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Luteinizing hormone receptors are expressed in rat myenteric neurons and mediate neuronal loss

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ABSTRACT

Background: Clinical observations have suggested repeated gonadotropin-releasing hormone (GnRH) exposure to cause intestinal dysfunction and loss of enteric neurons. This has been further studied and confirmed in a rat in vivo model involving iterated GnRH treatments. Mechanisms behind are enigmatic since no GnRH receptors are found to be expressed in enteric neurons neither in man nor rat. Both species, however, harbor substantial subpopulations of luteinizing hormone (LH) receptor-immunoreactive myenteric neurons which suggests that intestinal GnRH-induced neuropathy may be mediated by LH release.
Aims: To reveal if exposures of GnRH or LH to rat myenteric neurons in vitro cause neuronal loss.
Methods: Primary cultured adult rat myenteric neurons were exposed to single or repeated treatments of the GnRH analog buserelin or the LH analog lutrotropin alpha, and neuronal survival was determined by cell counting. Possible presence of GnRH- or LH receptor -immunoreactive neurons was determined by immunocytochemistry.
Results: Exposure to the LH, but not the GnRH, analog caused significantly reduced neuronal survival. LH, but not GnRH, receptors were found to be expressed on cultured myenteric neurons.
Conclusion: Myenteric neurons express LH receptors in vitro and LH exposure causes reduced neuronal survival. This suggests that GnRH-induced enteric neuropathy in vivo is mediated by way of LH release and activation of enteric neuronal LH receptors.

1. Introduction

Gastrointestinal (GI) dysfunction with enteric neuropathy and neurodegeneration are often identified in patients diagnosed with e.g. enteric dysmotility (ED), chronic intestinal pseudo-obstruction (CIPO) and diabetes (Gabbard and Lacy, 2013; Knowles et al., 2013; Yarandi and Srinivasan, 2014).

Gonadotropin releasing hormone (GnRH) stimulates secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) through GnRH receptor activation (Roch et al., 2014). Based on the observations that functional dysmotility is more common in women and that symptoms worsen during post luteal phase, pregnancies and when on oral contraceptives, GnRH analogs were put forward as a candidate for treating severe GI symptoms (Mathias et al., 1998). Women with functional GI disorders were evaluated in a double-blind placebo-controlled study involving continuous treatment with the GnRH analog leuprolide (Mathias et al., 1998). The analog reduced symptom scores for nausea and abdominal pain, and improved GI motility was found. Doubts on the use of GnRH agonists for treating IBS were, however, raised (Farthing, 1998). Such disbeliefs were reinforced when a woman undergoing multiple in vitro fertilization (IVF) with GnRH analogs was diagnosed with chronic intestinal pseudo obstruction (Ohlsson et al., 2007). Histological examination of the small intestine showed a reduced number of myenteric neurons. Additional cases of abdominal dysfunction after GnRH analog treatment were to follow (Hammar et al., 2013).

Investigations into the underlying mechanisms, using an animal model mimicking IVF procedures, showed administration of the GnRH analog buserelin to rats to cause a significant loss of enteric neurons all along the GI tract after multiple (3–4) exposure periods (Sand et al., 2013a). Further T-lymphocyte infiltration in myenteric ganglia (Ohlsson et al., 2014) and reduced fecal weight with increased amount of fecal fat were identified (Sand et al., 2014).

Throughout the GI tract in both man and rat no GnRH receptor expression is found (Sand et al., 2013b). Therefore, the marked loss of enteric neurons in response to GnRH administration is most likely indirectly mediated; possibly through LH or FSH release. To date no FSH receptor expression has been identified in the GI tract of neither man nor rat, while LH receptors are expressed in myenteric neurons in both (Sand et al., 2013b).

This study aim to investigate if GnRH analog-induced enteric neuronal death, noted in vivo, is mediated by direct or indirect mechanisms,

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using in vitro cultures of primary rat myenteric neurons. Enteric neuronal survival was examined after single or repeated exposures of GnRH analog (buserelin) or recombinant human LH (lutropin alfa). In addition, the possible presence of GnRH and/or LH receptors on cultured myenteric neurons was investigated.

2. Methods

2.1. Animals

Twenty female Sprague–Dawley rats (140–160 g, Charles River, DE) and two male Sprague–Dawley rats (300 g, Charles River, DE) were used. The rats were allowed to acclimatize to the climate- and light-controlled animal facility for at least 5 days before sacrifice. Standard rat chow (4% fat/g; Lactamin R36, SE) and water were supplied at all times. All experimental procedures were approved by the animal ethics committee in Lund and Malmö, Sweden. The animals were used in accordance with European Communities Council Directive (2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:534). The female rats were used for primary culturing of myenteric neurons while tissues from male rats were used for antibody evaluation.

2.2. Myenteric neuronal cultures

Primary cultures of myenteric neurons were performed using a previously described method (Voss and Ekblad, 2014). In brief anaesthetized rats had their small intestine exposed. The longitudinal muscle with attached myenteric ganglia were stripped, without penetrating the gut mucosa. The rats were killed by heart puncture after tissue sampling. The tissue was cut into pieces (2 × 2 mm), washed in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS, Life Technologies, SE), treated with collagenase II (1.5 mg/ml, Life Technologies SE) and protease (1.5 mg/ml, Sigma-Aldrich, SE), for 25 min at 37 °C. Trypsin (1.25 mg/ml, BioChrom, DE) and EDTA (0.01%, ethylenediaminetetraacid, Sigma-Aldrich, SE) were added and incubated 20 min. Trypsin inhibition was by addition of 50% fetal calf serum (FCS, Life Technologies, SE). The cell suspension was centrifuged and washed in HBSS three times. The pellet was diluted in 2.6 ml Neurobasal A (NBA) culture medium containing 10% FCS, 0.5 mM L-glutamine, 50 U penicillin G sodium and 50 μg streptomycin sulphate per ml (Life Technologies, SE). Cultures were prepared by seeding 50 μl of the constantly mixed cell suspension into 8-well chambers (BD Falcon, VWR, SE) prefilled with 450 μl NBA culture media and incubated in a humidified incubator holding 5% CO_2. From each animal six 8-well chambers (each well 69 mm^2) were prepared. Cultures were never prepared by pooling cell suspensions from different rats. After 4 days in vitro (4 D*IV*), experimentation was initiated.

2.3. Experimental setup

All agents were diluted in NBA cell culture medium; a flow chart of the experimental design is illustrated in Fig. 1. For all experiments untreated controls receiving NBA culture medium, were run in parallel. Single exposure experiments were performed by adding buserelin (10^{-10} – 10^{-6}M Suprefact®, Sanofi-Aventis, SE) or lutropin alfa (10^{-10} – 10^{-7}M, Luveris®, MerckSerono, SE) to cultures followed by a 4 day incubation (DIV 4 + 4). The repeated exposure experiments were performed by adding buserelin (10^{-8} – 10^{-6} M) or lutropin alfa (10^{-10} – 10^{-8} M) to cultures every other day for eight days (DIV 4 + 8). On day 5, 7, 9, and 11 cultures were exposed to pharmacological agent, on day 6, 8, 10, and 12 cultures were exposed to NBA culture medium.

The possible expression of GnRH- and LH receptors on cultured myenteric neurons studied on six separate cultures, cultured for 8 days in NBA only (DIV 4 + 4), medium was changed on the 4th day.

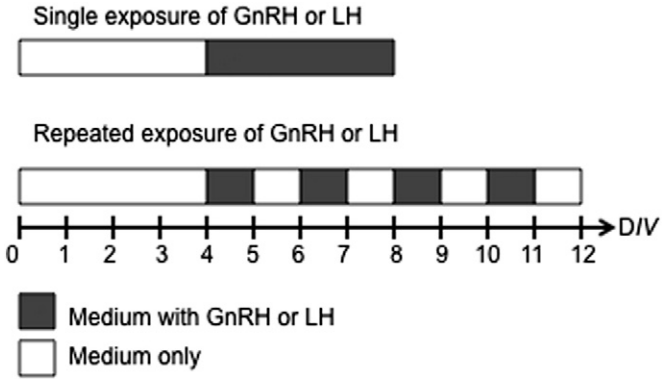

Fig. 1. Schematic illustration of the experimental setup. Single exposure was by the addition of either the GnRH analog buserelin (10^{-10} – 10^{-6} M) or the LH analog lutropin alpha (10^{-10} – 10^{-7}M) (grey boxes) to cultures after a 4 day incubation in NBA medium (white boxes) post seeding. Exposure time was 4 days followed by fixation. Repeated exposure started after a 4 day incubation in NBA medium only and was by the addition of buserelin (10^{-8} – 10^{-6}M) or lutropin alpha (10^{-10} – 10^{-8} M) on days 5, 7, 9 and 11. In between the cultures were grown in NBA medium only.

All cultures were fixed in Stefanini's fixative for 30 min and rinsed in Tyrode's solution 2 × 15 min. In order to enhance antibody penetration, the cultures were frozen in −20 °C for at least 1 h before being processed for immunocytochemistry.

2.4. Immunocytochemistry

Antibodies raised against the human neuronal marker (HuC/D) were used as a pan-neuronal marker. The cultures were rinsed in phosphate buffer containing 0.25% Triton X-100 (PBS-T), incubated with primary antibodies against HuC/D (dilution 1:400, code A-21271, produced in mouse; Life Technologies, SE; (Lin et al., 2003)) over night at 4 °C. For visualization, cultures were washed in PBS-T and exposed to DyLight TM 488-conjugated goat anti-mouse IgG antiserum (dilution 1:1000; Jackson Immunoresearch Laboratories, USA) for 1 h.

Analysis on possible neuronal GnRH- or LH receptor expressions was by double immunolabeling using antibodies against HuC/D (dilution 1:400) and GnRH-receptor (dilution 1:800, produced in goat, code no Sc-8682, Santa Cruz Biotech Inc., USA; (Sand et al., 2013b)) or HuC/D (dilution 1:400) and LH receptor (dilution 1:1600, produced in rabbit, code no L6792, Sigma Aldrich; (Sand et al., 2013b)). For visualization, cultures were exposed to DyLight TM 594-conjugated donkey anti-mouse IgG in combination with either DyLight TM 488-conjugated donkey anti-goat IgG (for GnRH receptor visualization) or DyLight TM 594-conjugated donkey anti-rabbit IgG (for LH receptor visualization) for 1 h (all 1:1000; Jackson Immunoresearch Laboratories, USA). Mounting was in PBS:glycerol 1:1 followed by fluorescence microscopy using appropriate filter settings.

2.5. Controls used in the immunocytochemical procedures

Absorption control was performed using the primary antiserum against GnRH receptors inactivated by the addition of 100 μg of synthetic peptide (code no Sc-8682P, Santa Cruz Biotech Inc. USA) per mL diluted antiserum. Controls did not exhibit any immunostaining. Synthetic antigens for testing specificities of the HuC/D and the LH-receptor antibodies are not commercially available, why omission of the primary antibodies was used as control.

For further characterization of GnRH- and LH receptor antisera, positive control tissues from rat were used. Pituitary and testis from two naïve male rats were fixed in Stefanini's overnight, rinsed three times in Tyrode's solution containing 10% sucrose, mounted in Tissue-Tek (Histolab, SE), frozen on dry ice, and sectioned (10 μm). Cryo sections from the pituitary were exposed to the GnRH receptor