Developmental pattern of three vesicular glutamate transporters in the myenteric plexus of the human fetal small intestine

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Summary. Three vesicular glutamate transporters (VGLUT1-3) have previously been identified in the central nervous system, where they define the glutamatergic phenotype, and their expression is tigtly regulated during brain development. In the present study we applied immunocytochemistry to examine the distribution of the immunoreactivity of all three VGLUTs during prenatal development of the myenteric plexus in the human small intestine. We also investigated changes in their localization in the different segments of the small intestine and in the different compartments of the developing myenteric ganglia. Immunoreactivity against all three VGLUTs was found predominantly in the ganglionic neuropil, interganglionic varicose fibers and perisomatic puncta, but cytoplasmic labeling with different intensities also occurred. Each transporter displayed a characteristic spatiotemporal expression pattern, with the transient increase or decrease of immunoreactive cell bodies, varicosities or perisomatic puncta, depending on the fetal age, the gut segment or the ganglionic compartment. Throughout gestational weeks 14-23, VGLUT1 immunoreactivity always predominated over VGLUT2 immunoreactivity, though both peaked around week 20. VGLUT3 immuno-reactivity was less abundant in the developing myenteric plexus than those of VGLUT1 and VGLUT2 immunoreactivity. It was mainly expressed in the ganglionic neuropil and in the perisomatic puncta throughout the examined gestational period. Neuronal perikarya immunoreactive for VGLUT3 were restricted to between weeks 18 and 20 of gestation and exclusively to the oral part of the small intestine.

Key words: VGLUT1-3, Myenteric plexus, Human, Fetal development

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Introduction

There is increasing evidence that glutamate (Glu), the major central excitatory neurotransmitter, also plays a role in the enteric nervous system (ENS) (Kirchgessner, 2001). Glu is first synthesized in the presynaptic cytoplasm before being loaded into the synaptic vesicles. The synaptic vesicle loading is mediated by transport proteins known as vesicular Glu transporters (VGLUTs). VGLUT1 was first recognized as a brain-specific inorganic phosphate transporter protein (Ni et al., 1994; Bellocchio et al., 1998) and was later characterized as the first VGLUT (Bellocchio et al., 2000; Takamori et al., 2000). To date, two other isoforms, VGLUT2 and VGLUT3, have been identified and functionally characterized in mammals (Aihara et al., 2000; Fremeau et al., 2001, 2002). VGLUT1 and VGLUT2 are largely segregated in the brain (Herzog et al., 2001; Fremeau et al., 2004), expressed exclusively in Glu-ergic neurons, and are reliable markers for the Glu-ergic phenotype. The distributions of VGLUT1 and VGLUT2 appear to include all the Glu-ergic pathways in the mammalian CNS, and the expression of VGLUT3 overlaps with them in some brain areas (Takamori et al., 2002). VGLUT3 is distributed only sparsely throughout the brain and is co-localized with GABA-ergic, serotonergic and cholinergic markers (Fremeau et al., 2002; Herzog et al., 2004). Recent studies suggested that the expressions of VGLUT1 and VGLUT2 are tightly regulated during development and that the two isoforms are occasionally co-expressed in the same synaptic terminals in the cerebellum (Hisano et al., 2002; Hioki et al., 2003). The VGLUTs are also widely distributed in the peripheral nervous system (PNS), e.g. in the motor endplates of the striated esophageal muscles (Kraus et al., 2004, 2007), the intraganglionic laminar endings (IGLEs) of the esophagus (Raab and Neuhuber, 2003, 2004) and the enteric neurons (Tong et al., 2001; Ewald et al., 2006). IGLEs derived from nodose ganglia are the

most frequent afferent endings throughout the vagally innervated gastrointestinal tract (Berthoud et al., 1997). The presence of VGLUT2 in IGLEs points to a role of L-Glu as a local signal molecule probably released from IGLEs effecting myenteric neurons. VGLUT3 is also expressed in the liver and kidney, which suggests that VGLUT3 functions as a component of the peripheral Glu-ergic system (Fremeau et al., 2002).

Glu not only plays an essential part in adult neural signaling, but also serves an important role in the development of the brain by modulating neuronal migration (Gudz et al., 2006; Manent et al., 2006) and the outgrowth of neuronal processes from the larval lamprey (Ryan et al., 2007), and it promotes refinement of the synaptic connections in the developing brain (van Kesteren and Spencer, 2003).

While these findings suggest the importance of Glu in the development of the brain, as far as we are aware the developmental pattern of the VGLUTs has not been analyzed to date in any part of the PNS. We have therefore studied the expression patterns of all three VGLUT isoforms in the myenteric plexus (MP) during prenatal development of the human small intestine.

Materials and methods

Materials

Intestinal segments from 14, 18, 20, 22 and 23-week-old human fetuses were obtained immediately after legally approved abortions. The crown-heel length was used to assign gestational age. Three fetuses were analyzed per gestational age (Table 1). Segments of the oral part of the small intestine were excised at 2-5 cm aboral from the pylorus, while segments of the aboral part were excised at 2-5 cm oral from the junction of the cecum.

The experiments were performed in full accordance with the declaration of the Medical World Federation proclaimed in Helsinki in 1964.

Tissue handling

Intestinal segments were ligated and distended, then fixed with 4% paraformaldehyde buffered with phosphate-buffered saline (PBS; 0.1 M, pH 7.4) overnight at 4°C. After rinsing with PBS, tissue pieces were further processed for paraffin embedding and/or whole-mount preparations.

Demonstration of VGLUT immunoreactivity in whole-mounts and tissue sections

All immunocytochemical incubations were carried out at room temperature. The primary antibodies, used in the present study together with their respective dilutions, are listed in Table 2. Sections and whole-mount preparations of the small intestine were preincubated in PBS containing 0.1% bovine serum albumin and 10% normal goat serum for 30 min. The preincubation solutions for wholemounts were supplemented with 0.5% Triton X-100. The preparations were then exposed overnight to the primary antibodies listed in Table 2. After washing with PBS, the preparations were incubated with biotinylated anti-rabbit IgG (Amersham, Buckinghamshire, UK, final dilution 1:100) for 6 h, followed by overnight incubation in streptavidin-biotinylated horseradish peroxidase (Amersham, final dilution 1:100). Peroxidase activity was revealed in diaminobenzidine.

Control preparations were performed by omission of either the primary or the secondary antibodies and/or by blocking the primary antibodies with the relevant control proteins (listed in Table 2). Preadsorption of the

Table 1. Human fetuses used throughout this study.

Fetal age (weeks)	External features of the fetus	Indication of the abortion
14	normal	trisomy 21
14	normal	trisomy 13
14	normal	psychiatric
18 (n=3)	normal	psychiatric
20	giant hydrocephalus	clinical
20	normal	endocardial fibroelastosis
20	normal	psychiatric
22	normal	mitral and tricuspidal insufficiency
22	normal	Wolf-Hirschhorn syndrome
22	normal	psychiatric
23	normal	univentricular fetal cardiac syndrome
23 (n=2)	normal	psychiatric

The psychiatric indication refers to suicide attempts by the mother. No chromosome aberrations or cardial abnormalities were known that would have influenced on the enteric development.

Table 2. List of primary antibodies and corresponding fusion proteins used for immunohistochemistry or preadsorption controls.

Primary antiserum	Host	Dilution	Source
Anti-VGLUT1 Polyclonal affinity-purified	rabbit	1:1000	Synaptic System, Göttingen, Germany, Code 135003
Anti-VGLUT2 Polyclonal affinity-purified	rabbit	1:500	Synaptic System, Göttingen, Germany, Code 135403
Anti-VGLUT3 Polyclonal affinity-purified	rabbit	1:500	Synaptic System, Göttingen, Germany, Code 135203

Antigens	Code
Recombinant protein containing amino acid residues 456-560 of rat VGLUT1/BNPI	135-3P
Recombinant protein containing amino acid residues 510-582 of rat VGLUT2/DNPI	135-4P
Recombinant protein containing amino acid residues 530-589 of rat VGLUT3	135-2P

respective antibody was performed by using the method of Ewald et al. (2006) when no specific staining was observed.

Paraffin sections and whole-mounts were mounted on gelatin-coated slides in glycerol-PBS, viewed and photographed with an Olympus DP70 digital camera attached to an Olympus BX60 light microscope. Stained whole-mounts were used for quantitative and semiquantitative analyses.

Quantitative analysis

Ten digital photographs of identical magnification, size and resolution were taken from randomly selected areas (0.188 mm^2) at 400-fold magnification of each stained whole-mount via an Olympus BX60 light microscope equipped with an Olympus DP70 digital camera. The coordinates of the nuclei of the VGLUT-positive neurons were digitalized by the Plexus Pattern Analysis software developed in our laboratory (Roman et al., 2004), which counted the labeled VGLUT-immunoreactive neurons.

Statistical analysis was performed by using one-way ANOVA with GraphPad Prism software. A probability of $P<0.05$ was set as the level of significance in all analyses. Data were expressed as means±SE.

Semiquantitative analysis

VGLUT-IR puncta were counted in randomly selected ganglia and interganglionic fibers. Care was taken to ensure that the neurons in the full depth of the ganglia were counted. Data from 15 ganglia and 15 interganglionic fibres per intestinal segment per fetal intestine per age group were included in the present study.

Puncta counts were assessed on a 5-grade scale; mean grades are given in Table 3.

Results

To study the spatiotemporal pattern of the three VGLUT isoforms in the MP of the human fetal small intestine, we analyzed their immunoreactivity between weeks 14 and 23 of gestation. No specific immunreactivity was found in control specimens that were processed without primary or secondary antibodies or with antisera preadsorbed with the fusion protein used as antigen (not shown). Immunoreactivity for all three VGLUT isoforms was revealed in the MP of the developing human fetal small intestine (Figs. 1, 2). Specific immunoreactivity for each transporter isoform appeared as early as gestational week 14. VGLUT1-IR perikarya and even perisomatic puncta appeared frequently at this age, although the premature ganglia were separated from each other; the internodal connections were not yet completely formed (Fig. 1).

From this age on there was a gradual increase in the intensity of immunoreactivity and in the number of immunopositive nerve structures in the MP.

From week 22 of gestation onwards, VGLUT-IR cell bodies and interganglionic fibers also appeared in the submucous plexus, particularly after VGLUT1 immunostaining (Fig. 2F).
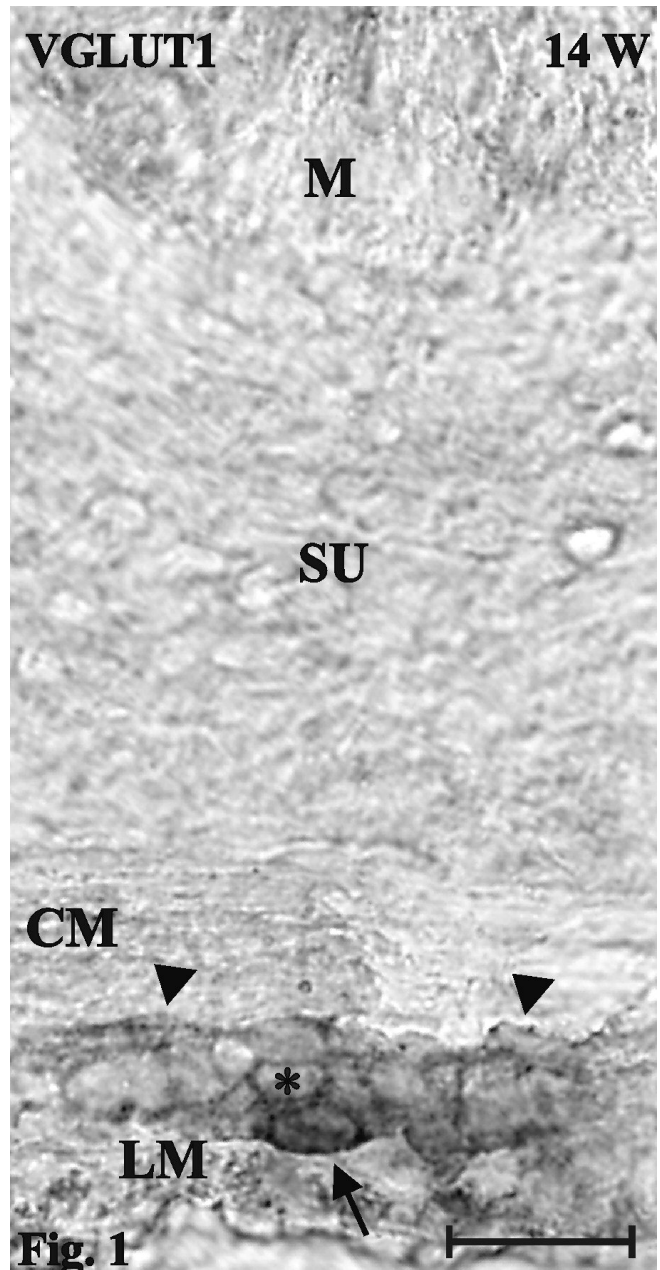

Fig. 1. Cross-sections of paraffin-embedded human embryonic small intestine at week 14 of gestation, immunolabeled for VGLUT1. Aggregates of myenteric neurons are situated between the well-developed circular (CM) and poorly-developed longitudinal (LM) muscle layers. The arrow indicates a VGLUT1-IR perikaryon, the arrowheads indicate immunostained perisomatic puncta and the asterisk indicates neuron surrounded by stained puncta. M: mucosa, SU: submucosa. Bar: 30 μm
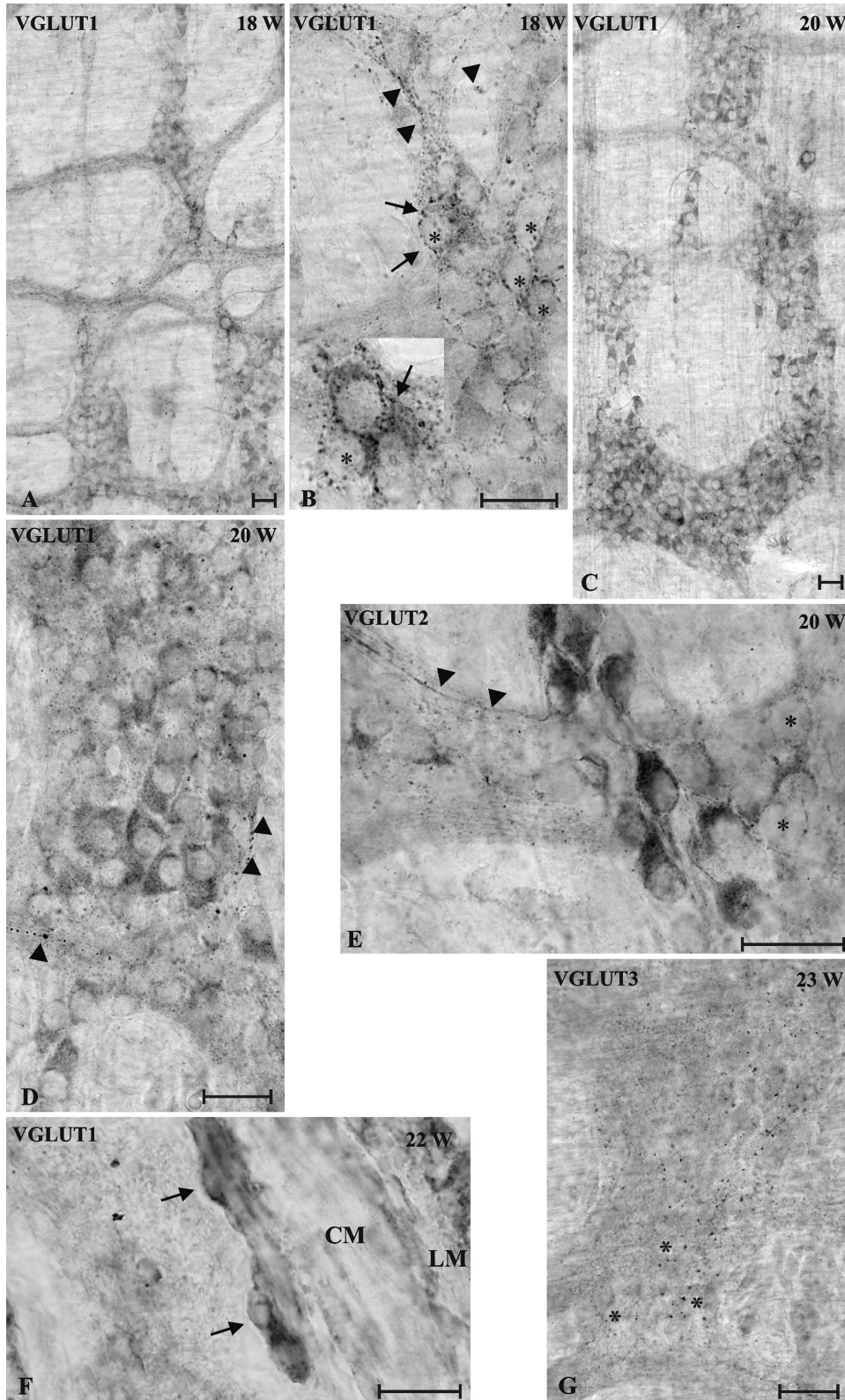
Fig. 2. Representative light microscopic images illustrating VGLUT1 (**A-D and F**), VGLUT2 (**E**) and VGLUT3 (**G**) immunoreactivity in the myenteric plexus of the small intestine of the human fetus between weeks 18 and 23 of gestation. At week 18 of gestation (**A, B**), VGLUT1-IR cell bodies, varicosities and perisomatic puncta are abundant. Perisomatic puncta sometimes appear even around cell bodies immunolabeled for VGLUT1 (**B**, arrows). At higher magnification (**B**, insert), the punctate staining pattern in the cytoplasm is obvious (**B**, insert, arrows). In the oral region of the small intestine, the cytoplasmic signal increases by fetal week 20 (**C, D**), although the perisomatic puncta have almost completely disappeared by this age (**D**). Moderate labeling of the ganglionic neuropil is always present. Widespread cytoplasmic staining is characteristic in the aboral region of the small intestine at week 20 of gestation (**E**). Labeling of the interganglionic fibers, the ganglionic neuropil and also the perisomatic puncta is seen. Submucous ganglia on the outer surface of the CM show VGLUT1-IR (arrows, **F**) in the cross-sections of paraffin-embedded human embryonic small intestine at week 22 of gestation. VGLUT3-IR perisomatic puncta and labeled puncta in the ganglionic neuropil are present in the oral part of the small intestine at week 23 of gestation (**G**). Asterisks indicate nuclei of neurons surrounded by perisomatic puncta; arrowheads indicate VGLUT signals in interganglionic fibers. CM: circular muscle layer. LM: longitudinal muscle layer, Bars: 30 μm

Wholemounts prepared from intestinal segments of fetuses at between 18 and 23 weeks of gestation were suitable for the quantitative analysis of stained cell bodies and a semiquantitative estimation of the immunoreactive puncta (Fig. 2). Characteristic age-related developmental patterns were revealed for each transporter. However, the quantitative predominance of VGLUT1-IR nerve cell bodies and puncta was unambiguous at all fetal ages and in both intestinal segments. The detailed description of the quantitative changes for each transporter between weeks 18 and 23 of gestation is given separately. The quantitative and semiquantitative results are summarized in Fig. 3 and Table 3.

Table 3. Developmental patterns of VGLUT1, VGLUT2 and VGLUT3-immunoreactive puncta in the MP of the human fetal small intestine.

VGLUT isoform	VGLUT1			VGLUT2			VGLUT3		
Fetal week	18	20	23	18	20	23	18	20	23
ORAL									
Perisomatic	++++	+	++++	+	++	+	++	+	+++
Ganglionic neuropil	++	+	+	++	++	++	++	++	+
Interganglionic fibers	+++	+++	+++	+	+	-	++	+	-
ABORAL									
Perisomatic	+	+	++++	++	+	+	+	+	+
Ganglionic neuropil	+	+++	+	++	++++	+	-	-	+
Interganglionic fibers	+++	+++	+++	+	+++	+	-	-	-

The relative density of immunoreactivity is based on semiquantitative analysis (-, no immunoreactive puncta; ++++, highest density of immunoreactive puncta).
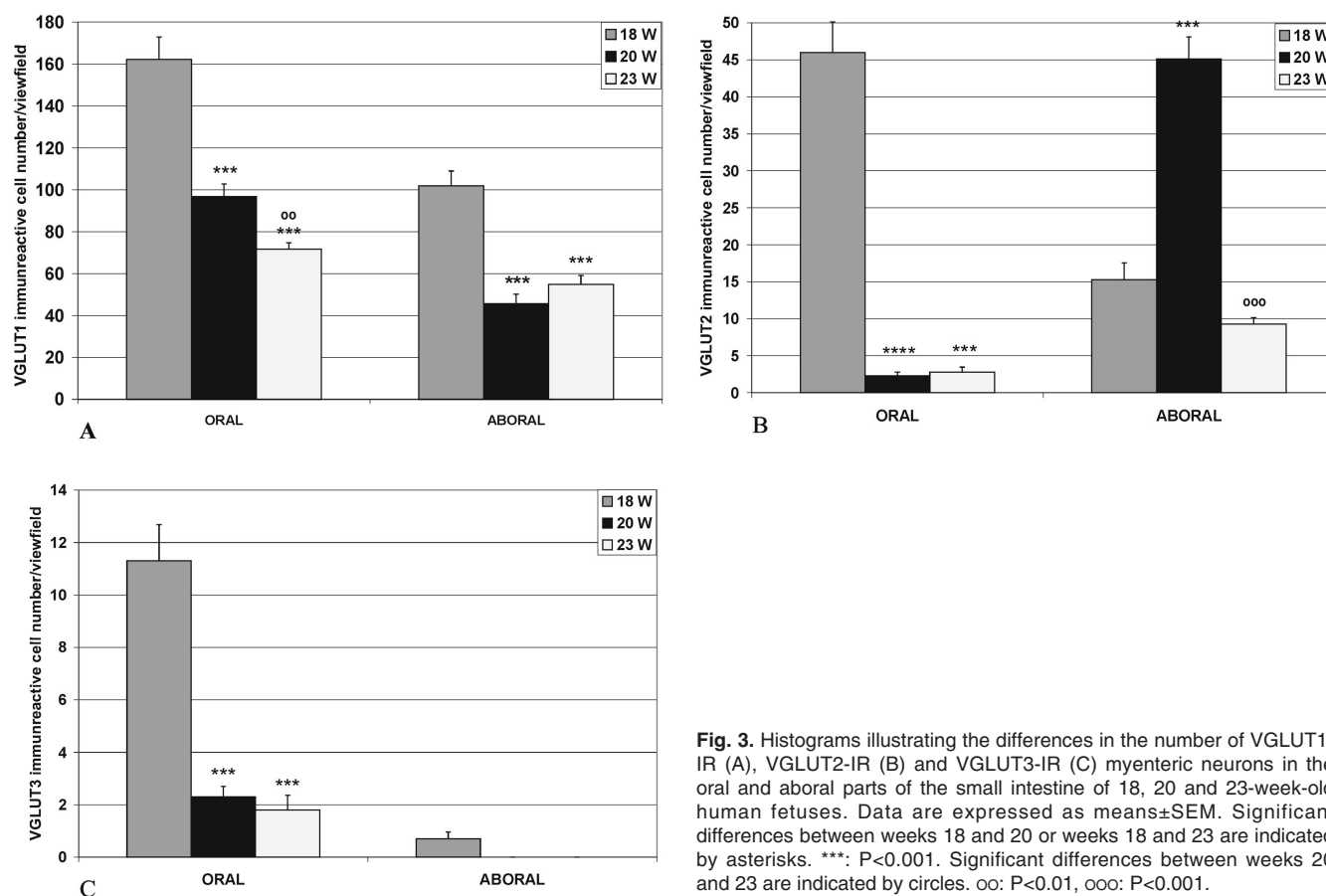

Fig. 3. Histograms illustrating the differences in the number of VGLUT1-IR (A), VGLUT2-IR (B) and VGLUT3-IR (C) myenteric neurons in the oral and aboral parts of the small intestine of 18, 20 and 23-week-old human fetuses. Data are expressed as means±SEM. Significant differences between weeks 18 and 20 or weeks 18 and 23 are indicated by asterisks. ***: $P<0.001$. Significant differences between weeks 20 and 23 are indicated by circles. oo: $P<0.01$, ooo: $P<0.001$.

VGLUT1 immunoreactivity in the developing MP

In the MP of the small intestine of the 18-week-old human fetus, VGLUT1-IR was well pronounced throughout the whole length of the small intestine. Immunoreactive cell bodies were spread within the ganglia, and puncta were scattered along the interganglionic fibers and within the ganglionic neuropil, while many of them were arranged in a perisomatic location (Fig. 2A,B and Table 3). Analysis at higher magnification demonstrated that VGLUT1-IR in the cytoplasm has a punctate staining pattern (Fig. 2B insert). In the oral part of the small intestine, the quantitative predominance of stained puncta was obvious in the perisomatic location at week 18 (Fig. 2B and Table 3). This was in contrast with what was seen at week 20, when the VGLUT1-IR puncta in the perisomatic compartment had almost completely disappeared from this intestinal segment (Fig. 2D and Table 3), though this was followed by a renewed increase, which peaked at week 23 (Table 3). The labeling of the ganglionic neuropil in the oral region was always moderate (Fig. 2C,D and Table 3). In the aboral region of the small intestine, the number of VGLUT1-stained perisomatic puncta was increased by week 23 of gestation, this being preceded by an increase in the stained puncta in the ganglionic neuropil at week 20 (Table 3). In the oral region of the small intestine, the number of VGLUT1-IR cell bodies decreased by approximately 50% from gestational week 18 to 20, and by more than 70% from week 18 to 23. In the aboral region of the small intestine, the pattern of developmental changes between gestational week 18 and 20 was similar to that seen in the oral region (Fig. 3A), but a significant increase in the number of stained cell bodies was noted thereafter (Fig. 3A).

VGLUT2 immunoreactivity in the developing MP

The number of VGLUT2-IR cell bodies at week 18 of gestation was only one-quarter of the number counted after VGLUT1 immunohistochemistry (Fig. 3B). It then further decreased greatly from week 18 to 23. The number of VGLUT2-IR cell bodies at gestational week 23 was only one-tenth of the number at week 18 (Fig. 3B). A slight but significant increase in labeled cell numbers was noticed thereafter. The pattern of the quantitative changes was basically different in the aboral intestinal segment. Here, only a few VGLUT2-IR cell bodies were seen at week 18, but there was an enormous increase from week 18 to 20 (Fig. 3B). This increase was transient, only a few labeled cell bodies were counted on gestational week 23 (Fig. 3B). Semiquantitative analysis of the puncta revealed a moderate labeling of all the ganglionic compartments in the oral part (Table 3). A transient increase in the number of stained puncta in the ganglionic neuropil and also in the internodal segments in the aboral part of the small intestine was pronounced

at week 20 (Table 3).

VGLUT3 immunoreactivity in the developing MP

Like those of the other two transporter isoforms, VGLUT3-IR was identified in the MP as early as week 14 of gestation (not shown). However, throughout the fetal period under investigation, both the cytoplasmic and the punctate signals were restricted almost exclusively to the oral part of the small intestine (Fig. 3C and Table 3). With the exceptions of the several VGLUT3-IR cell bodies in the aboral region at week 18 of gestation, VGLUT3 immunoreactivity was virtually absent from this intestinal segment (Fig. 3 and Table 3).

Discussion

In the present work, we examined the spatiotemporal distribution of VGLUT1-3-IR in the MP of the developing human fetal small intestine between weeks 14 and 23 of gestation. The quantitative predominance of VGLUT1 immunoreactivity over VGLUT2 immunoreactivity was characteristic at all fetal ages in both intestinal segments. A quantitative predominance of VGLUT1 was similarly demonstrated previously as concerns the mRNA levels in the cerebellar granule cells *in vitro* (Hallberg et al., 2006) and also in neocortical circuits (De Gois et al., 2005). However, in the adult human intestines, VGLUT2 was the only existing isoform (Tong et al., 2001). The main findings of the present study were that all three VGLUT immunoreactivities are present in the different ganglionic compartments of the human fetal MP, and each displays a unique and characteristic spatiotemporal distribution. Since peristalsis of the human fetal small intestine has been recorded from week 12 of gestation (Stach, 1989; Montgomery et al., 1999), we presume that the VGLUT-IR perisomatic puncta demonstrated in the myenteric ganglia of the 14-week-old fetuses are a morphological indication of the Glu-ergic neurotransmission acting in the early intestinal transit. The morphological maturation of MP is still in progress at this fetal age (Fekete et al., 1995).

The appearance of the VGLUT1-IR submucosal ganglion cells after week 22 of gestation might be related to the mucosal secreto-motor function, which starts after a prolonged maturation of the mucosal layer at around this fetal age (Bagyánszki et al., 2002).

Even though the VGLUTs are primarily localized to synaptic vesicles, VGLUT protein is also found in cell bodies, probably representing protein internalized from the cell surface after endocytosis (Fremeau et al., 2001). Quantitative analysis of the VGLUT-IR cell bodies and semiquantitative estimation of the immunolabeled puncta in the different ganglionic compartments (perisomatic, ganglionic neuropil and interganglionic fibers) in the oral and aboral segments of the developing small intestine revealed age-related changes in VGLUT-

IR, including the intensity and location of the immunohistochemical signals. The different time courses of the expressions of the different transporters suggests that the VGLUTs in the ENS, just as in the CNS (Hallberg et al., 2006; Liguz-Lecznar and Kramska, 2007), are tightly regulated during development.

Since VGLUT1 and VGLUT2 are reliable markers of Glu-ergic neurons in the CNS (Fremeau et al., 2004; Takamori et al., 2002), we postulate that VGLUT1 and VGLUT2 immunoreactivity indicate Glu-ergic neurons in the fetal MP too. Electrophysiological studies support the conclusion that the Glu-containing varicosities in the gut represent true synapses, and the myenteric neurons contain functional Glu receptors (Liu et al., 1997; Kirchgessner, 2001). It is well established that both N-methyl-D-aspartate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors regulate the formation of a precise pattern of synaptic connectivity, and also neural cell death in the developing brain (Ikonomidou et al., 2000; Adams et al., 2004; Hetman and Kharebava, 2006). We presume, therefore, that in the fetal intestine Glu released from the perisomatic terminals may bind to the Glu receptors of the myenteric neurons and activate them. The activated receptors in turn may generate signals that ensure the survival of the neurons with activated receptors, which successfully form synapses in the later development. The developmental consequences of the transiently high cytoplasmic VGLUT2 expression at weeks 18 and 20 of gestation are difficult to assess. We conceive that VGLUT2 expressed in a critical developmental period in regions outside the synaptic terminals may modulate the Glu signaling by acting as part of a larger Glu reservoir.

In contrast with the two major VGLUT isoforms, VGLUT3 is distributed very sparsely throughout the brain and co-localizes with markers for other transmitters (Fremeau et al., 2002; Herzog et al., 2004). VGLUT3 immunoreactivity was also less abundant in the developing MP as compared with VGLUT1 immunoreactivity and VGLUT2 immunoreactivity, and was always restricted to a subset of myenteric neurons localized specifically to the oral region of the small intestine. Thus, VGLUT3 immunoreactivity covered only a limited portion of the possible Glu-ergic pathways in the human fetal small intestine.

Despite our lack of knowledge concerning the functional differences between the VGLUT isoforms, the unique developmental pattern of nerve elements immunoreactive for the three VGLUTs revealed in the present study suggests functional differences between them in the developing MP. Since data concerning the development of the VGLUTs at the periphery are not available from other studies for a closer understanding of the functional significance of each individual isoform, many issues remain to be solved. In particular, we plan further studies to determine the precise localization of the VGLUT isoforms at the subcellular level, and to establish the neurochemical code of the enteric neurons, which might interact with the VGLUT-IR nerves through the perisomatic puncta.

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