker genes such as Mash1, Ret, p75, nestin, Phox2b and eventually may become more committed neurons (positive for β-tubulin III, PGP9.5, HuC/D) or, with slight delay, glial cells (positive for B-FABP, S100, Sox10), respectively (Young et al., 2003, 2004; Ruhl, 2005). More recently, protocols have been established allowing the generation of NCSCs from embryonic stem cells (Lee et al., 2007; Kawaguchi et al., 2010). This will certainly accelerate further insights into molecular pathways and factors important for NCSC specification, expansion and differentiation. Noteworthy, many of the markers found in neural crest-derived

stem cells are also present in the glial lineage such as Sox10, Notch or EdnrB (Paratore et al., 2002; Okamura and Saga, 2008). A subpopulation of enteric glia also maintain their ability to divide and, as mentioned above, show a great plasticity in postnatal gut during pathological processes (e.g. inflammation) indicated by upregulation of certain neurotrophic factors (e.g. GDNF, NGF and others) and expression of markers for neural stem cells (e.g. nestin, GFAP), respectively (von Boyen et al., 2004; Schafer et al., 2009). Whether these cells can act as "true" stem cells is still unclear but these findings suggest many parallels to the glia and neural stem cells in the CNS which has also been discussed elsewhere (Laranjeira and Pachnis, 2009; Schafer et al., 2009). Interestingly, until the early 1990's neurogenesis in the CNS was also believed to end shortly after birth. Meanwhile, many studies in mammals, including humans, consistently confirm the persistence of CNS adult stem cells in defined neural stem cell niches of the hippocampus and subventricular zone/olfactory bulb which were classically considered as astrocytes (Kriegstein and Alvarez-Buylla, 2009). In response to relevant stimuli, neural stem cells i.e. a GFAP-expressing subpopulation of astrocytes (which correspond to radial glial cells in the embryo) are able to differentiate into neurons and glia *in vitro* and *in vivo* which is in stark contrast to the classical concept that separated glial and neuronal cell origins (Reynolds and Weiss, 1992). Prompted by these findings many questioned if a stem cell niche in the postnatal ENS may also exist? As mentioned above the onset of enteric neurogenesis begins at the earliest embryonic stages of gut colonization and continues during fetal gut development. Some neuronal subtypes, however fully appear first in the very late fetal and postnatal gut stage such as CGRP neurons, suggesting ongoing neurogenesis after birth, too (Young et al., 2003). Further, at birth up to 5% of cells within enteric ganglia still remain negative for both, pan-neuronal and glial markers, depending on the gut region. Although this value further decreased to less than 1% in the adult gut, the authors could not rule out the presence of undetected immature cells outside the analyzed ganglia. The hypothesis of the existence of an undifferentiated postnatal enteric cell reserve pool was also supported in various experimental animal disease models with impaired ENS (Cracco and Filogamo, 1997; Poli et al., 2001; Hanani et al., 2003). The authors describe signs of ENS neuroplasticity and reversibility of plexus disruption based on histological analyses and electronmicroscopical observations. A recent *in vivo* study in adult mice more clearly specified postnatal neurogenesis residing within germinal niches between the myenteric plexus and the longitudinal muscle layer (Liu et al., 2009). After BrdU co-labeling, incorporation of newly generated enteric neurons into enteric ganglia between week 16 and 24 after their birth was described. These observations were strongly dependent on 5-HT_4 (serotonin) receptor activation which confirms earlier studies in the rat hippocampus, where similar effects have been observed (Lucas et al., 2007). Accumulating evidence for a persistent postnatal ENS stem cell niche also arose from many *in vitro* stem cell culture studies that started almost 10 years ago. Kruger and colleagues successfully adapted protocols from NCSCs cultures and thus were able to demonstrate multipotent neural stem cells isolated from postnatal rat gut (Kruger et al., 2002). Neural cell clones were isolated using flow-cytometry and p75 and α4-integrin antibodies and eventually reflected the characteristic properties of neural stem cells *in vitro* i.e. they were able to self-renew and differentiated into large colonies containing neurons (including nitric oxide synthase/NOS, vasoactive intestinal peptide/VIP, neuropeptide Y/NPY-positive neuronal subtypes), glia and myofibroblasts. However, in contrast to their fetal counterparts, postnatal cells revealed significant changes in self-renewal capacity, neuronal subtype potential (loss of serotonergic and noradrenergic potential) and responsiveness to lineage determination factors (i.e. BMPs), showing the same trend as other stem cell types including CNS stem cells (Maslov et al., 2004). In the following years other groups were able to confirm the existence of multipotent neural progenitors in postnatal gut tissue of mice. In these studies, enrichment of progenitors was achieved by flow sorting of retroviral GFP-transfected cells and/or selective culture growth conditions, mainly by adding FGF-2 and EGF, promoting cell expansion either as monolayer cultures or stem cell-containing aggregates termed neurosphere-like bodies (NLBs) in a manner similar to CNS neural stem cell cultures established earlier (Reynolds and Weiss, 1992; Bondurand et al., 2003; Suarez-Rodriguez and Belkind-Gerson, 2004). In addition to their bi- or

multipotentiality shown in clonal cell culture assays, authors further demonstrated the capability of murine progenitors to colonize wild-type and aganglionic gut tissue *in vitro* (Bondurand et al., 2003). More recently, Anitha and colleagues established immortalized fetal and postnatal enteric cell lines of transgenic mice that have characteristics similar to the primary cultures and therefore offer an alternative "off-the-shelf" cell resource to study the ENS and diseases that affect it (Anitha et al., 2008). This study also utilized functional tests after transplantation *in vivo* for the first time. Thus, after injection into transgenic mouse models of aganglionosis a significant improvement of neuronal function was reported by measuring contractile response and colonic transit time. These data are in line with earlier *in vivo* studies in the gut using embryonic CNS-derived stem cells and providing the "proof of principle" of alternative cell-based treatments for neurogastrointestinal disorders (Micci et al., 2005; Liu et al., 2007; Dong et al., 2008). In further exciting studies, a significant progress has been made in the identification and harvesting of ENS stem and progenitor cells from postnatal human gut (summarized in Table I and illustrated in Fig. 1). In analogy to the studies in animals, the first human study (patient age up to 5 years after birth) could successfully generate progenitor-containing NLBs (positively immunostained for nestin) from full-thickness gut samples which were able to differentiate into neurons and glial cell *in vitro* (Rauch et al., 2006). Using a similar approach, another group reported the exponential growth of dissociated primary neonatal human NLBs as secondary and tertiary NLBs which continuously expressed characteristic markers to identify ENS progenitors (p75, BrdU) as well as a panel of neuronal subtype markers (nitric oxide synthase/NOS, choline acetyl transferase/ChAT, vasoactive intestinal peptide/VIP, substance P/SP and calcitonin gene-related peptide/CGRP) following induction of NLB differentiation (Almond et al., 2007; Lindley et al., 2009). After injection into aganglionic murine gut explants (E11.5), human NLBs differentiated to neurons and glia and improved gut functionality *in vitro* as demonstrated by calcium imaging, contractility measurement and electron microscopy (Lindley et al., 2008). More recently the expansion and differentiation of neural progenitors isolated from human adult small and large intestinal full-thickness specimen (patient age up to 89 years, mean age ~55 years) was shown (Metzger et al., 2009). Dependent on tissue age and quality, an effective expansion of adult progenitors as NLBs was possible under serum-free conditions, however medium supplementation with conditioned medium of fast-growing fetal mouse cultures was crucial, indicating the need of still undetermined secreted growth and/or survival factors. In addition to an immunohistochemical analysis of NLBs and mature neural cells, for the first time *in vitro* differentiated neurons were also characterized by electrophysiological studies revealing voltage-dependent sodium channels in more than 50% of all analyzed cells. Furthermore, real neo-differentiation of enteric neurons from dividing adult progenitors was demonstrated by BrdU co-labeling experiments and quantitative PCR analysis. Noteworthy, as in earlier studies also smooth muscle-like cells were identified, however it was unclear if these cells arose from a common neural stem cell or from another, still unidentified myogenic progenitor cell pool. Another recent human study represented a significant practical advance towards the development of potential stem cell-based therapies for ENS disorders such as Hirschsprung's disease. Thus, mucosal gut biopsies from postnatal gut, taken via minimally invasive endoscopic techniques, were demonstrated as a viable source of ENS stem cells (Metzger et al., 2009). In particular, NLBs could be generated from biopsies of children (patient age up to 17 years, mean age ~9 years), including those with HSCR, in a similar way as from full-thickness samples. In line with previous studies, these NLBs showed phenotypic markers characteristic for ENS progenitors including Sox10 expression and when transplanted into explants of aganglionic chicken or human HSCR gut, plexus-like structures were observed. In addition to BrdU-colabeling assays, for the first time bipotentiality of human cells could be verified in clonogenic cultures with a frequency of ~4%. The precise tissue origin of these stem cells was unclear but given the fact that most of the biopsies were free of submucosal tissue, and putative stem cell markers (p75, Sox10) were present within the mucosa, it was hypothesized that these stem cells might derive from a not yet identified niche within the mucosa. However, the possibility of transdifferentiation of cells from other stem cell pools such as epithelial or bone-marrow-derived stem cells can not be ruled out and should clearly be addressed in future studies.

Table I. - Summary of experimental studies establishing enteric stem and progenitor cells from postnatal gut tissue. Abbreviations: N, neurons; G, glia; MF, myofibroblasts; ND, not determined; wog, weeks of gestation.

Species	Age	Cell propagation	Differentiation	Recipient tissue	Functional tests	Reference
Rat	E14.5-P110	Monolayer (p75/ α4 integrin+ cells)	N + G + MF	Chick embryo hindlimb bud somites in ovo	ND	Kruger et al., 2002
Mouse	E11.5-P14	Neurospheres (GFP+ cells)	N + G	Aganglionic gut explant from Ret-/- mouse embryo *in vitro*	ND	Bondurand et al., 2003
Mouse	P3-12 months	Monolayer	N + G + MF	ND	ND	Suarez-Rodriguez and Belkind-Gerson, 2004
Human	9 wog-5 years	Neurospheres	N + G	Human gut explant *in vitro*	ND	Rauch et al., 2006
Mouse/ Human	E11.5/3 weeks-7 months	Neurospheres	N + G	Aganglionic gut explant from E11.5 mouse embryo *in vitro*	ND	Almond et al., 2007
Rat	P7-P14	Neurospheres	N + G	ND	ND	Silva et al., 2008
Mouse transgenic	E13-P2	Monolayer, cell line (p75+ cells)	N + G + MF	Colon from nNOS-/- and Ednrb+/- mice *in vivo*	Isometric muscle recording and colonic motility measurement	Anitha et al., 2008
Mouse/ Human	E11.5/neonatal	Neurospheres	N + G	Aganglionic gut explant from E11.5 mouse embryo *in vitro*	Bowel contractility and calcium imaging	Lindley et al., 2008
Human	Neonatal	Neurospheres	N + G	Aganglionic gut explant from E11.5 mouse embryo *in vitro*	ND	Lindley et al., 2009
Human	8 wog-17 years	Neurospheres	N + G	Aganglionic gut explant from HSCR patient and E5 chick gut *in vitro*	ND	Metzger et al., 2009a
Human	26-89 years	Neurospheres	N + G + MF	E18 organotypic slice culture and aganglionic gut explant from E11 mouse embryos *in vitro*	ND	Metzger et al. 2009b

Conclusions

In the past years astonishing knowledge about the biology of ENS and its involvement in numerous aspects of intestinal function and diseases have been achieved. However, the functional significance of neural crest-derived stem cells in the postnatal gut remains unclear and demands more work in order to elucidate whether these cells permanently perform neurogenesis and gliogenesis or whether and how they can be affected by injury, ageing or external stimuli, respectively. Nevertheless, the recent advances in molecular biology and enteric stem cell technology seems promising for future alternative treatment options of genetic and acquired ENS disorders. Clearly, before stem cell therapies can be translated into clinical practice further extensive fundamental research is needed in this field. This includes issues

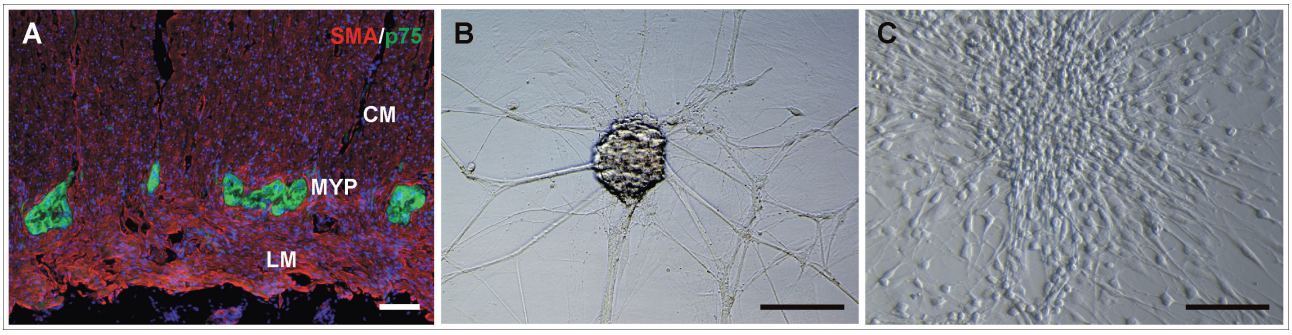

Fig. 1. - Enteric stem and progenitor cells can be isolated from human postnatal gut. (A) Cryosection through a full-thickness gut sample (obtained from neonatal human tissue) showing the presence of myenteric plexus (MYP, green, stained with p75 neurotrophin receptor) between the circular (CM) and longitudinal smooth muscle layers (LM) (red, stained with smooth muscle actin/SMA). Dapi staining (blue) marks the cell nuclei. (B) Stem cell containing NLBs can be generated from postnatal human gut tissue. Neural cells start to spread out after induction of differentiation. (C) After 2-3 weeks of differentiation dense colonies can be observed showing characteristic neural morphology under phase contrast. Scale bars: 100 μm.

about the ideal stem cell source, improved protocols for cell isolation and propagation *in vitro*, practical challenges such as establishing optimal routes of cell delivery, analysis of functional cell integration and safety. The many parallels and previous successes in CNS stem cell research should encourage ENS researchers to further advance their scientific concepts. All in all, the field is at a promising juncture and there is no doubt that the near future will reveal many interesting new aspects of the "2nd brain".

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