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Nociceptin/orphanin FQ and stress regulate synaptophysin expression in the rat fundic and colonic mucosa

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ABSTRACT

Nociceptin/orphanin FQ (N/OFQ), the endogenous ligand of the N/OFQ peptide (NOP) receptor, is a neuropeptide regulating gastrointestinal functions. The present study investigated the influence of acute cold-restraint stress and of short- and long-lasting peripheral infusion of N/OFQ on the level of synaptophysin, an exocytotic protein involved in neural plasticity. Exposure to cold-restraint stress for 3 h or subcutaneous infusion of N/OFQ, 1 μg/kg/h for 4 h, induced a significant increase of the area of synaptophysin-immunoreactive nerve fibers in the fundic mucosa, while prolonged subcutaneous infusion of N/OFQ, 1 μg/kg/h for 52 h and for 14 days, did not modify the synaptophysin-immunostained fibers. In the colonic mucosa stress exposure and subcutaneous infusion of N/OFQ, at any time point considered, had no significant effect on the area of synaptophysin-immunoreactive nerve fibers. Synaptophysin immunoreactive nerve fibers were decreased in knockout rats for the NOP receptor gene both in the fundic and colonic mucosa. Synaptophysin-immunoreactivity was demonstrated in cells located in the basal portion of the fundic mucosa. Our study is the first to show that the N/OFQ/NOP receptor system influences the expression of synaptophysin and hence the process of exocytosis both in nerve terminals and in cells.

1. Introduction

Synaptophysin is a membrane protein distributed in nerve terminals and in a variety of neuroendocrine cells and it is implicated in the final steps of exocytosis (Bennett and Scheller, 1993; Navone et al., 1986; Wiedenmann and Franke, 1985). This protein is involved in neural plasticity. Expression of synaptophysin appears to be altered in response to stress in the rat central nervous system. Exposure to acute and repeated restraint stress in adult rats caused a significant decrease of synaptophysin expression in hippocampus and cerebral cortex (Thome et al., 2001; Xu et al., 2004). Similarly the expression of synaptophysin was reduced in the hippocampus and cortex following pre- and postnatal stress (Koo et al., 2003). Synaptophysin-immunoreactivity was found in endocrine cells and in varicose fibers innervating the glands of the rat gastric mucosa (Andersson et al., 2005; Navone et al., 1986; Zhao et al., 1997) and it has also been identified in the rat colonic mucosa (Barreau et al., 2008). Evidence is provided that synaptophysin is overexpressed in the colonic mucosa of maternally deprived rats (Barreau et al., 2008), and in human colonic mucosa in association with inflammatory bowel diseases (Strobach et al., 1990) or in acute appendicitis (Xiong et al., 2000). Expression of synaptophysin in ileocecum and colon is altered in *Trichinella spiralis*-infected rats, suggesting a role in the formation of visceral hypersensitivity (Yang et al., 2009).

The heptadecapeptide nociceptin/orphanin FQ (N/OFQ), the endogenous ligand of the N/OFQ peptide (NOP) receptor, could be considered a component of the interaction neuropeptides-stress in the gastrointestinal tract. The peptide affects mucosal integrity and functions of rat gastrointestinal tract in resting and stress-challenged conditions (Agostini et al., 2009; Broccardo et al., 2007, 2008; Grandi et al., 2007, 2010, 2011a,b; Morini et al., 2005). In the

Abbreviations: N/OFQ, nociceptin/orphanin FQ; NOP, N/OFQ peptide.

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present study we have used fluorescence immunohistochemistry to investigate the effects of acute cold-restraint stress and of short- and long-lasting peripheral infusion of N/OFQ on the level of synaptophysin in the fundic and colonic mucosa of male Wistar rats. The recent availability of NOP (−/−) rats (Homberg et al., 2009) afforded us the opportunity to study the level of synaptophysin in absence of the NOP receptor.

2. Materials and methods

2.1. Animals

Experiments were conducted using male Wistar rats (9 weeks old, 180–200 g; Harlan, San Pietro al Natisone, UD, Italy). Additional experiments were conducted using four male NOP (+/+) and six male NOP (−/−) littermates rats supplied by GenOway (Lyon, France). These rats were generated in a Brown Norway background and subsequently backcrossed on a Wistar background for four generations as described (Homberg et al., 2009). They were 9 weeks old on arrival and they were sacrificed 1 week later. All rats were maintained under controlled temperature (25 ± 1 °C), humidity (60 ± 5%) and light conditions (12:12 light dark cycle). Rats were housed in individual cages with free access to food and water. They were deprived of food but not of water for 24 h before the experiments. All the experiments were approved by the Italian Animal Care and Committee.

2.2. Alzet osmotic minipump insertion and N/OFQ delivery

N/OFQ was synthesized as salt derivative at the Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, Italy. All other reagents were commercially available. Alzet osmotic minipumps (Cupertino, CA), model 2001, flow rate 1 μl/h (4 and 52 h treatment) or model 2ML2, flow rate 5 μl/h (14 day treatment) were filled with either 0.9% saline (control) or N/OFQ (dissolved in 0.9% saline to deliver at the rate of 1 μg/kg/h) under sterile conditions. The Alzet pumps were then primed in vials containing saline solution at 37 °C overnight before implantation. The dose of N/OFQ was selected on the basis of previous dose–response studies (Grandi et al., 2010).

2.3. Experimental protocol

Wistar rats were randomized into six groups ($n = 6$ per group). On the day 0, the rats were anaesthetized by intramuscular injection of tiletamine hydrochloride (200 mg/kg) and zolazepam hydrochloride (200 mg/kg) and implanted subcutaneously in the interscapular region with the primed minipumps. Rats recovered from anesthesia within 1 h after the operation. Rats infused with N/OFQ, 1 μg/kg/h, were sacrificed 4 h, 52 h and 14 days after the minipump implantation. A group, implanted with saline-filled minipump was subjected to cold-restraint stress, 1 h after operation. Rats were restrained into individual wire-mesh restraint cages and placed in a cold room at 3 °C for 3 h. The rats were sacrificed at the end of the restraining period. The control group of rats was sacrificed 4 h and 14 days after the saline-filled minipump implantation ($n = 3$ rats for each time point). NOP (−/−) rats and their littermates were left untreated and not subjected to minipump implantation. All rats were sacrificed between 1 pm and 3 pm.

2.4. Tissue preparation

On sacrifice, the abdomen was opened. The stomach and colon were removed from the peritoneal cavity. The stomach was opened along the lesser curvature and a strip was excised from the fundus, 3–4 mm below and parallel to the limiting ridge, so that the greater curvature was approximately located in the middle of the strip. Segments of the distal colon (3–6 cm to the anus) were collected and opened along the mesenteric border. Tissue samples were taken, fixed in 10% buffered formaldehyde overnight and paraffin-embedded. Tissue samples from stressed rats were taken from areas that were normal upon gross examination. Serial section, 5 μm thick and perpendicular to the mucosal surface, were cut from each block and processed. One section from each block was stained with hematoxylin and eosin and additional sections were cut for immunohistochemistry.

2.5. Immunohistochemistry

Sections were incubated overnight at 4 °C with rabbit polyclonal antibody to synaptophysin (1:50, Cell Marque, Rocklin, CA, USA). After three 15-min rinses in 0.01 M PBS, the sections were subsequently incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:70, Chemicon, Temecula, CA, USA). Sections were then washed in PBS, dried and mounted (Vectashield). As negative control, the primary antibody was replaced by PBS in one section for each animal. No staining was observed in these preparations.

2.6. Image analysis

Images of well-oriented sections were taken with a color camera (Nikon DXM1200F) attached to a fluorescence microscope (Nikon Eclipse 80i). Images were analyzed using a color image analysis software system (NIS-Elements AR, Nikon Laboratory Imaging, Japan). Nerve fibers immunoreactive to synaptophysin were traced in the lower half (10 μm from the muscularis mucosae) and upper half of the fundic mucosae and in the middle portion of the colonic mucosae (Karam and Leblond, 1992). Results were expressed in μm^2 of surface occupied by synaptophysin immunoreactivity in nerve fibers per 100 μm^2 of mucosa. The number of cells immunoreactive to synaptophysin was evaluated in the fundic mucosa and results were expressed as number of positive cells per 100 μm^2 of mucosa. Quantitations were performed on at least nine areas of each portion of the fundic mucosa and of the colonic mucosa from three different sections for each rat. Mean values for each rat were determined and used to determine mean values for each experimental group.

2.7. Statistical analysis

Results were expressed as means ± SEM and were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. General observations

No significant differences in body weights were observed between NOP (−/−) rats and their littermates or saline-treated controls. The body weights were not significantly changed following continuous subcutaneous infusion of N/OFQ, compared with the corresponding saline-treated controls. Fundic and colonic mucosa had a normal histological appearance and oxyntic and colonic mucosal thickness was similar in the different experimental groups.

3.2. Density of synaptophysin immunoreactivity in nerves of the fundic and colonic mucosa

Immunoreactivity to synaptophysin was present in nerve fibers of both fundic and colonic mucosa. Varicose nerve fibers positive