Author's response to reviews

Title: Antibodies against gonadotropin-releasing hormone (GnRH) and destruction of enteric neurons in 3 patients suffering from gastrointestinal dysfunction

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Author's response to reviews: see over
Answer to the reviewers

We thank all the reviewers for their valuable criticism of our manuscript. We have revised the paper according to your suggestions, and think that it has been improved. Please therefore kindly reconsider this manuscript, 5206547702654597 – entitled “Antibodies against gonadotropin-releasing hormone (GnRH) and destruction of enteric neurons in 3 patients suffering from gastrointestinal dysfunction”, for publication in BMC Gastroenterology.

Reviewer 1

We thank the reviewer for the positive comments.

Reviewer 2

Major comments

**Question:** Case presentation 1 is a little confusing. The patient is described to have a longstanding history of nausea, vomiting and severe abdominal pain in association with weight loss. However, he is labeled as having IBS based on ROME II criteria. This patient does not appear to have IBS. His clinical presentation as well as his gastric emptying scan are most consistent with gastroparesis. The authors even describe the small the intestine as macroscopically normal and with normal peristalsis. At this point I would have expected that they would have obtained a gastric biopsy but they go on to describe that the small intestine was biopsied and that histopathology suggested ganglioneuritis. In the end, this patient ends up being labeled as having an enteric neuropathy.

It is unclear why the biopsies were obtained from the small bowel if the clinical symptoms and the gastric emptying suggest gastroparesis. It is hard to understand the significance of the histopathological findings in a small intestine that apparently demonstrated normal peristalsis. The biopsies should have focused on the stomach.

Case 2 may have represented a case of IBS although reflux esophagitis with secondary dysmotility cannot be ruled out based on the limited description. Case 3 is a case of an endstage diabetic with gastroparesis who ends up dying. Clearly, this patient was severely ill with multiple medical problems and the only case with high CD40 levels. Only serum from this case altered neuronal cell survival and this could be due to a variety of unknown factors.

The rationale for looking at CD40 levels should be introduced in the introduction. No rationale is provided.

The authors stated that the number of surviving neurons was counted. However, how does that have any meaning if we do not know with how many neurons they started out with. Although the authors state that surviving neurons were compared to controls, this would only be acceptable is the same number of neurons was present in all wells at the onset of the experiment.

The authors assessed the effect of human sera on cultured rat neurons. What is the percent homology between the human and rat GNRH. What was the hypothesis behind this experiment in which case sera (with the only common denominator the presence of GNRH antibodies) was cultured with rat neurons?
The authors stated that GNRH antibodies raised in rabbit (presumably against rat?) did not alter neuronal survival. Why did they even do this experiment? The cultured data were not at all helpful and their rationale remains elusive in the current version of the manuscript.

Answer: Generally, we have extended the three case presentations in order to make them easier to understand (M&M, page 4-8). Case 1 has a long history of gastrointestinal symptoms. During the first years, he fulfilled the criteria for IBS according to the ROME II criteria (Thompson 1999) as he had typical symptoms and normal examinations including laboratory analyses. However, after some years the symptoms aggravated and changed in character. His clinical presentation suggested more severe dysmotility. This could not only be due to gastroparesis as there were symptoms also from disturbed bowel habits. As the clinical presentation suggested severe dysmotility we performed a diagnostic laparoscopy to exclude any organic etiology to the dysmotility. As no organic reason to the pain and disturbed motor function of the bowel was found, a full-thickness biopsy was taken to confirm the suspected enteric dysmotility. The criteria for enteric dysmotility are clinical findings of dysmotility combined with pathological findings in the ENS and abnormal contractility or transit (Wingate 2002). The method where in the gastrointestinal tract it is best to take biopsies to evaluate the ENS is not clearly established (Knowles 2009). In Stockholm, biopsies have been standardized to jejunum. In the Malmö/Lund region it is standardized to ileum, one meter proximal to the ileocecal valve. This is to be able to compare the different patients to each other and to normal patients. In the clinical practice, gastric biopsies are seldom taken to study the ENS, but it occurs. The reason to standardise it to the jejunum-ileum region is that the ENS is most well-developed in this segment and this operative technique is most safe. The estimation of peristalsis during surgery is very rough and does not reveal slighter disturbances. Antroduodenal manometry is necessary to perform to find disturbed MMC complexes. However, we found this examination unnecessary as the clinical picture together with histopathology and delayed ventricular emptying gave us the diagnosis. A correlation between the degree of macroscopic peristalsis and morphology in ENS is not known from the literature.

Case 2 represents an IBS patient. EGD was normal excluding an esophagitis. For further information, see page 6-7.

Case 3 could of course have a lot of other factors as well in sera. This study does raise new hypothesis for future studies, as suggested page 16, second section . However, sera from other diabetic patients, with and without gastroparesis and inflammation, did not affect neuronal survival, page 15, line 21.

The rationale for looking at CD40 levels was that this is a key factor in inflammation and autoimmunity. Our hypothesis is that autoimmunity might be one of the causes of dysmotility. This is now included in the Background, first section.

Estimation of surviving neurons was made possible by counting the number of neurons in each culture dish. The numbers of neurons after the various treatments were compared to controls run in parallel. The number of neurons seeded in each dish is impossible to estimate since immunostaining techniques utilising neuronal markers are required for the purpose of visualising the neurones, page 9, line 20. For each experiment the same volume of dissociated neurons is added to each culture dish and consequently the same numbers of neurons are added as well. This makes numbers of neurons after the culture period comparable. The cultures run in parallel are set to 100%.
Addition of human sera on basis of their titre of GnRH antibodies to cultured rat neurons may rightly be questioned, however human and rat GnRH show 100% homology. The amino acid sequence of GnRH is identical in all mammals: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH(2). The antibodies are raised in rabbit but they are tested to be specific for GnRH in human, bovine, mouse and rat. The rationale for doing this experimentation was to study a possible direct effect (the cultures are devoid of immune cells) of the GnRH antibodies on neuronal cell death, page 4, line 10 and page 15, line 14. The outcome of these studies was not clear cut; only serum from the case 3 patient caused neuronal cell death. The rest of the human sera as well as the antiserum raised in rabbit against GnRH were ineffective in enhancing neuronal cell death. Taken together these experiments indicate that additional factors in sera from patients with dysmotility are needed in order to decrease neuronal survival.

This issue has been clarified on page 16, first and second section and under conclusion.

Minor comments

DIV is an abbreviation of “days in vitro”. Introduced in Results, page 9, line 9.

Figure 3 is now available on line. We apologize for not being available earlier.

The histopathology findings have been condensed.

1. Is the question posed by the authors well defined? The study is descriptive and raises the hypothesis about what role GnRH play in the gastrointestinal tract. It raises a hypothesis, but can not answer this or give any sure conclusions. Further studies in the same field have to answer this. This is just a first description of a phenomenon that may be important to further study in the future.

2. Are the methods appropriate and well described? The description of the methods have been improved, page 8-10.

3. Are the data sound? The cases are now better described and further clinical data is added. We have earlier published a case where a patient who received GnRH analogues repeatedly because of in vitro fertilization (IVF), developed severe gastrointestinal dysmotility and antibodies against GnRH (Ohlsson 2007). The 3 cases in this manuscript are all cases with idiopathic dysmotility who have never ever received any treatment with GnRH analogues. This is described in the Background, last section. We here raise different hypothesis, whether the GnRH antibody development is primary or secondary in the disease development. This is in the Discussion, page 16, first section.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? No comments are needed.

5. Are the discussion and conclusions well balanced and adequately supported by the data? The discussion is now almost completely rewritten and modified. We can not draw any sure conclusions from these results, only raise different hypotheses, page 16, line 3 and on and page 17 under conclusion.

6. Are limitations of the work clearly stated? Limitations of the work is stated, page 16, second section. It is a limitation that not all cases had the same effect on cell cultures, and that not all cases went trough full-thickness biopsies.

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? No comments are needed.
8. Do the title and abstract accurately convey what has been found? The findings do not lead to a solid conclusion, but we think that here is comprehensive hypotheses that hang together in some way.

9. Is the writing acceptable? No comments are needed.

Reviewer 3
General comments

**Question:** This report of 3 cases investigates the interesting hypothesis that circulating autoantibodies against gonadotropin-releasing hormone (GnRH) may lead to neuron degeneration in the gut and consequent dysmotility in a subset of patients with diverse forms of idiopathic gastrointestinal dysmotilities. The study builds on a previous case study by the same group showing dysmotility and degeneration of GnRH+ neurons in a patient who had developed anti-GnRH autoantibodies from buserelin treatment. In this paper the authors also attempt to establish a causal relationship between the autoantibodies and neuron loss by using a rat enteric neuronal culture indicator system. Strengths of the paper are the interesting hypothesis, the demonstration of anti-GnRH antibodies in the sera of patients with dysmotilities and the inclusion of the mechanistic, in vitro studies. However, the results from the latter do not support the main conclusion of the paper and the mechanistic experiments are incomplete. The authors did not attempt to discuss and interpret the intriguing findings that the majority of enteric neurons express GnRH and about one-half express receptors for GnRH. Most unfortunately, the authors also failed to ensure that their submission is complete and Figure 2A and the entire Figure 3 are missing and could not be evaluated.

**Answer:** The whole Discussion is rewritten and the main conclusion is revised as we do not know the exact mechanism of the neuron degeneration. Other factors in addition to GnRH antibodies may be involved, page 16, second section. We can not explain why there is less immunostaining of the GnRH receptor than the GnRH peptide. In the patient, the amount of staining was equal. One possible explanation to the lesser amount of GnRH receptors may be down-regulation due to prior stimulation of the peptide. This is a well-known effect on many receptors after secretion of a peptide, page 16, two last sentences. We apologize for missing Fig 2A and Fig 3.

**Major Compulsory Revisions**
1. The paper cannot be properly evaluated without the missing figures, which must be replaced. This is a mistake that the figure is missing. We apologize for this and do now include them.

2. Only serum from Case 3 affected neuron survival in vitro and commercial anti-GnRH sera were also ineffective. Despite these negative findings, the authors claim that the anti-GnRH autoantibodies in Case 3 were the most likely cause of neuron degeneration and symptoms. Based on the data, this claim is not justified. It is equally or even more likely that inflammatory cytokines in the serum of this diabetic patient with high degree of inflammation were responsible for the described neurotoxicity. To positively link the neuron loss to the
anti-GnRH antibodies, the authors need to demonstrate that the neurotoxicity can be eliminated or significantly reduced by pre-absorbing the patient’s serum with GnRH or a suitable analog. The neuroprotective effect of buserelin (applied alone) does not constitute sufficient evidence in this regard.

It is not the GnRH antibodies alone that destroy the enteric neurons. This is apparent as commercial GnRH antibodies were without effects. Further unknown factors are needed to be present in the sera to destroy the antibodies. The claim is now justified, page 16, second section.

3. The authors should discuss their intriguing observation that a very high percentage of enteric neurons express GnRH or GnRH receptors. How rigorously has their immunostaining been evaluated? Have they performed pre-absorption studies? Have they tested multiple antibodies? Is there evidence of GnRH neurosecretion or neurotransmission in the enteric nervous system? What function(s) do they think GnRH could perform in the ENS?

Different immunostainings are routine work at the Department of Pathology and are widely used in the evaluation of different biopsies. Pre-absorption has been performed by addition of the GnRH peptide to the antibody solution and the test of various antibody concentrations has also been done prior the staining of the sections. We have tested only one antibody. This is now added in the method section, page 10, second section.

We have earlier described the presence of GnRH in the human enteric nervous system (Ohlsson 2007). In rats, GnRH mRNA has been found in parietal cells of gastric glands, the epithelium of the small and large intestine, and in parasympathetic ganglion cells of the myenteric plexus. In addition, the GnRH receptor has been found in the epithelium of gastric pits (Huang 2001) and GnRH receptor mRNA in the myenteric neurons in the rat (Ho 1996). GnRH has also been detected in rat pancreas (Soldani 1982). In dog, GnRH has been shown to inhibit the release of gastric secretions and gastrin release (Schally 1999), possibly due to diminished vagal activity. To what extent this peptide is secreted or transmitted is not known, only the locations have been described.

Laboratory studies have shown that ovarian products such as progesterone, luteinizing hormone and human chorionic gonadotropin are neural antagonists of gastrointestinal motility (Ducker 1996, Wang 2003). GnRH binds to specific receptors on the gonadotropes and controls the secretion of these sex hormones (Hazum 1988). By stimulation of leuprolide, the hypothalamic-pituitary-gonadal axis is downmodulated and the gonadal hormone production inhibited (Rabin 1980). The other hypothesis is that leuprolide by acting on the GnRH receptors on myenteric neurons (Ho 1996) are effective neural modulators in disorders like functional bowel diseases, through regulating the voltage-gated calcium channels and the endoplasmic reticulum calcium pump, resulting in the movement and control of intracellular and extracellular calcium (Stojikovic 1996). This assumption is strengthened by the finding that leuprolide restored normal motor function to the gastrointestinal tract in female rats and a patient suffering from chronic intestinal pseudobstruction (CIPO) (Khanna 1992, Mathias 1992, Ducker 1996), while administration of the same drug into the intraventricular system of the rat brain had little if any effect (Heiner 1989).

We have now added some information in the discussion about the effects of GnRH in the gastrointestinal tract, page 14, last section.
Minor essential revisions

1. The space between gastro and paresis is deleted. Anti-LHRH receptor is written in the earlier 5th line from the bottom, now page 10, line 14.

DISCRETIONARY REVISION

1. The authors should consider including images of GnRH+ and GnRH receptor+ neurons in Figure 3. Images of GnRH+ and GnRH receptor + is now added in the manuscript, Fig 1A and B.

References


