

# Pattern of distribution and co-localization of NOS and ATP in the myenteric plexus of human fetal stomach and intestine

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The pattern of distribution and co-localization of nitric oxide synthase (NOS) and quinacrine fluorescence (indicative of vesicular adenosine 5'-triphosphate, ATP), and co-localization of NADPH-diaphorase (NADPH-d) activity and NOS-immunoreactivity in the myenteric plexus of pre-term human fetal (6–17 weeks of gestation) stomach and small intestine was examined using immunohistochemical and histochemical techniques. In all stages of gestation investigated, NOS-immunoreactive and NADPH-d-reactive myenteric neurons and nerve fibres were seen in the fetal intestine and stomach. However, in fetuses of 6–10 weeks of gestation, only 15% of the NADPH-d-positive myenteric neurons were NOS-immunoreactive, whereas a 100% co-localization was found in samples of 12–17 weeks of gestation. Quinacrine fluorescent myenteric

neurons and nerve fibres were found only in the fetal intestine of 12–17 weeks of gestation, of which 25% of the NADPH-d-positive myenteric neurons in these samples were quinacrine fluorescent. These findings demonstrate the presence and co-localization of markers for nitric oxide (NO)- and ATP-utilizing myenteric neurons and nerve fibres in the early stages of gestation, suggesting possible co-transmitter and/or trophic roles of ATP and NO in the process of development and maturity of human myenteric neurons. In addition, the fact that only a small percentage of NADPH-d-reactive myenteric neurons express NOS immunoreactivity at 6–10 weeks of gestation confirms that NADPH-d-reactivity does not always represent NOS activity. *NeuroReport* 11:5–8 © 2000 Lippincott Williams & Wilkins.

**Key words:** ATP; Fetal gut; Myenteric plexus; NADPH-d; NO; NOS; Quinacrine

## INTRODUCTION

Non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmission in the gastrointestinal tract (GIT) has been accepted to be mediated by more than one neurotransmitter. Pharmacological and physiological studies have so far provided evidence for the involvement of adenosine 5'-triphosphate (ATP), vasoactive intestinal polypeptide (VIP) and its related peptide pituitary adenylate cyclase activating peptide (PACAP), and nitric oxide (NO) [1–15]. Immunohistochemical and histochemical investigations have further substantiated the proposed role of these neurotransmitters by localising vesicular ATP, VIP and NO in enteric neurons and nerve fibres along the regions of the GIT of various mammalian and non-mammalian species [16–23]. An earlier study on human fetal gut has reported the appearance of VIP-immunoreactive enteric neurons in the intestine of 15–18 weeks of gestation [24], although other investigators have reported the presence of VIP-mRNA in the ganglion cells as early as 9 weeks of gestation [25]. A study by Timmermans and colleagues [26]

has revealed the presence of NO-utilizing enteric neurons in human fetal gut as early as 18 weeks of gestation. In the present study, we examined the presence, pattern of distribution and co-localization of nitric oxide synthase (NOS, a marker for NO-utilizing neurons) and quinacrine fluorescence (a marker for vesicular ATP) in myenteric neurons and nerve fibres in the intestine and stomach of preterm human fetuses (6–17 weeks of gestation) using immunohistochemical and histochemical techniques.

## MATERIALS AND METHODS

Fetal tissue (6–17 weeks of gestation) consisted of stomach and segments of small intestine which were obtained from elective terminations of pregnancy. This investigation was covered by the approval of the Joint University College/University College Hospital Committee on Ethics of Human Research. The stomach and segments of intestine were rinsed in 0.01 M phosphate buffered saline (PBS), cut open and stretched out onto strips of Sylgard silicone rubber (Dow Corning, Seneffe, Belgium) with mucosal side down-

wards. Some of the stretched intestine and stomach were used fresh for quinacrine labelling followed by fixation and NADPH-diaphorase (NADPH-d) staining, while others were directly fixed and used for immunohistochemical and histochemical localization of NOS and the general neuronal marker protein gene product 9.5 (PGP). The stretched specimens were fixed in 4% paraformaldehyde in PBS for 2 h at 4°C. The fixed tissues were then washed three times each for 10 min with PBS containing 0.1% Triton X-100 and the outer muscular coat (together with the myenteric plexus) was separated from the mucosal layer of the gut, immunolabelled either for NOS or PGP as described previously [19]. To verify that NADPH-d activity represent NOS, after taking photographs, the NOS immunolabelled tissue samples were removed from the glass slides, placed on strips of silicone rubber with identical orientation, washed and stained for NADPH-d. NADPH-d staining was performed by incubating tissues with 1.2 mM  $\beta$ -NADPH, 0.24 mM nitroblue tetrazolium and 0.1% Triton X-100 in 0.1 M Tris-HCl (pH 7.4) for 1 h at 37°C.

Quinacrine fluorescence staining was performed on stretched segments of fresh tissue with mucosal layer carefully peeled away from the outer muscular coat of the gut. The tissue samples were incubated with  $10^{-7}$  M quinacrine hydrochloride in PBS at 37°C for 30 min, transferred onto clean glass slides and mounted with PBS. The quinacrine fluorescent neurons and nerve fibres were visualized and photographs were taken using a Zeiss microscope. To investigate possible co-existence of NOS and quinacrine fluorescence, the quinacrine-labelled tissues were removed from the glass slides, washed and fixed as described above. The fixed tissues were first immunolabelled for PGP to assess the percentage of quinacrine fluorescent neurons and stained for NADPH-d as described above.

## RESULTS

Quinacrine fluorescent myenteric neurons and nerve fibres were seen only in fetal intestine of 12–17 weeks of gestation (Fig. 1a), but not in the intestine at earlier stages of development nor in the fetal stomach of any stages investigated. Co-localization of quinacrine-fluorescence and NADPH-d reactivity was found in about 25% of NADPH-d-positive myenteric neurons (Fig. 1a,b). There were also quinacrine fluorescent, but NADPH-d-negative, myenteric neurons. Despite the fact that the images shown in Fig. 1a and Fig. 1b are of the same magnification, the neurons in the former look larger than those in the latter. This is a common artefact encountered when co-localization of antigens in fresh and fixed tissues is investigated.

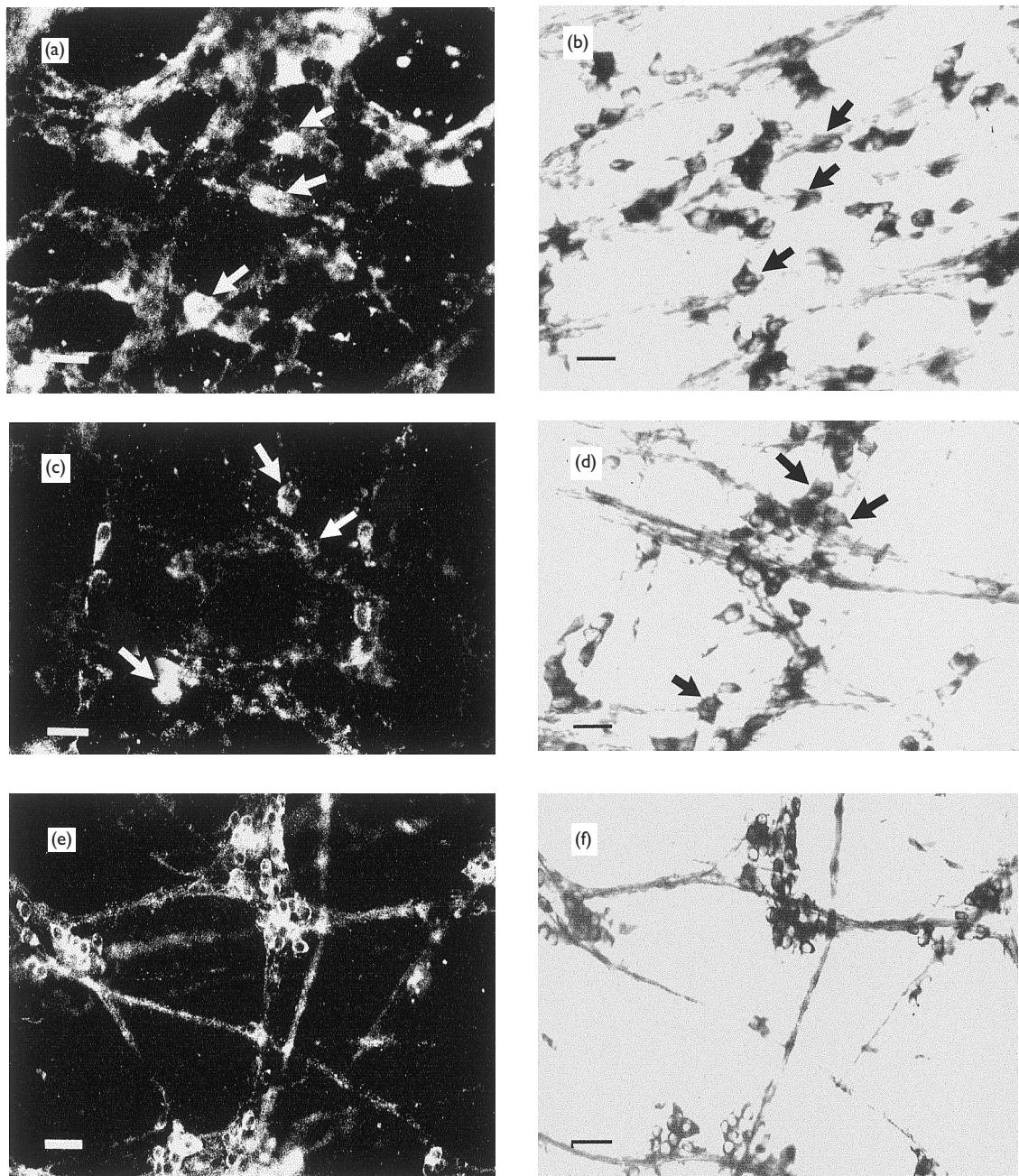
In the myenteric plexus of fetal stomach and intestine of all stages of gestation investigated there were NOS-immunoreactive and NADPH-d-reactive neurons and nerve fibres (Figs. 1c–f). In the myenteric plexus of fetal stomach and intestine of 6–10 weeks of gestation, only about 15% of NADPH-d-reactive myenteric neurons were also NOS-immunoreactive (Fig. 1c,d), whereas there was 100% co-localization of NOS immunoreactivity and NADPH-d reactivity in the myenteric neurons and nerve fibres of fetal stomach and intestine of 12–17 weeks of gestation (Fig. 1e,f). Myenteric neurons, which were NOS-immunoreactive but not NADPH-d-reactive, were also found in the stomach and intestine in earlier stages of gestation (Fig. 1c,d).

## DISCUSSION

The present findings have revealed an interesting pattern of distribution and co-localization of vesicular ATP- and NOS-utilizing myenteric neurons and nerve fibres in pre-term human fetal stomach and intestine. Myenteric neurons and nerve fibres of both stomach and small intestine contain NOS immunoreactivity as early as 7 weeks of gestation, although a 100% co-localization of NOS immunoreactivity and NADPH-d reactivity was apparent only at 12–17 weeks of gestation. At the earlier gestations there were more NADPH-d-positive neurons, with an average of only 15% of these neurons being NOS-immunoreactive. In addition, quinacrine fluorescent myenteric neurons and nerve fibres were seen in the small intestine but not stomach of fetuses of only 12–17 weeks of gestation and about 25% of the NADPH-d-reactive neurons were also found to be quinacrine positive.

It is now well accepted that in GIT, NANC inhibitory neurotransmission is mediated by ATP, VIP and NO [7,8,11–15,19,20,27–29], although the relative degree of importance of each mediator along the regions of GIT and various animal species remains to be established. The evidence for multi-neurotransmitter NANC inhibitory neurotransmission in the GIT has been mainly based on findings from animal studies. The results of the present study have demonstrated that a neurochemical pattern similar to that found in the myenteric plexus of laboratory animals also occurs in human fetal stomach and small intestine. In addition, the presence of two of these NANC inhibitory mediators as early as preterm stage of fetal development implies that ATP and NO may play an important role in the process of development and maturity of human myenteric neurons. We have already reported co-localization of quinacrine fluorescence and NADPH-d reactivity in the myenteric neurons and nerve fibres of ileum, proximal colon and annococygeus muscle of the rat [23]. In the proximal colon of the rat, the percentage of myenteric neurons showing co-localization of quinacrine fluorescence and NADPH-d reactivity was higher than in the ileum [23]. This differential distribution may be indicative of the relative degree of importance of ATP and NO as NANC neurotransmitters along the large and small intestine of the rat. Although such a comparison between the small and large intestine was not made in the human fetal intestine, the percentage of myenteric neurons expressing NOS immunoreactivity and quinacrine fluorescence in the fetal ileum is similar to that found in the rat small intestine [23]. The lack of quinacrine-positive myenteric neurons in the fetal stomach of at least all the stages of gestation investigated in the present study further strengthens the suggestion that the relative importance of each neurotransmitter varies along the different regions of the GIT. This however, does not rule out the possibility that ATP-utilising neurons may occur at other stages of development or in adult human stomach.

Although the functional implications of the presence of vesicular ATP- and NOS-containing myenteric neurons in the human fetal intestine needs to be determined, the possibility that both ATP and NO may play a role in the process of development and maturation of human myenteric neurons cannot be ruled out. In fact, both ATP and NO have been suggested to play an important role in the



**Fig. 1.** Micrographs showing co-localization of quinacrine fluorescence (**A**) and NADPH-d activity (**B**); NOS immunoreactivity (**C,E**) and NADPH-d activity (**D,F**) in the human fetal small intestine (**A–D**) and stomach (**E,F**). Images in (**A,B,E**) and (**F**) were from fetal tissues of 12–17 weeks of gestation, whereas those in (**C**) and (**D**) are from fetal tissues of 6–10 weeks of gestation. Arrows indicate neurons containing both markers. Bars = 15  $\mu$ m (**A–D**), 25  $\mu$ m (**E,F**).

process of development of neuronal and non-neuronal tissues. In the chick embryo, a G protein-coupled receptor for extracellular ATP (chick  $P_{2Y1}$ ) was found to be expressed during the first 10 days of embryonic development [30]. The receptor was expressed in a developmentally regulated manner in the limb buds, mesonephros, brain, somites and facial primordia, suggesting possible role in the development of each of these systems [30]. Earlier studies have revealed that extracellular ATP is involved in

the regulation of the development of the nervous system [31] and neuromuscular junctions [32] in *Xenopus* embryos. We have described earlier a possible contribution of NO in the process of development of enteric neurons in the rat intestine [33] and guinea-pig gall bladder [34].

#### CONCLUSION

The present findings provide evidence that a subpopulation of myenteric neurons from preterm human fetal

stomach and small intestine contain NOS and ATP as early as 6–17 weeks of gestation. These results raise the possibility that, in addition to a cotransmitter role in NANC neurotransmission, ATP and NO may have important roles in the process of development of human myenteric neurons.

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