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Postnatal maturation of the gastrointestinal tract: A functional and immunohistochemical study in the guinea-pig ileum at weaning

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ABSTRACT

Gastrointestinal motility is mainly controlled by the myenteric plexus. The longitudinal muscle-myenteric plexus (LMMP) preparation from the guinea-pig ileum is the best characterised adult gastrointestinal preparation; it has also been studied in old and neonatal animals, but not at weaning, when milk is substituted with the food typical of adult animals. We used LMMP preparations from weanling and adult guinea-pigs to study different functional parameters and immunohistochemically identified subpopulations of myenteric neurones, including the excitatory motor neurones to the longitudinal muscle (LM-EMN). Excitatory stimuli (low-frequency electrical stimulation, acetylcholine, substance P, and naloxone in morphine-tolerant preparations) produced similar responses in weanling and adult guinea-pigs. The endogenous cannabinoid anandamide, but not the synthetic cannabinoid agonist WIN 55,212-2 or the opioid morphine, inhibited the electrically stimulated twitches less efficaciously, and in vitro tolerance to morphine was also lower in weanling compared to adult animals. The packing densities of the calbindin-immunoreactive neurones (sensory neurones) and of neurones immunoreactive to both calretinin (CR) and neurofilament triplet protein (NFT; ascending interneurones) were slightly but significantly lower in weanling animals, whereas those of the neurones immunoreactive to CR but not NFT (LM-EMN) or immunoreactive to nitric oxide synthase (mainly inhibitory motor neurones) were comparable to the adult. Although guinea-pigs are relatively mature and can even ingest solid food at birth, their myenteric plexus is still not fully mature at the standard time of weaning. The nutritional, behavioural and environmental changes associated with weaning may be essential to attain full maturation of the myenteric plexus and gastrointestinal motility.

The enteric nervous system controls gastrointestinal functions, including motility. Appropriate neurotransmission from the myenteric neurones to the muscle layers of the gut wall is crucial for normal gastrointestinal motility [16]. Different functional and structural alterations occur in the myenteric plexus of old and neonatal animals; these may underlie the modifications in GI motility encountered at both paediatric [5,14] and old [2–4,9,26] ages.

The myenteric plexus of the neonate shows functional and immunohistochemical differences compared to that of the adult animal. Clinical reports showed that constipation is more prevalent in neonates [5,14]. Inhibitory neuromuscular transmission was shown to be relatively more effective in the small intestine of neonatal guinea-pigs, whereas the maturation of excitatory motor pathways was delayed [6]. Structural and proteomic analyses confirmed these functional results [6,19,22]. Studies in humans [30] also suggest that the ENS continues to mature beyond birth.

Weaning is an important postnatal event at which milk is replaced by an adult diet. The changes associated with weaning, like new food composition or maternal separation, might be essential for the completion of postnatal development. Although no functional study was performed, the immunohistochemical analysis of the myenteric plexus revealed some differences between weanling and adult rats [22,29], suggesting that the myenteric plexus might not be fully mature at weaning.

The aim of this work was to determine the functional and immunohistochemical characteristics of the myenteric plexus at weaning as compared to the adult age, using guinea-pig ileum (GPI) longitudinal muscle-myenteric plexus (LMMP) preparations. This is the best characterised adult gastrointestinal preparation [12] and it has also been studied in old [2–4] and neonatal [6] animals but, to our knowledge, not in weanling guinea-pigs.

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Female Dunkin–Hartley guinea-pigs were used. These were 23 days (weanling, weight 200–300 g; standard weaning was performed 21 days postnatally) or 2–3 months (adult, weight 330–450 g) old. All experimental protocols were approved by the Ethical Committee at the Universidad Rey Juan Carlos and performed in strict accordance with the EC regulations for care and use of experimental animals (EEC No. 86/609). Animals were killed by cervical dislocation. Segments of the distal ileum, at least 10 cm oral to the ileocaecal junction, were opened along the mesenteric border, rinsed and pinned flat on a Sylgard-coated Petri-dish filled with modified Krebs solution (mM: NaCl 118, KCl 4.75, NaH_2PO_4 1.0, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11; pH 7.4). Whole-mount LMMP preparations were obtained and used in two different sets of experiments, using methods described elsewhere [2–4]. In each experiment, at least four preparations from different adult and weanling animals were used.

For the functional studies, LMMP preparations were suspended under a tension of 1 g in an organ bath containing Krebs solution (36 °C) and aerated with carbogen (95% O_2 to 5% CO_2). Contractile activity was recorded using an isometric transducer connected to a PowerLab/4e system. Field electrical stimulation (pulses of 2 ms in duration, 0.3 Hz and increasing voltage until supramaximal stimulation was reached) was used to induce atropine-sensitive twitches in the preparations. The preparations whose contractions did not reach 0.5 g were discarded. No age-related differences were apparent in the number of usable preparations (85% for both age groups).

The functional parameters were as follows: (1) the minimum voltage that produced a stable contractile response of the preparations (threshold); (2) the force of the contraction elicited by supramaximal electrical stimulation; (3) the force of the contractile responses induced by in vitro non-cumulative administration of the excitatory agonists acetylcholine (ACh) and substance P (SP) (10^{-8} to 5×10^{-6} M), in the absence of electrical stimulation; (4) the force of the in vitro sign of opioid withdrawal induced by administration of the opioid antagonist naloxone (10^{-6} M) on non-stimulated LMMP preparations pre-incubated with the opioid agonist morphine (5×10^{-7} M) for 90 min; (5) the effect of cumulative doses of morphine (5×10^{-8} to 1.6×10^{-6} M) administered at intervals of 3 min to electrically stimulated LMMP preparations, either naïve (acute effect) or pre-incubated with morphine (5×10^{-7} M) for 90 min (development of tolerance); and (6) the effect of the cannabinoid agonists anandamide (endogenous CB1 ligand) and WIN 55,212-2 (WIN, non-selective synthetic cannabinoid drug), added in increasing cumulative concentrations (10^{-8} to 2.4×10^{-6} M) at intervals of 15 min to electrically stimulated LMMP preparations. The effect of morphine (5) and cannabinoids (6) was evaluated as the percentage inhibition of the initial twitch amplitude. At the higher concentration used, the cannabinoid vehicle produced a slight inhibition (less than 10%) of the electrically stimulated contractions.

ACh, SP and morphine were purchased from Sigma–Aldrich and dissolved in distilled water. WIN and anandamide were purchased from Tocris and dissolved as previously described [25].

For the immunohistochemical study, each LMMP preparation was stretched to its maximal extension, fixed in Zamboni's fixative, cleared with dimethylsulfoxide, and washed with phosphate buffered saline (PBS). Adjacent tissues were incubated overnight at room temperature (RT) with a mixture of either mouse anti-CB (calbindin, 1:500, Sigma, C9848) and sheep anti-NOS (nitric oxide synthase, 1:500, Chemicon, AB1529), or mouse anti-NFT (neurofilament triplet, 1:500, a generous gift from Dr. Vitadello, Padova, Italy) and goat anti-CR (calretinin, 1:1000, Chemicon, AB1550). After washing with PBS (3× 10 min), tissues were exposed (90 min, RT) to a mixture of the following, corresponding secondary antibodies: donkey anti-mouse-CY2 (1:500, Jackson, 715-225-150), donkey anti-sheep-CY3 (1:500, Jackson, 713-165-147) and donkey anti-goat-RRG (1:500, Jackson, 705-295-147). Some additional tissues were labelled with the pan-neuronal marker HuC/D (Hu), by using mouse streptavidin-conjugated anti-Hu (1:1000, Molecular Probes, A21272) as the primary antibody, and the avidin-AlexaFluor 488 complex (1:1000, Molecular Probes, A21275) to develop the reaction. All antibody mixtures were diluted with hypertonic PBS (1.7% NaCl). The preparations were observed under a fluorescent microscope (Nikon Eclipse TE2000-U, with appropriate filters). The immunohistochemical analysis was performed in a blinded fashion. Thirty-two micrographs were taken randomly at 20× magnification, using a DXM1200 camera (Nikon, Spain). The packing density (number of neurones per ganglionic surface unit: [2,20]) and proportion vs. the general, Hu-immunoreactive population of myenteric neurones were evaluated for neurones immunoreactive to CB, NOS, CR and NFT. Packing density and proportions were considered to be 100% in adult animals, so that the differences at weaning in the following four populations of immunohistochemically identified myenteric neurones [4,12,13] could be compared: sensory neurones (CB^+), inhibitory motor neurones (NOS^+), ascending interneurones (CR^+-NFT^+) and excitatory longitudinal muscle motor neurones (LM-EMN: CR^+-NFT^-). Controls for double-labelling were performed by pairing the wrong primary and secondary antibodies or by bypassing exposure to the primary antibody. No specific labelling was observed in either case.

Statistical analysis was carried out using GraphPad Prism® (GraphPad Software, Inc.). All results are expressed as mean ± SEM; n refers to the number of preparations and, unless otherwise stated, is equivalent to the number of experimental animals (N). Differences between groups were analysed using either an unpaired Student's t-test or a one-way or two-way ANOVA followed by a post hoc Bonferroni multiple comparison test. Values with $p < 0.05$ were considered to be statistically significant.

In preparations from adult guinea-pigs, the threshold for the induction of stable electrically stimulated contractile responses and the amplitude of these responses were 1.97 ± 0.02 mV and 0.75 ± 0.12 g ($n = 12$), respectively. In comparison, no significant difference was found in preparations from weanling animals (1.99 ± 0.01 mV and 0.74 ± 0.10 g, respectively; $n = 13$). The cholinergic (ACh) and tachykinergic (SP) excitatory agonists induced concentration-dependent contractions of the non-stimulated preparations; these were not statistically different between weanling and adult guinea-pigs (Fig. 1A and B). Similarly, in morphine-incubated preparations, naloxone induced a contraction (in vitro opioid withdrawal sign) with a force that was not significantly different between weanling (0.51 ± 0.07 g; $n = 12$, $N = 6$) and adult animals (0.64 ± 0.08; $n = 12$, $N = 5$).

The electrically stimulated contractions were dose-dependently inhibited by both cannabinoid agonists in both adult and weanling preparations. However, in weanling animals, anandamide was significantly less efficient than it was in adult guinea-pigs, whereas no significant difference was found for WIN (Fig. 1C and D). Morphine also inhibited the contractions of the naïve LMMP preparations with less efficiency in weanlings than in adults but the difference was not statistically significant (Fig. 1E). However, in morphine-incubated LMMP preparations, the inhibitory effect of morphine was greater in weanling guinea-pigs (Fig. 1E).

Only minor immunohistochemical differences were found between weanling and adult animals (Figs. 2 and 3). Thus, the packing density of myenteric neurones immunoreactive to CB (sensory neurones, Fig. 2A), NFT (Fig. 2D) or both CR and NFT (ascending interneurones, Fig. 3B) was significantly lower, and the percentage of CR^+ vs. Hu-immunoreactive neurones was higher in weanlings (Fig. 2C′). Among the neurones that were immunoreactive to CR, the proportion that was also immunoreactive to NFT (ascending interneurones) was significantly lower in weanling guinea-pigs