Program # 639.1

While morphology and development of the zebrafish enteric nervous system (ENS) are known, neurochemical coding of enteric neurons is still incomplete. Using immunofluorescence, we aimed to unravel the neurochemical coding of zebrafish enteric neurons. Tyrosine hydroxylase (TH), VIP and PACAP were observed in nerve fibres, while other markers were detected in neuronal cell bodies. In embryos, all markers, except for choline acetyltransferase (ChAT) and TH, were present from 72 hpf on. Nitrergic neurons, which were evenly distributed and remained constant in time, constituted a major subpopulation. The neuronal proportions of the other markers increased during development. These results support previous data that the ENS is well-developed before the onset of feeding. In the adult, all markers were expressed in the ENS. A major percentage of neurons displayed calbindin and calretinin, while serotonin was the only marker showing significant distribution differences in the three intestinal regions. Serotonin was not coexpressed with any of the other markers. At least five neuronal subpopulations were determined. A functional classification of the subpopulations has been suggested.

Neurochemical coding of enteric neurons in embryonic and adult zebrafish

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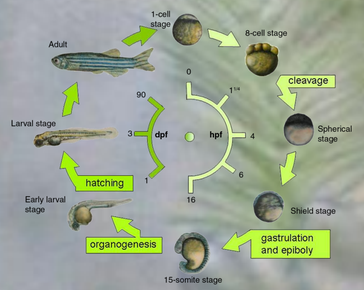
Introduction

Zebrafish emerged as a model organism in experimental research, including studies of congenital gastrointestinal diseases. While general morphology and development of the enteric nervous system (ENS) are already known, knowledge concerning the specific characteristics of enteric neurons is still incomplete. We aimed to unravel the neurochemical coding of specific subpopulations of zebrafish enteric neurons.

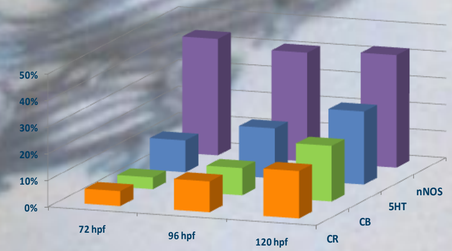


Methods

Using immunological staining methods on isolated intestines from adult and larval (72-96-120 hpf) zebrafish, we demonstrated and quantified the expression of different neurochemical markers in the three functional intestinal segments: the proximal, middle and distal intestine (PI; MI; DI). An antibody against Hu was used as a pan-neuronal marker.

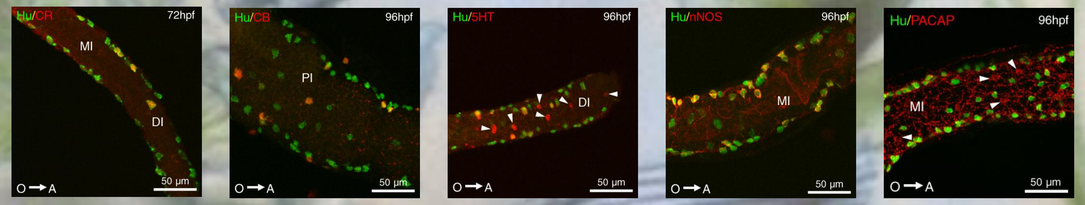
Abbreviations
5HT: serotonin; CB: calbindin; ChAT: choline acetyl transferase; CR: calretinin; DI: distal intestine; dpf: days post-fertilization; ENS: enteric nervous system: hpf: hours post-fertilization; IR: immunoreactivity; MI: middle intestine; nNOS: neuronal nitric oxide synthase; PACAP: pituitary adenylate cyclase activating peptide; PI: proximal intestine; VIP: vasoactive intestinal peptide

Neurochemical coding in zebrafish embryo's

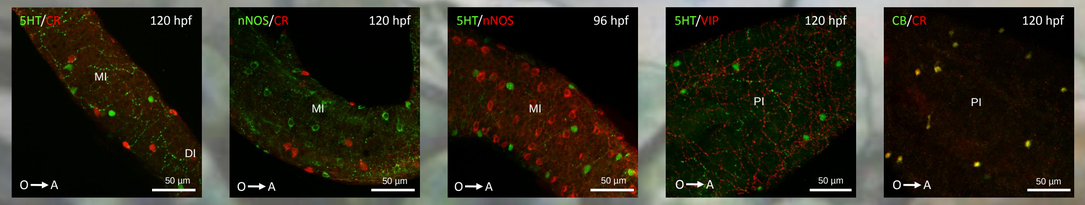


	72 hpf	96 hpf	120 hpf
CR	6%	12%	18%
CB	4%	10%	21%
5HT	12%	19%	27%
nNOS	44%	41%	42%

CR-, CB-, 5HT- and nNOS IR were present from 72 hpf on in neuronal cell bodies. More than 40% of the enteric neurons were nNOS-positive, their proportion remaining constant over time. The proportions of CR, CB and 5HT neurons increased in the later stages. In the intestinal wall, Hu-negative 5HT-positive cells (*arrowheads*), being enteroendocrine cells, were also found. VIP and PACAP IR showed a granular appearance and was only detected in enteric nerve fibers from 72 hpf on and in some mucosal cells (*arrowheads*).



Multiple immunolabelings using different neuronal markers revealed a complete overlap between CB and CR, but no overlaps were found between the other neuronal markers.

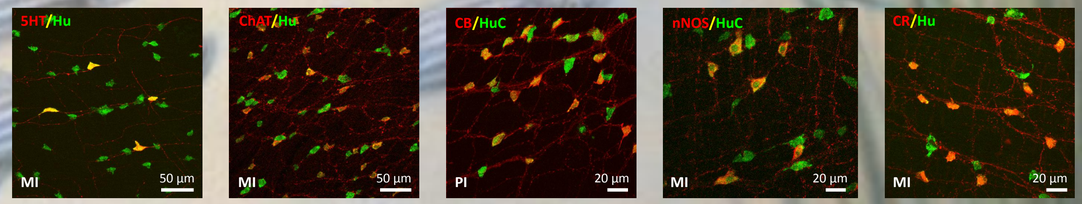


Conclusion:

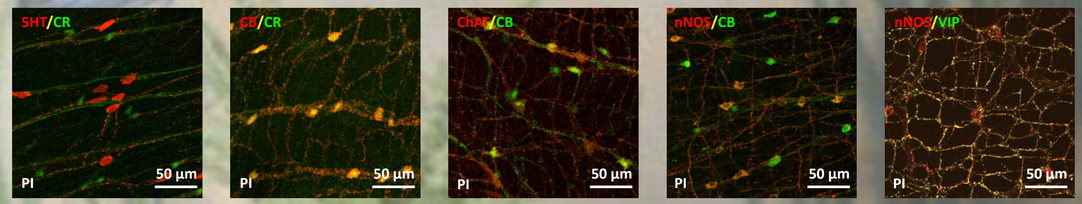
The results indicate that at the beginning of enteric neuronal differentiation, several neuronal markers are expressed. They are suggested to play a role in the spontaneous motility activity of the gut between hatching (2-3 dpf) and first feeding (5-6 dpf). These data support previous reports that the ENS is well-developed before the onset of feeding. nNOS has been suggested to play a role in the development of the ENS.

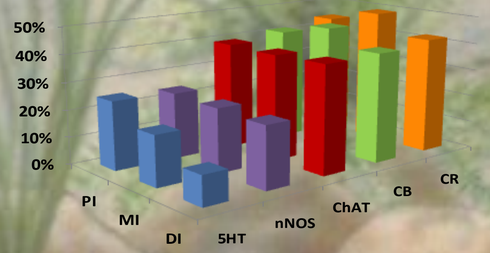
Neurochemical coding in adult zebrafish

IR for ChAT, CR, CB, 5HT and nNOS was predominantly found in neuronal cell bodies. The proportion of nNOS, ChAT, CB and CR showed no regional differences, while the percentage 5HT-positive neurons significantly decreased along the length of the intestine.



Quantification and combination of these neuronal markers revealed five different subpopulations. VIP and PACAP IR were only observed in varicosities of nerve fibres; showing a complete overlap and colocalization with nNOS.





Proportions of neurochemical contents expressed in the ENS of adult zebrafish

Colocalization with...	CR	CB	ChAT	nNOS	5HT
CR	---	100%	±94%	100%	0%
CB	100%	---	±93%	100%	0%
ChAT	±85%	±86%	---	±11%	0%
nNOS	±39%	±37%	±7%	---	0%
5HT	0%	0%	0%	0%	---

Percentages of colocalization between the different markers tested

Conclusion:

At least five neuronal subpopulations were determined: a serotonergic (5HT *± 18%*), a nitrergic non-cholinergic (CB/CR/nNOS *± 15%*), two cholinergic non-nitrergic subpopulations (ChAT *± 5%*; CB/CR/ChAT *± 35%*), and one subpopulation expressing both ChAT and neuronal nitric oxide synthase (CB/CR/ChAT/nNOS *± 3%*).

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