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Phenotype and distribution pattern of nestin-GFP-expressing cells in murine myenteric plexus

David Grundmann[1] · Franziska Markwart[1] · Anja Scheller[2] · Frank Kirchhoff[2] ·
Karl-Herbert Schäfer[1]

Abstract The enteric nervous system has to adapt to altering dietary or environmental conditions and presents an enormous plasticity that is conserved over the whole lifespan. It harbours neural-crest-derived neurons, glial cells and their precursors. Based on a nestin-green fluorescent protein (NGFP) transgenic model, a histological inventory has been performed to deliver an overview of neuronal and glial markers for the various parts of the gastrointestinal tract in newborn (postnatal day 7) and adult mice under homeostatic conditions. Whereas NGFP-positive glial cells can be found in all parts of the gut at any individual age, a specific NGFP population is present with both neuronal morphology and marker expression in the myenteric plexus (nNGFP). These cells appear in variable quantities, depending on age and location. Their overall abundance decreases from newborn to adults and their spatial distribution is also age-dependent. In newborn gut, nNGFP cells are found in similar quantities throughout the gut, with a significantly lower presence in the duodenum. Their expression increases in the adult mouse from the stomach to the colon.

All of these nNGFP cells expressed either (but not both) of the glia markers S100 or glial fibrillary acidic protein (GFAP). In the S100-positive glia population, a subset of cells also shows a neuronal morphology (nS100), without expressing nestin. Thus, the presence of premature neurons that express NGFP demonstrates that neurogenesis takes place far beyond birth. In enteric neurons, NGFP acts as a marker for neuronal plasticity showing the differentiation and change in the phenotype of neuronal precursor cells.

Introduction

Because the enteric nervous system (ENS) seems to be associated with an increasing number of diseases (Bassotti et al. 2010; Butler Tjaden and Trainor 2013; Chandrasekharan et al. 2010; Derkinderen et al. 2011; Semar et al. 2013; Villanacci et al. 2008), the investigation of its development, plasticity and aging becomes of greater importance. Depending on species, the ENS consists in 2–4 ganglionated networks, mainly the myenteric plexus (MP) and submucous plexus (SMP), which are both composed of glial and neuronal cells. Additionally, several fibrous networks occur within the smooth muscle, serosal or mucous layer (Furness 2012). The neural cells originate from neural-crest-derived stem cells, which migrate and colonise along the gut during embryonic development (Heanue and Pachnis 2007). Neural progenitor cells can be identified by specific markers such as p75 and nestin (Heanue and Pachnis 2007; Rauch et al. 2006).

Nestin is an intermediate filament class VI protein that can be found during development in healthy or diseased cells (Dahlstrand et al. 1992, 1995; Florenes et al. 1994,

✉ David Grundmann
david.grundmann@hs-kl.de

✉ Karl-Herbert Schäfer
karl-herbert.schaefer@hs-kl.de

[1] ENS Group, University of Applied Sciences Kaiserslautern, Amerikastraße 1, 66482 Zweibrücken, Germany

[2] Department of Molecular Physiology, Medical Faculty of the University of Saarland, Homburg/Saar, Germany

Frojdman et al. 1997; Lendahl et al. 1990; Mizuno et al. 2006; Rauch et al. 2006; Tsujimura et al. 2001). It is prominently present in uncommitted neural progenitor cells of the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus but also occurs in neurons in other parts of the central nervous system (Dahlstrand et al. 1995; Hendrickson et al. 2011). A deficiency of nestin leads to the decreased survival and self-renewal of neural stem cells (NSCs), whereas the integrity of the cytoskeleton is unaffected (Park et al. 2010). Nestin has been reported in diverse cell populations such as glial cells, neurons, pericytes, endothelial cells and interstitial cells of Cajal in the gastrointestinal tract (Belkind-Gerson et al. 2013; Schäfer et al. 2009; Vanderwinden et al. 2002).

The development of neurons in the ENS takes place not only very early when enteric neural-crest-derived stem cells (eNCSC) populate the foregut but also after birth. Enteric neurons are derived from proliferating neuroblasts during embryonic development of the ENS (Hao and Young 2009). The earliest detectable neuronal markers are pan neuronal markers such as Hu or PGP 9.5, which can be revealed at an early developmental stage of the ENS when the vagal-derived enteric neural crest cells populate the foregut (Hao and Young 2009). The next neuronal markers in this time-line are acethylcholine at embryonic day 11 (E11) and calbindin and nitric oxide synthase 1 (NOS1) at E11.5. Other markers arise at later developmental stages, such as 5HT at E18 or calretinin at postnatal day 1 (P1; Hao and Young 2009).

Obviously, neurogenesis can continue far beyond birth and strongly correlates with the conditions within the gut. Thus, the number of neurons is decreased in germ-free postnatal mice in both the ileum and jejunum but not in the duodenum. In addition, the chemical coding is changed indicating the influence of the microbiome on neuronal development or survival at an early postnatal stage (Collins et al. 2014). More recently, cell-fate mapping in a Dhh-Cre mouse has shown that Schwann cell precursor cells (SPCs) serve as neuronal precursor cells in the postnatal ENS. The SPCs colonise the gut at the embryonic stage and give rise to calretinin- and neural NOS (nNOS)-positive neurons at the postnatal stage (Uesaka et al. 2015).

Moreover, the presence of ENS progenitor cells in the postnatal and adult ENS differentiating into neurons has been shown by numerous in vitro studies (Metzger et al. 2009; Rauch et al. 2005; Suarez-Rodriguez and Belkind-Gerson 2004).

Several experimental models for neurogenesis are available, such as incomplete stenosis or BAC injury, which demonstrate neurogenesis in adult rodents in vivo from postmitotic glial cells (Filogamo and Cracco 1995; Hanani et al. 2003). Furthermore, neurogenesis has been demonstrated in vivo in response to the treatment with 5-HT agonist from neuronal precursor cells located outside the myenteric ganglia (Liu et al. 2009) and during intestinal inflammation in a colitis mouse model in which glial

cells serve as neuronal precursor cells by transdifferentiation (Belkind-Gerson et al. 2015). In human adult tissues, neuronal hyperplasia has also been reported in inflammatory bowel disease (Geboes and Collins 1998). The persisting plasticity and neurogenesis throughout its lifespan makes the ENS a suitable autologous neural stem cell source for cell-based therapies to cure neural disorders, such as Hirschsprungs disease or brain injury (Belkind-Gerson et al. 2016; Hagl et al. 2013; Hotta et al. 2013; Rauch et al. 2005; Jiang et al. 2003).

The aim of our study was to investigate the stem cell and neurogenic potential of ENS based on the expression of nestin-green fluorescent protein (GFP) after birth. We used a nestin-GFP (NGFP) transgenic mouse model in order to reveal the progenitor cell potential in the various parts of the gastrointestinal tract in postnatal and adult mice. The colocalisation of neuronal and glial markers with NGFP-expressing cells was investigated in the MP. NGFP was found in both neurons and glial cells in the postnatal and adult stages. We suggest that NGFP in this transgenic mouse model is a marker for neuronal plasticity rather than for real stemness, when premature neurons undergo differentiation. Moreover, our study supports the strong neurogenic potential of the glial cells in the postnatal MP under physiological conditions because of the presence of glial-neuron intermediate cells. These cells are mainly found in the early postnatal gut under homeostatic conditions.

Materials and methods

Animals

Balb/c mice of both sexes and at various ages (postnatal P7 and adult ≥12 weeks) were used. Heterozygous BL6/J NGFP (Yamaguchi et al. 2000) and FVBN TgN[hGFAP-EGFP(GFEC)] (Nolte et al. 2001) were used to visualise nestin and glial fibrillary acidic protein (GFAP) expression. Animal dissections were conducted under the guidelines of the local ethics committee and in accordance with the animal protection laws in Rhineland-Palatinate, Germany.

Whole-mount immunofluorescence staining

For whole-mount staining, formalin-fixed tissue was used from each investigated part of the gastrointestinal tract (glandular stomach, duodenum, jejunum, jejunum/ileum transition zone, ileum, caecum, proximal and distal colon). The intestinal tissue was fixed in 4 % formaldehyde at 4 °C directly after dissection from mice. The postnatal gastrointestinal tract was fixed for 3 h. The adult gastrointestinal tract was perfused with 4 % formaldehyde and post-fixed for 24 h at 4 °C. Parts of the adult caecum were fixed for 6 h only to allow the use of more sensitive markers that do not work in heavily fixed tissue, such

as nNOS, calbindin and calretinin. GFP localisation was enhanced by anti-GFP immunohistochemistry. The muscle layer was dissected from the submucous/mucous layer by means of a watchmakers forceps. Whereas muscle layers from the postnatal gastrointestinal tract were dissected after the staining procedure, those from adult gut were dissected prior to the staining procedure. Prior to the first staining steps, tissues were permeabilised at room temperature (RT) with Triton (Carl Roth) for 20 min (postnatal 2.5 %, adult 0.5 %), followed by a blocking step (1 h at RT) with 10 % normal goat serum (PAA) or donkey serum (Millipore), respectively. Whole-mounts were incubated for up to 48 h with primary antibodies (Fig. S1). After the tissues had been rinsed (3 × 20 min at RT) with phosphate-buffered saline (PBS; PAN BIOTECH), the secondary antibodies were incubated with the tissues for up to 24 h (Fig. S1). Whole-mounts were rinsed with PBS (3 × 20 min at RT) and counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). Finally, the whole-mounts were mounted on microscopic slides (Carl Roth) in mounting medium (Dako Cytomation). Detection and image processing were performed with CellOberserver Z1 by using ApoTome technology and Axiovision software (Zeiss).

Cell quantification and statistics

Neuronal NGFP (nNGFP) cells were quantified in the MP in the stomach, duodenum, jejunum, ileum, caecum, proximal and distal colon. The nNGFP cells were quantified within 10 ganglia per intestinal region from five different Bl6/J NGFP mice at the postnatal (~P7) stage and adult (≥12 weeks) stage. The absolute number of nNGFP cells, the percentage of ganglia containing nNGFP cells and the ratio of nNGFP cells to the whole NGFP-expressing population within the ganglia were analysed.

The analysis of S100 cells with a neuronal morphology (nS100) was carried out in the stomach, duodenum, jejunum, jejunum/ileum transition zone, ileum, caecum, proximal and distal colon of five different postnatal mice (P7). The nS100 cells were quantified within 50 ganglia under homeostatic conditions. Large numbers of ganglia were necessary because the nS100-containing ganglia were often far away from each other. The absolute number, the percentage of ganglia containing nS100 cells and the proportion within the S100 population were analysed.

For statistical analysis, the Mann–Whitney U test was applied by using GraphPad Prism Software 6. Data are displayed as means ± SEM and P-values (exploratory results) < 0.05 (*) and < 0.01 (**) were considered as statistically significant.

Results

Analysis of the NGFP transgenic mice revealed that nestin-positive cells were present in the myenteric ganglia

throughout the gut in both postnatal and adult mouse (Fig. 1, Fig. S2, S3). Moreover, NGFP-expressing cells were also found in the SMP as demonstrated in the proximal colon (Fig. S4). The majority of these NGFP cells presented a glial morphology in the MP and were stained either by S100 (Fig. 1, Fig. S2) or by GFAP (Fig. 1) as shown in both newborn and adult mice.

In addition to NGFP-expressing cells with glial morphologies and staining patterns, a population of NGFP-expressing cells with a neuronal morphology (nNGFP) could be found in the MP in both postnatal and adult mice (Fig. 2, Fig. S3). The postnatal nNGFP cells developed one or several axons, whereas the adult nNGFP cells predominantly exhibited one axon only.

These cells expressed the pan-neuronal markers PGP9.5 and HuC/D wherever investigated in both postnatal and adult (Fig. 2) mice. nNGFP cells were present in most segments of the gastrointestinal tract that were investigated. Their distribution varied significantly depending on age and location (Fig. 3). Interestingly, no nNGFP cells were detected in the adult stomach. In the newborn stomach, 14.9 % (±3.3) of the NGFP population presented a neuronal morphology and staining pattern (Fig. 3a). The amount of nNGFP cells dropped dramatically to 1.6 % (±1.2) in the duodenum. The highest proportion of nNGFP cells could be found in the jejunum (22.08 % ±1.77) and decreased from there continuously to 8.7 % (±0.8) in the distal colon.

Interestingly, the absolute nNGFP cell numbers gave a different impression in postnatal mice compared with relative numbers (Fig. 3b). Here, the maximum absolute number of cells could be seen in the proximal colon. In general, starting from the duodenum, a cell gradient of the absolute cell number occurred with an increase towards the proximal colon and a further decrease to the distal colon (stomach 15.40 ± 1.83, duodenum 1.40 ± 0.93, jejunum 6.60 ± 0.51, ileum 12.60 ± 1.63, caecum 12.80 ± 1.11, proximal colon 16.60 ± 1.91, distal colon 8.60 ± 0.60).

nNGFP cells could not be found in every single ganglion in either postnatal or adult mice (Fig. 3c, f). In parallel to the distribution of the absolute numbers of nNGFP cells, the percentages of nNGFP-harbouring ganglia in postnatal mice showed a similar distribution. Moreover, the lowest percentage of ganglia containing nNGFP cells was found in the duodenum (14.00 ± 9.27 %), whereas up to 72 % of the ganglia (stomach 66.00 ± 6.78 %, jejunum 48.00 ± 2.00 %, ileum 64.00 ± 6.00 %, caecum 72.00 ± 3.74 %, proximal colon 72.00 ± 3.74 %, distal colon 60.00 ± 4.47 %) contained nNGFP cells in the rest of the gastrointestinal tract in postnatal mice.

The distribution of the nNGFP cells in the adult gut was different from that seen in the postnatal gut. The proportion of nNGFP/PGP9.5-positive cells within the overall NGFP population showed a slight increase from the duodenum to the

Fig. 1 S100 and glial fibrillary acidic protein (GFAP) expression of nestin-expressing cells in the myenteric plexus. Nestin-green fluorescent protein (*Nestin-GFP*; **a–d**) cells expressed S100 (**a', b', a", b"**) and GFAP (**c', d', c", d"**) in the jejunum (*JE*) of postnatal and adult mice as shown by immunofluorescence staining (*grey arrows* double-positive cells, *blue arrows* S100-positive cell with neuronal size and morphology, *DAPI* blue nuclear staining). *n* = 5. Magnification ×200. *Bar* 50 μm
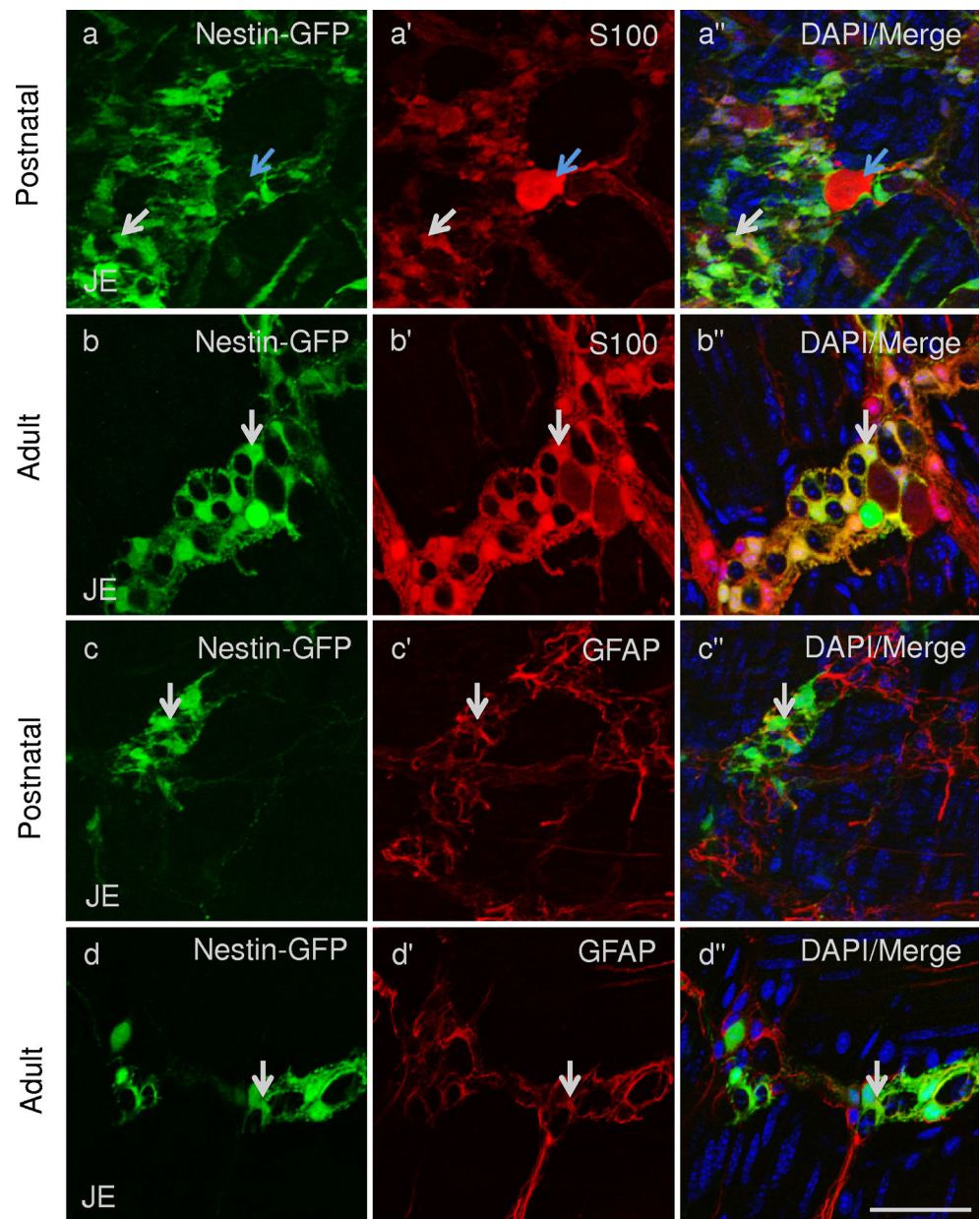

distal colon but at very low rates (stomach 0.00 %, duodenum 0.89 ± 0.89 %, jejunum 0.48 ± 0.48 %, ileum 0.90 ± 0.38 %, caecum 1.16 ± 0.48 %, proximal colon 2.10 ± 0.71 %, and distal colon 2.13 ± 0.73 %). The highest proportion of nNGFP cells could be found in the distal colon (2.1 ± 0.7 %, Fig. 3d), whereas the highest absolute cell numbers could be seen in the proximal colon (8.0 ± 2.86 %, Fig. 3e).

Although the distribution patterns of absolute cell numbers (stomach 0.00, duodenum 0.40 ± 0.40, jejunum 0.40 ± 0.40, ileum 1.0 ± 0.45, caecum 2.40 ± 0.93, proximal colon 8.00 ± 2.86, distal 3.20 ± 1.02) and percentages of nNGFP-containing ganglia (stomach 0.00 %, duodenum 4.00 ± 4.00 %, jejunum 4.00 ± 4.00 %, ileum 10.00 ± 4.47 %, caecum 18.00 ± 6.63 %, proximal colon 40.00 ± 12.25 %, distal colon 26.00 ± 8.12 %) were very similar (Fig. 3e, f), their absolute abundance was much lower in adult mice compared with postnatal mice.

A more detailed analysis of the phenotype of nNGFP cells was performed in the caecum in postnatal and adult mice. The caecum was chosen because of its enormous content of microbiota. Whereas chemical codings had been revealed previously in the small intestine, the caecum was neglected (Qu et al. 2008). A subgroup of nNGFP cells was found to be positive for nNOS (Fig. 4) in postnatal mice. In the adult caecum, the nNGFP cells were generally positive for nNOS. Moreover, we investigated the neuronal subtype markers calbindin and calretinin (Fig. 5). Calbindin was clearly detected only in one single cell within the analysed nNGFP cells in all five investigated postnatal mice. Only four nNGFP cells were positive for the neuronal marker calretinin in two of the five

Fig. 2 Nestin-GFP cells express neuronal markers (nNGFP) in the myenteric plexus of postnatal and adult caecum. As shown by immunofluorescence staining, the nNGFP cells (**a–d**) were positive (**a"–d"**) for the pan neuronal markers PGP9.5 (**a', b'**) or HuC/D (**c', d'**) in the caecum (*CA*) of postnatal and adult mice (*grey arrows* double-positive cells). PGP9.5 $n = 5$, HuC/D $n = 3$. Magnification ×200. *Bar* 50 μm
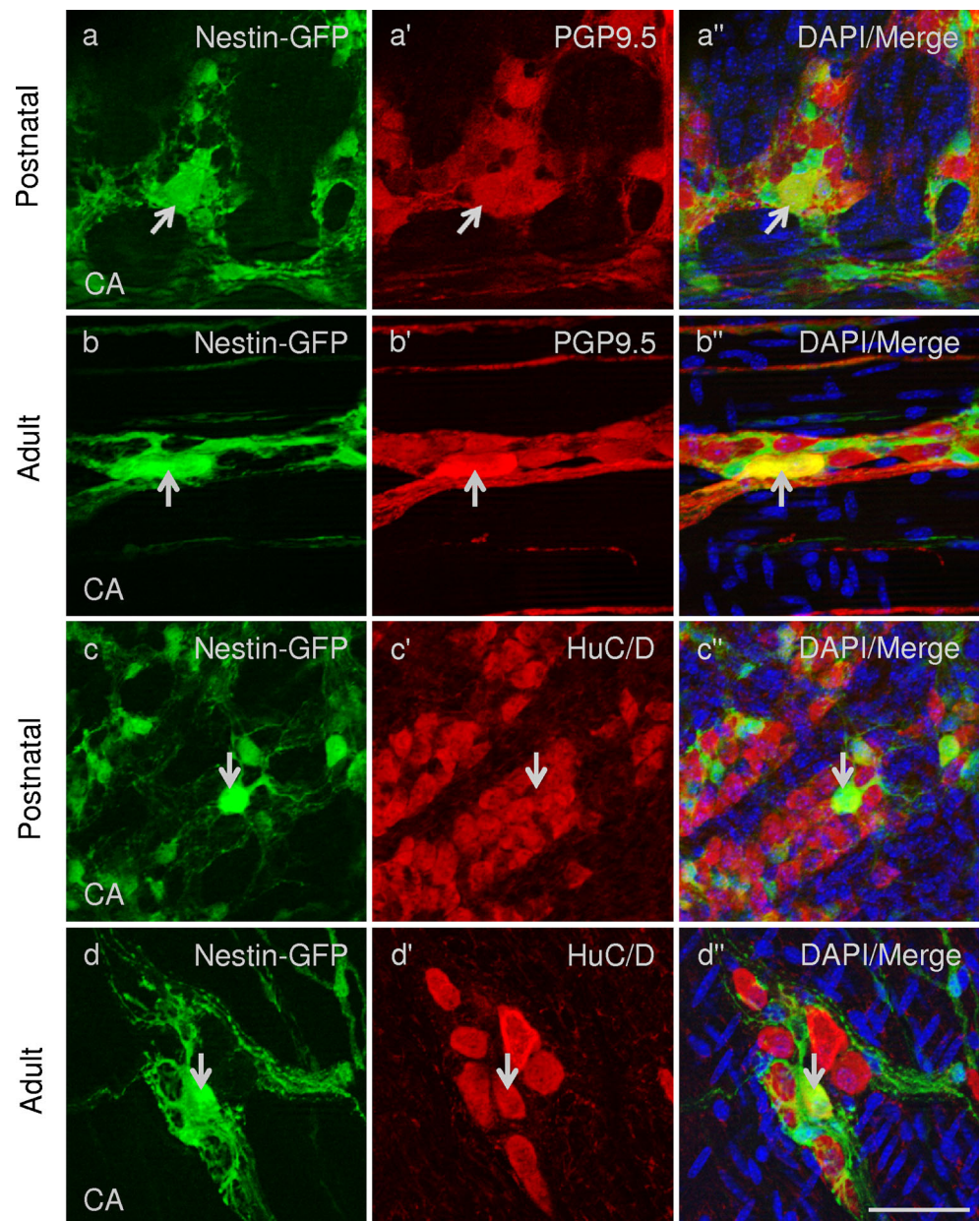
investigated postnatal mice. Adult nNGFP cells were partly positive for both neuronal markers calbindin and calretinin, especially when the nNGFPs were located within small myenteric ganglia, in all analysed mice. Neither of the glial markers S100 and GFAP were expressed by nNGFP cells (Fig. 6). Immunofluorescence staining against Ki67 revealed that some of the NGFP cells were in a proliferating stage during postnatal development, whereas the NGFP cells had left the cell cycle at the adult stage (Fig. 7).

Based on a detailed investigation of the S100-positive glial cells throughout the gastrointestinal tract, we were able to find a subset of cells that combined glial staining for S100 with a neuronal morphology (nS100) mainly in postnatal mice. The nS100 cell morphology was predominantly characterised by a large cell body and up to two axons (Fig. 8). The nS100-

positive cells were negative for the neuroepithelial stem cell marker nestin, as revealed by the transgenic NGFP mouse model (Fig. 1). The neuronal phenotype was confirmed by the expression of the early pan neuronal marker PGP 9.5 (Fig. 8) in the jejunum and distal colon. The nS100 cells could mainly be found in myenteric ganglia of the jejunum and, to a lesser extent, in the duodenum and transitional zones towards the ileum and in the distal colon. The nS100 cells were localised within or associated with the enteric ganglia. The overall amount of nS100 cells did not even reach 2 % of the total S100 glial population (Fig. 8). The maximum percentages within the S100 population could be seen in the jejunum (1.16 ± 0.15 %). The duodenum and jejunum/ileum transition zone showing similar values (0.79 ± 0.06 % in the duodenum, 0.77 ± 0.10 % in the jejunum/ileum transition zone). In the
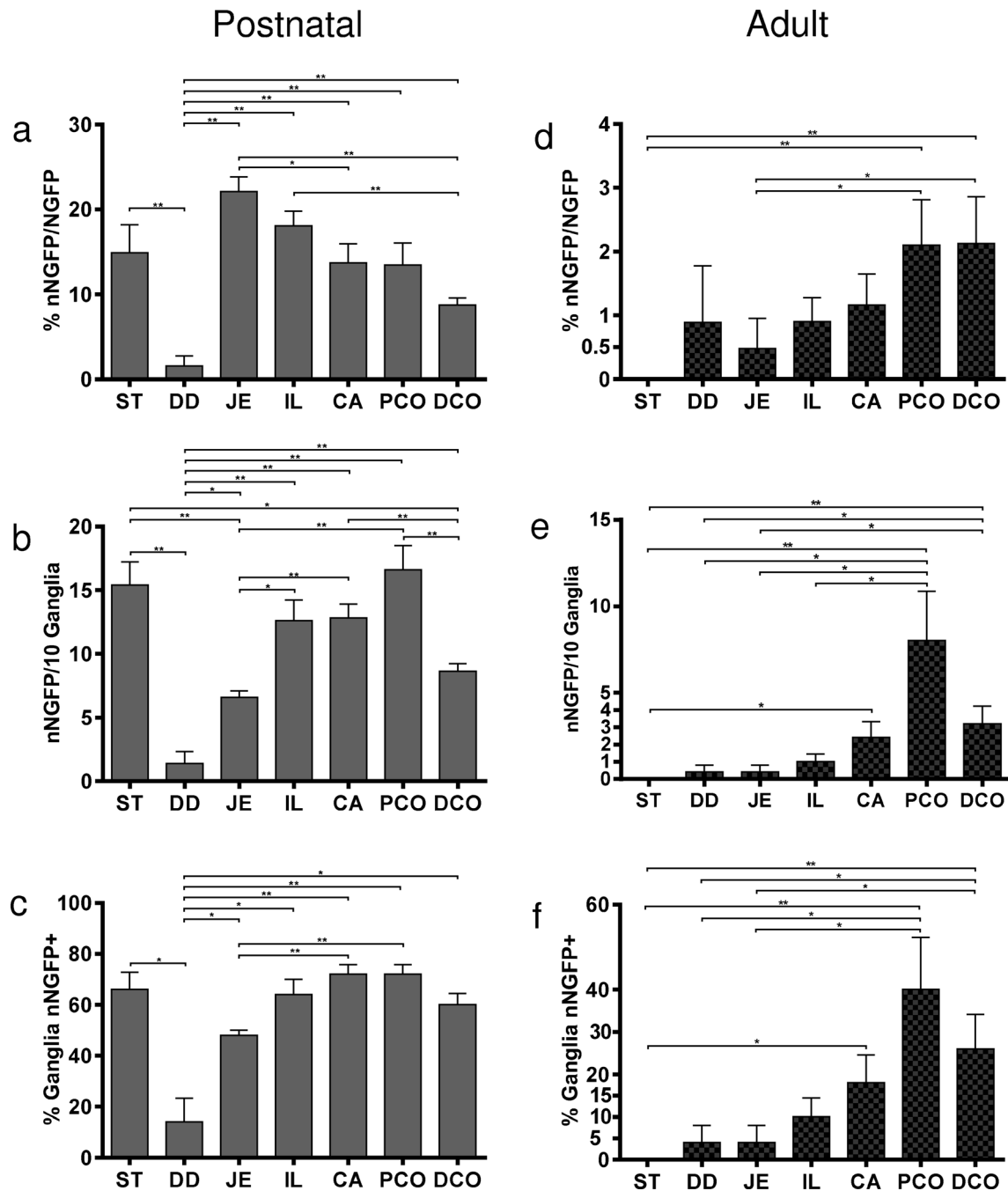
Postnatal

Adult

Fig. 3 Distribution pattern of NGFP-expressing neuronal cells (*nNGFP*) in the gastrointestinal tract. The proportion of nNGFP cells within the whole NGFP population (**a**, **d**), the absolute cell number (**b**, **e**) and the percentage of nNGFP+ cells per ganglia (**c**, **f**) were quantified per 10 ganglia of the stomach (*ST*), duodenum (*DD*), jejunum (*JE*), ileum (*IL*),

caecum (*CA*), proximal colon (*PCO*) and distal colon (*DCO*) of 5 post-natal (**a**–**c**) and adult (**d**–**f**) mice. nNGFP cells were mostly present in the proximal colon at both ages, with a decrease of nNGFP cells from the postnatal to adult stage. Means ± SEM, *P < 0.05, **P < 0.01, n = 5

large intestine, these cells could only be detected in small percentages in the distal half of the colon (0.18 ± 0.06 %).

Additionally, the absolute number of these cells was analysed (Fig. 8) in order to exclude a strong influence of the increase of the S100 cells per 50 ganglia from the proximal small intestine to the distal colon. The absolute number of nS100 cells increased from the duodenum (5.6 ± 0.61) to the

jejunum (9.60 ± 1.03). In the jejunum/ileum transition zone, the absolute number was again reduced (7.20 ± 0.86) compared with the jejunum. Whereas no nS100 cells could be found in the terminal ileum and proximal colon, they reappeared in the distal half (1.8 ± 0.66) of the colon. The distribution patterns of both the absolute cell numbers and percentages of nS100-containing ganglia were identical and

Fig. 4 Neuronal nitric oxide synthase (*nNOS*) is expressed in Nestin-GFP-expressing neuronal cells (nNGFP) in the myenteric plexus of postnatal and adult caecum (*CA*). The nNGFP cells (**a**, **b**) were at least partly positive (**a"**, **b"**) for the neuronal marker nNOS (**a'**, **b'**) in the caecum of postnatal mice as shown by immunofluorescence staining. The analysed nNGFP cells showed general expression of nNOS in the adult caecum (*grey arrows* double-positive cells). *n* = 3. Magnification ×200. *Bar* 50 μm
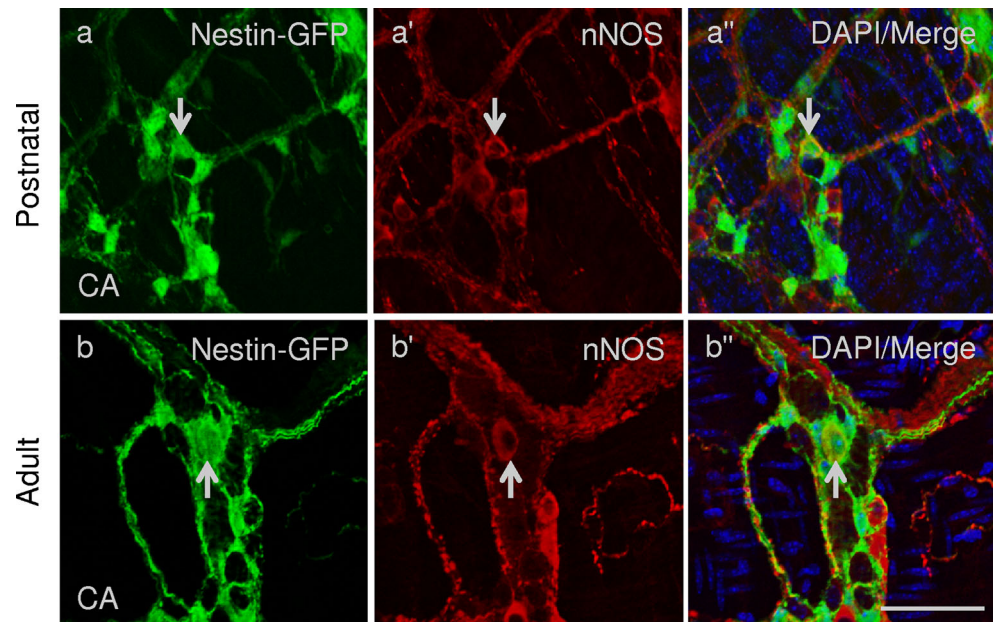
were similar to the percentage of nS100 within the S100 population. The percentages of nS100-containing ganglia were 10.0 ± 0.63 % in the duodenum, 18.4 ± 2.5 % in the jejunum, 13.60 ± 1.47 % in the jejunum/ileum transition zone and 3.60 ± 0.75 % in the distal colon (Fig. S5).

For a detailed phenotypic analysis, we used tissue from the jejunum. The highest expression of nS100 cells was found in this region. In addition to PGP9.5, the nS100 cells expressed the pan neuronal marker HuC/D (Fig. 9). Because of the filamentous expression pattern of GFAP by immunostaining, clear colocalisations with S100 were often difficult to find (Fig. 9). We therefore additionally used the hGFAP-GFP transgenic mouse model to confirm the findings of the immunofluorescence staining. Here, we did not see any colocalisation of GFAP and S100 within the nS100 cells (Fig. 9). In postnatal mice, the presence of Sox2 and Sox10 was reported in glial cells and neural precursor cells (Heanue and Pachnis 2011; Laranjeira et al. 2011). With our staining method, nS100 cells were negative for both the glial/progenitor cell markers Sox2 and Sox10 (Fig. 10).

Moreover, nS100 cells were found at a later postnatal stage at 11 days after birth (Fig. S6). In the adult mouse, nS100 cells were only rarely found in the jejunum and caecum. Colocalisation with PGP9.5 in the caecum could be demonstrated, as could the lack of NGFP expression in the jejunum (Fig. S6).

Discussion

Although the neural stem cell marker nestin can be found in enteric glial cells in both postnatal and adult MP, a subset of neurons also shows strong NGFP expression. The presence of nNGFP cells demonstrates a continuous supply with new differentiated neurons after birth. The morphology of the postnatal nNGFP cells differs amongst each other indicating that several neuronal subtypes can differentiate from the nNGFP population. The number and phenotype of neurons within the ENS is most probably influenced by the bacterial population. Recently, neuronal numbers have been demonstrated to be reduced in the ileum but not in the duodenum of germ-free mice. The duodenum rarely harbours bacteria (Collins et al. 2014). These findings correspond to our data in which NGFP cells that express a neuronal character are rarely to be found in the duodenum but are increased in the jejunum and ileum. The high number of nNGFP cells in the postnatal stomach can also be explained by the influence of bacteria that enter the stomach via breast milk (Jost et al. 2013, 2015). In this study, we used the caecum for a detailed investigation of the nNGFP cells due to the high number of nNGFP cells and because the caecum of mice more closely resembles the colon of humans with regard to many important properties. The fermentation by bacteria leading to the production of metabolites such as short-chain fatty acids takes place in the caecum in mice and in the colon in humans (Nguyen et al. 2015). In general, we found the neuronal subtype markers calbindin, calretinin and nNOS in the caecum, as described in mouse small intestine by Qu et al. (2008). Only very small amounts of calretinin- and calbindin-positive nNGP cells were present in the postnatal caecum. The decrease of the nNFGP cells and nNGFP-positive ganglia from postnatal to adult stage and the increase of, for example, calbindin provide evidence that neuronal differentiation occurs from postmitotic NGFP-positive neuronal progenitor cells at postnatal time points. This is supported by the finding that nearly all NGFP cells have left the cell cycle at the postnatal stage (P7) and that no proliferating

Fig. 5 Calbindin and calretinin are expressed in Nestin-GFP-expressing neuronal cells (nNGFP) in the myenteric plexus of postnatal and adult caecum (*CA*). The nNGFP cells (**a–d**) rarely express (**a"**, **b"**) calbindin (**a'**) and calretinin (**b'**) at the postnatal stage as shown by immunofluorescence staining. Calbindin (**c'**) and calretinin (**d'**) expression within the nNGFP (**c**, **d**) population was increased (**c"**, **d"**) in the adult mice (*grey arrows* double-positive cells). *n* = 5. Magnification ×200. *Bar* 50 μm
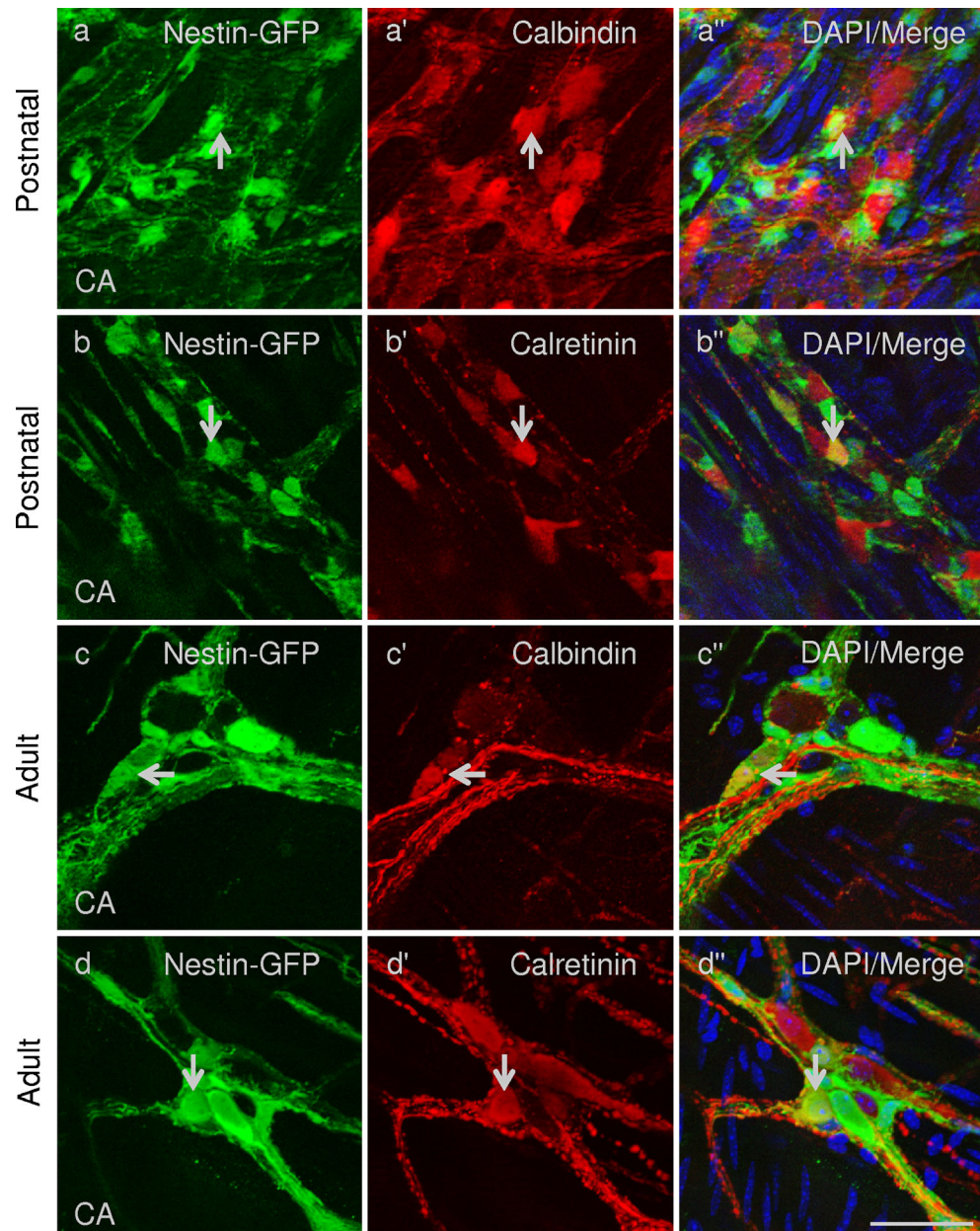

NGFP cells were found at the adult stage. We therefore suggest that NGFP in neurons in our transgenic mouse model represents a marker for plasticity during differentiation and not mandatorily the proliferative potential of these cells. This is in agreement with a study that revealed the expression of nestin in neurons derived from P19 cells and primary cerebellar granule cells from postnatal day 6 mice in vitro. Nestin is localised in the growth cones and is suggested to play a role in growth cone guidance during axon elongation (Yan et al. 2001). Moreover, the presence of nestin in non-dividing cells has also been shown in brain regions that are essential for cognitive functions. Here, the cells need an extreme cellular plasticity (Hendrickson et al. 2011). Another molecule that is related to plasticity and development is growth-associated-

protein-(GAP)-43. Whereas GAP-43 is found throughout the developing nervous system, it is found only in the hippocampus and also in the ENS, both regions with high plasticity, in the adult animal (Stewart et al. 1992).

Interestingly, some evidence has been presented that nestin has further distinct functional roles in addition to being involved in the survival of neural stem cells, i.e., in the brain (Park et al. 2010). Nestin has been demonstrated to have a key role in the regulation of Cdk5 kinase activity, which is associated with the maintenance and dispersion of acetylcholine receptors during development and in the adult mouse. Acetylcholine receptor clusters and the endplate architecture are altered in the neuromuscular junction area of nestin-deficient mice (Mohseni et al. 2011). Nestin might have

Fig. 6 Nestin-GFP-expressing cells with a neuronal morphology (nNGFP) expressed neither S100 nor GFAP in the myenteric plexus of postnatal and adult caecum (*CA*). nNGFP (**a–d**) cells did not express S100 (**a'**, **b'**, **a''**, **b''**) and GFAP (**c'**, **d'**, **c''**, **d''**) in postnatal and adult mice as shown by immunofluorescence staining (*grey arrows* nNGFP cells). *n* = 5. Magnification ×200. *Bar* 50 μm
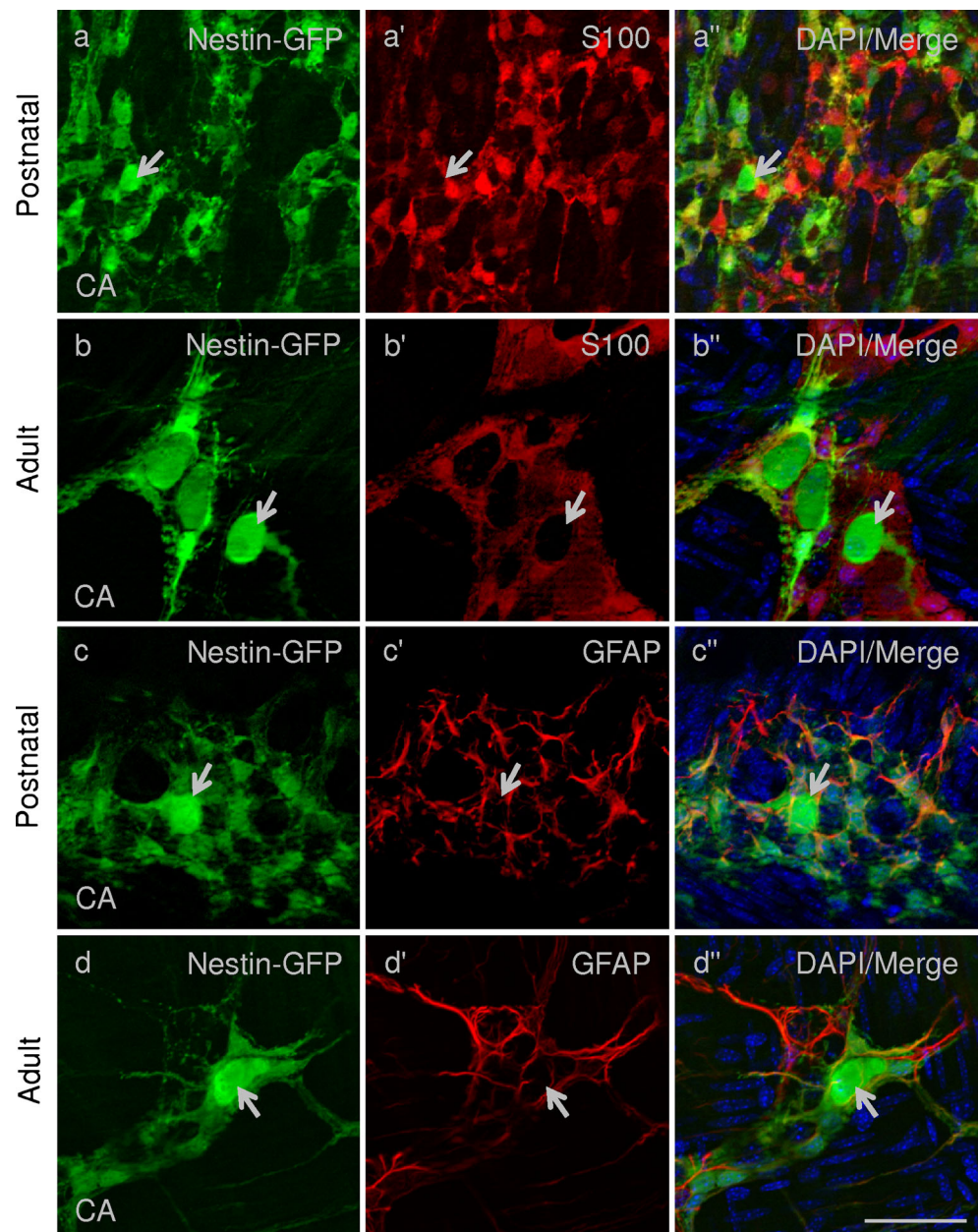
similar functions in the ENS by controlling the formation of neural circuit structures.

Nestin and PGP9.5 double-positive cells have previously been described in the embryonic MP of human tissue, being considered as a premature neuronal cell population (Rauch et al. 2006). Moreover, these cells have been shown to appear in the SMP but not in the MP of the human adult ENS (Azan et al. 2011). These findings might be related to the SMP being located much nearer to the gut lumen and, hence, the probability of a potential influence by environmental challenges or microflora changes is much greater. nNGFP cells were also found in the SMP in our transgenic NGFP mouse model (Fig. S4).

This study focused on neuronal differentiation but additional initial data indicate a higher number of NGFP positive enteric glial cells in areas with a higher amount and diversity of intestinal bacteria in adult mice (Fig. S7). Whether nNGFP cells are derived from NGFP glial cells or represent a distinct lineage of premature neurons derived from neural crest stem cells remains to be elucidated. The differentiation of neurons from nestin-expressing cells or glial cells in vitro has been shown by several studies (Belkind-Gerson et al. 2013; Suarez-Rodriguez and Belkind-Gerson 2004).

Furthermore, the postnatal MP harbours a group of S100 cells with neuron-glial intermediate properties (nS100) mainly in the MP of the duodenum, jejunum and transition zones towards the ileum and in the distal half of the colon. This very small subpopulation of S100-expressing cells support the finding that enteric

Fig. 7 Analysis of the proliferation of Nestin-GFP expressing cells in the myenteric plexus of postnatal and adult caecum (*CA*). Immunofluorescence staining against the proliferation marker Ki67 (**a'**, **b'**) revealed that postnatal Nestin-GFP-expressing cells (**a**, **b**) are rarely Ki67-positive (**a''**), whereas adult Nestin-GFP-expressing cells had left the cell cycle (**b''**). *Grey arrows* indicate a Nestin-GFP and Ki67 double-positive cell. Magnification ×200. *Bar* 50 μm
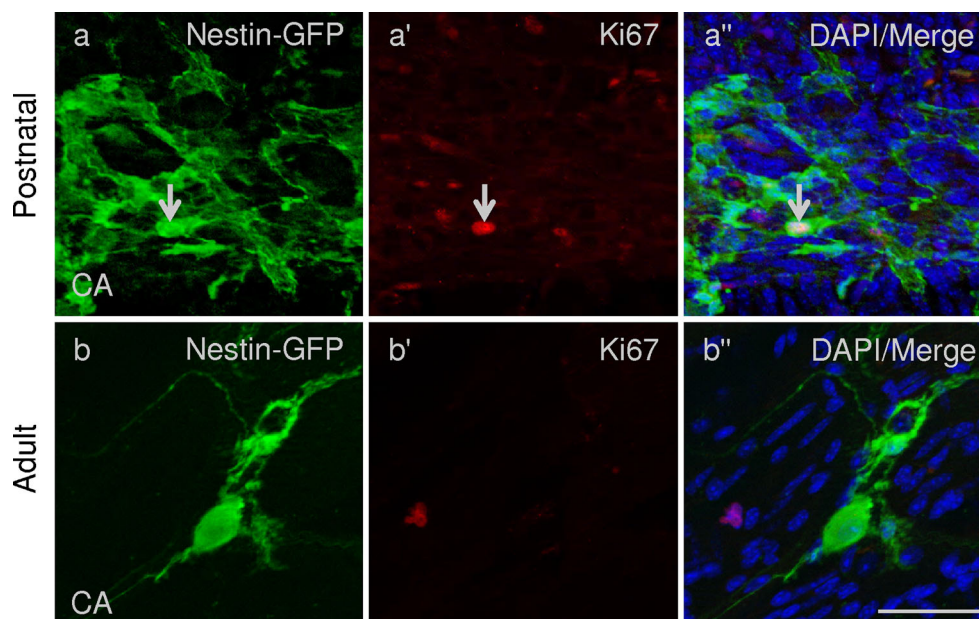
glial cells or SPCs serve as enteric neuronal precursor cells during homeostasis (Joseph et al. 2011; Uesaka et al. 2015) in the postnatal period when the development of the ENS is most probably influenced by environmental factors.

Uesaka et al. (2015) pointed out that further investigations are necessary to show whether differentiated Schwann cells can form neurons. Because S100 is not present on SPCs but on immature Schwann cells (Jessen and Mirsky 2005), the nS100 cells in our study might be immature Schwann cells that are derived from Dhh-positive SPCs.

In addition, enteric glial cells have recently been shown to have a different expression pattern of S100 and GFAP representing the high plasticity of enteric glial cells in vivo (Boesmans et al. 2014). S100-positive cells without GFAP expression have been found. Moreover, S100 is an earlier glial marker (Young et al. 2003) compared with GFAP (Rothman et al. 1986) and GFAP is considered as a marker for mature glial cells in the ENS (Jessen and Mirsky 1980). Probably, a subpopulation of S100-positve/GFAP-negative enteric glia cells represents a more premature stage of enteric glial cells with a neuronal linage potential. The absence of the progenitor markers nestin, Sox2 and Sox10 leads to the assumption that these cells have lost their proliferative potential and present instead a postmitotic progenitor cell line that undergoes neuronal transdifferentiation, accompanied by the expression of the two general neuronal markers PGP9.5 and HuC/D.

The nS100 cells are not evenly distributed along the gut axis. These cells were mainly found in the proximal half of the small intestine. The enzymatic breakdown and resorption of nutrients take place in the proximal small intestine.

These regions are also the areas with no or reduced microflora (Hayashi et al. 2005). nS100 cells might be generated in response to the absorption of nutrients in the small intestine at the postnatal stage. Evidence has been presented that breast milk proteins induce cellular mechanisms that can influence the enteric neural cells in vitro. Neurotrophic factors and cytokines from breast milk might serve as neuron-promoting factors leading to the glia-neuron switch (Fichter et al. 2011). Whether factors such as ciliary neurotrophic factor (CNTF) or glial-derived neurotrophic factor (GDNF), which have been found in breast milk, take part in these processes has still to be elucidated. GDNF, as found in breast milk but also in enteric glia in Crohn's disease, might be a potential candidate (von Boyen and Steinkamp 2011). Probably, a relationship exists between GDNF and the newborn neurons that arise from postmitotic cells in Crohn's disease (Geboes and Collins 1998).

In addition to the low intestinal distribution of the nS100 cells, an even smaller amount of nS100 cells is present in the distal colon. Whether this finding is related to the different functional aspects of the distal colon or to specific microbiome patterns or whether it is a sign of ongoing development in the ENS has to be investigated.

Single nS100 cells can be found in the postnatal proximal colon and stomach. Especially in the adult gut, this seems to be rather a focused event in response to local challenges, such as inflammation or the replacement of single neurons, because the gastrointestinal tract represents an important entry site for pathogens. Because of their morphologic properties, some of the nS100 cells could develop intrinsic primary afferent
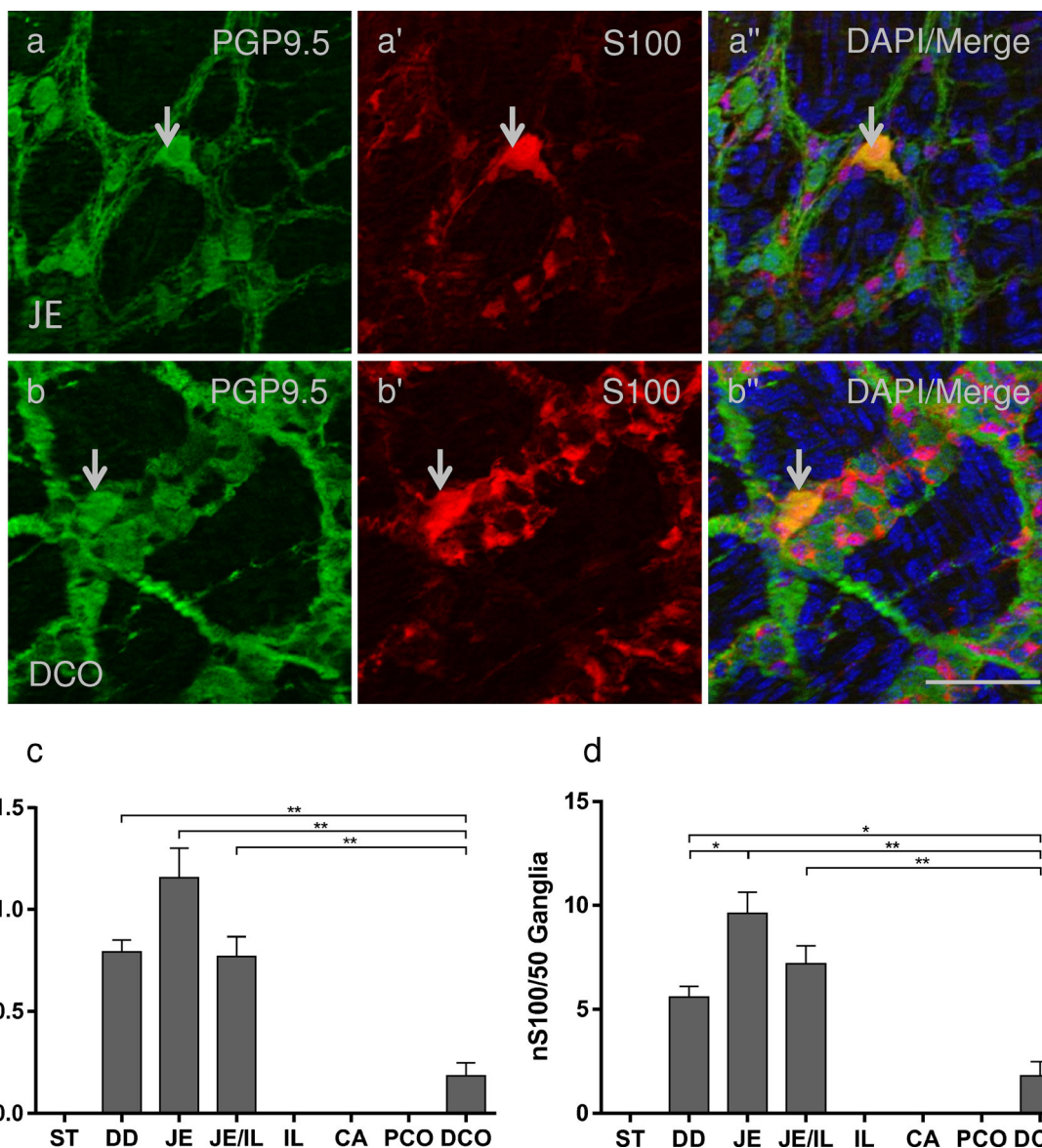
Fig. 8 Glia-neuron intermediate cells are present in the myenteric plexus in postnatal mice. Immunofluorescence staining showing a small number of S100 cells with a neuronal morphology (nS100) located mainly in the jejunum (*JE*) but with a lower frequency also in the distal half of the colon (*DCO*) of postnatal mice. These cells expressed the pan neuronal marker PGP9.5 (**a–a"**, **b–b"**). The proportion within the S100 population (**c**) and the absolute number (**d**) of nS100 cells were quantified within 50 ganglia. The stomach (*ST*), duodenum/jejunum (*DD/JE*), jejunum (*JE*), jejunum/ileum (*JE/IL*), ileum (*IL*), caecum (*CA*), proximal (*PCO*) and distal colon (*DCO*) were analysed in five postnatal mice. The proportion within the S100 population was approximately 1 % in the duodenum, jejunum and jejunum/ileum and decreased significantly to the distal colon. The highest absolute cell number of nS100 cells was found in the jejunum and was significantly decreased in duodenum/jejunum and distal colon. In the stomach, caecum and proximal colon, nS100 cells were not detected in the analysed ganglia (*grey arrows* double-positive cells). Means ± SEM, *$*P < 0.05$, **$**P < 0.01$. PGP9.5 staining: jejunum $n = 5$, distal colon $n = 3$. *Bar* 50 μm

neurons (IPANs) in response to environmental stimuli. IPANs are described as having large cell bodies and two axons in the small intestine (Qu et al. 2008).

In conclusion, NGFP in enteric neurons in our transgenic mouse model represents a marker for neuronal plasticity rather than stemness, especially at the postnatal stage when the enteric neurons undergo differentiation as shown above. Our data support recent findings that environmental factors such as the microbiome strongly influence neuronal differentiation and plasticity. Immunohistochemical analysis demonstrated the expression of S100 in cells with neuronal properties. Our data support recent findings that SPCs or enteric glial cells transdifferentiate into neurons at postnatal stages under physiological conditions or following intestinal disease or injury (Belkind-Gerson et al. 2015; Joseph et al. 2011; Laranjeira et al. 2011; Uesaka et al. 2015).

Fig. 9 S100 cells with a neuronal morphology (nS100) expressed a neuronal phenotype in the myenteric plexus of the jejunum (*JE*) in postnatal mice. Immunofluorescence staining against S100 in combination with the pan neuronal marker HuC/D (**a–a”**) confirmed the neuronal phenotype of the nS100 cells. Immunofluorescence staining against S100 in combination with the reactive glial marker GFAP (**b–b”**) showed no coexpression. Moreover, the hGFAP-GFP transgenic mouse model (**c–c”**) gave identical results (*grey arrows* nS100 cells). HuC/D and GFAP *n* = 5, hGFAP-GFP *n* = 3. *Bar* 50 μm
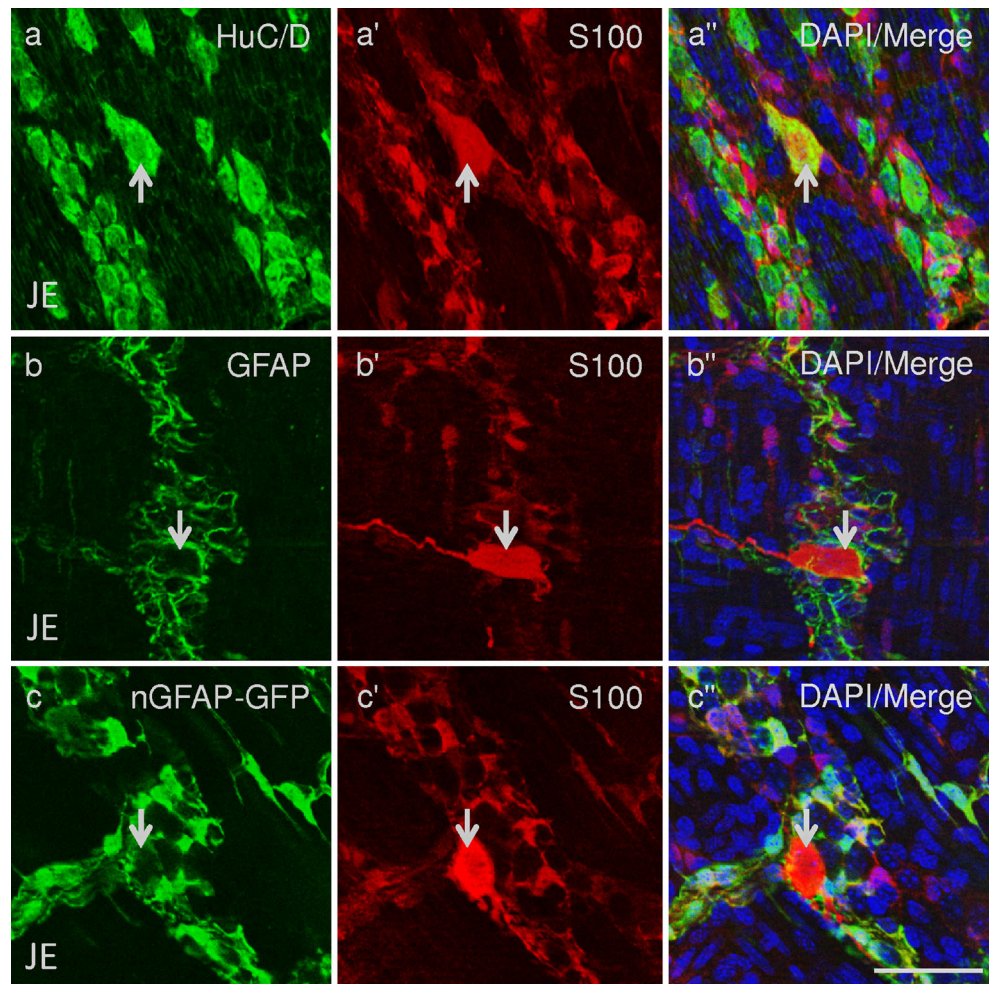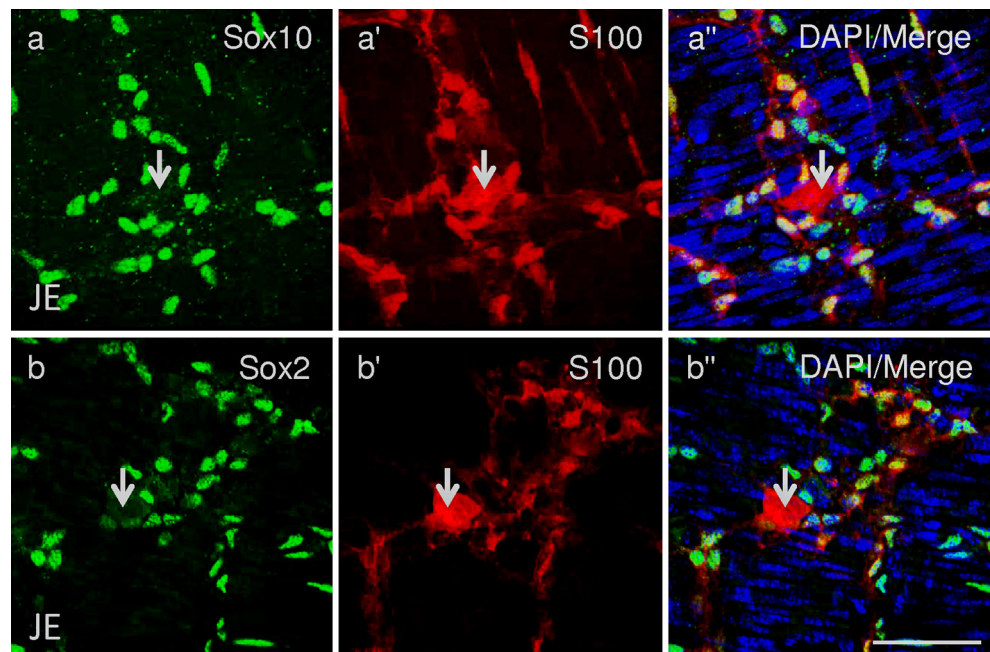

Fig. 10 In postnatal mice, S100 cells with a neuronal morphology (nS100) expressed no glial progenitor marker in the myenteric plexus of the jejunum (*JE*). Immunofluorescence staining against the progenitor marker Sox10 (**a–a”**) and Sox2 (**b–b”**) in combination with the glial marker S100 showed no coexpression within nS100 cells (*grey arrows* nS100 cells). *n* = 5. *Bar* 50 μm

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