**Comments from the Reviewers:**

**Reviewer 1**: The manuscript by Virtanen et al investigates a difficult and important question of whether variation in enteric nervous system (ENS) anatomy and cellular constitution is likely to correlate to interindividual gastrointestinal (GI) functional differences or even predisposition of dysfunction. The major finding is that GDNF signaling intensity is linked to both enhanced generic differentiation and specific differentiation of nitrergic neurons. The authors assemble RNA-seq data of human gut tissue from different individuals and identifies a positive correlation between GDNF and neuron markers (Pgp9.5 and Elavl4) and specific markers (NOS1, VIP, GAL), while Chat levels did not correlate to GDNF. The intestine of animals with an Gdnf-hypermorph had a denser network of neurons and a relative increase of NOS1 neurons. Interestingly, the GI-function of GDNF hypermorph mice was altered in relation to stool-size/water content, transit time, mucosal permeability and proinflammatory genes (increase). Based on previous scRNA-seq atlases from mouse and human embryos and postnatal stages, transcription factors involved in NOS1+ neuron differentiation was acquired and tested for their possible involvement in GDNF-induced ENS differentiation. Combinations of GDNF exposure in combination with shRNA-mediated suppression of candidate transcription factors demonstrated that Etv1 as a likely transcription factor acting downstream of Gdnf to promote NOS1+ neuron differentiation. This study is neat, clearly presented and shows that inter-individual levels of growth factors could play a role in the formation of the ENS cell type composition. Only relatively minor issues remain to be addressed as summarized below.

*ANSWER: We thank the reviewer for appreciating our work. Please see our point-by-point responses below.*

Issues:  
1) Considering the increase in neuronal mass in Gdnf hyperallelle mice it’s important to confidently ascertain that there is a relative increase of NOS1+ neurons in comparison to non-NOS1+ neurons. Thus, it would be appropriate to assess the percentage of HUD cells expressing NOS1 in the wt versus Gdnf hyperallelle mice (relates to Fig 1K-N).

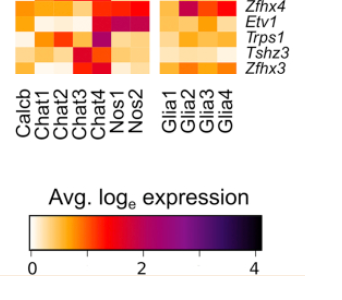
*ANSWER: We agree with the reviewer and have now done this analysis in adult mice. We observe 30% relative increase in NOS1+ neurons (Figure 1P). Additionally, we analysed Calretinin neurons, but found no relative increase (Supplementary Figure 1E).*

2) It is not completely clear why Etv3 was used as a negative control, given that the expression of this gene is very sparse in the developing and adult ENS.

*ANSWER: Etv3 was used as a negative control indeed for this reason, because of its sparse expression in the ENS as was highlighted by the reviewer. Similarly, we chose Casz1 as a negative control for denoting NOS+ population - it is sparsely expressed in NOS+ neurons (Figure 6A).*

3) Citations: Morarach et al (#24) recognized Etv1, Tbx3 and Casz1 as plausible regulatory genes in the neurogenic branching while the current manuscript only mentions Zeisel et al and Wright et al. Similarly, Wright et al recognized the correlation to Casz1 and cholinergic neurons (includes even an analysed Casz1 mutant mice), while this is not cited in the current manuscript. Moreover, Zfhx4 is not mentioned in Wright et al, only Zfhx3. Why was Zfhx4 picked and not Zfhx3?

*ANSWER: We agree with the reviewer and have now corrected the citation. Morarach et al is now included as suggested. We have now also further opened work with Casz1 in Wright et al as suggested. We acknowledge reviewers concern about not including Zfhx3. Based on Wright et al (Please see the screen capture of Figure 7 from Wright et al below) Zfhx4 is enriched in Nos1 neurons, but Zfhx3 is not which is why we included Zfhx4. Zfhx3 is enriched in a subset of cholinergic neurons and could have been used as a negative control. However, we already included a more general cholinergic marker, Casz1 (Figure 6A, D), as a negative control.*



4) Does GDNF give any increase in generic neural differentiation when supplemented to the ENS primary cultures, given that more neurons appear to form in the GDNF hypermorph animals?

*ANSWER: We thank the reviewer for this suggestion. This analysis has now been performed by measuring Pgp9.5 mRNA in our vitro experiment mRNA samples, thus under the following culture conditions: 24H 10% FBS, 24H N2 and then GDNF 24H🡪analysis. Data on PGP9.5 (Uchl1) expression with and without GDNF treatment has now been included to Figure 6C, the above details on the experiment have been added to the MM section.*

5) The titles of each subsections need revision. Some of the titles are very long, and some are not very informative.

*We have now tried to improve the information content of the subtitles and cut the length where possible.*

6) Material and Methods: It is not clear which method has been used for the transfection of shRNA constructs. Please give a more full-bodied description of the experiments.

*ANSWER: Those details have now been added into the MM section under* ***siRNA Transfection****. Briefly, we used DharmaFECT (lipid-based method) that was purchased from horizondiscovery.com.*

7) It is stated in the text that enteric neurons are transfected, however as the methodology of transfection is not described, it is hard to ascertain whether only neurons are transfected, or also progenitor cells, or perhaps only progenitor cells? For the transcription factor suppression to give an effect on NOS1+ neuron specification, it is likely that progenitors would need to be transduced.

*ANSWER: We agree.* *As the reviewer already pointed out it is the most likely that mainly the progenitors are transfected because Lipofection is not particularly effective in transfecting post-mitotic neurons* (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6632737)*. Details about the transfection kit have now* *been added into materials and methods under* ***siRNA Transfection****.*

8) Vincent et al, 2023 PNAS, demonstrated that fewer cells differentiate into Branch A neurons (ie NOS1+ cells) in Ret mutant mice. Ret/Gfra1 are the receptors for GDNF, hence the study would be complementary and in line with findings unravelled in the current study. Thus, it would be appropriate to cite and discuss this study in comparison to own data.

*ANSWER: We thank the reviewer for pointing this out. Indeed, this recent study by Vincent et al complements our work and we have now cited and discussed the results of Vincent et al in the revised manuscript Discussion section.*

**Reviewer 2:** In this study, Virtanen et al. addressed how endogenous GDNF levels vary amongst individuals and whether this GDNF variation influences ENS composition, size and function. The study was supported by the evidence that in the adult human colon, GDNF mRNA levels vary at least up to 5-fold among individuals and that GDNF levels correlate with pan-neuronal and nitrergic, but not cholinergic, marker expression. In mice, a similar upregulation of endogenous post-transcriptional GDNF expression occurs via Gdnf 3'UTR, (warranting increased expression only in naturally GDNF expressing cells) results in a similar increase in nitrergic marker gene expression and up to 3-fold increase in ENS ganglion size. Assessment of microRNAs miR-133b, miR-96 and miR-9, which bind to GDNF-encoding mRNA negatively regulate GDNF expression via defined binding sites, indicated a correlation between GDNF levels and their expression in the developing intestine. This suggests (although does not conclusively prove) that the negative regulation of GDNF levels impacts on ENS development and nitrergic innervation. A number of functional data support the increase in nitrergic innervation as indicated by delayed GI transit, increased stool size and water content although with minor changes to the epithelial barrier function. Finally, the authors also putatively identified the transcription factor responsible for GDNF activation which occurs via ETV1.  
  
This reviewer has no doubt that the paper by Virtanen et al. contains important data of interest to the CMGH neurogastroenterology community.

*ANSWER: We thank the reviewer for appreciating our work.*

However, several aspects require, in my opinion, careful re-evaluation.  
*We would like to thank the reviewer for constructive comments. Please see our point-by-point responses below.*

Comments:  
  
1. It is surprising that some experiments do not appear to be performed appropriately and even P values are not indicated (they are only in the figure legends). As an example, t-test or ANOVA would be fine for quantitative data of gene expression, assuming a normal distribution of the expression of that specific gene. However, for quantification of neurons, the use of non-parametric tests would be more appropriate to evaluate difference between groups.

*ANSWER: We agree with the Reviewer’s concern over the use of an appropriate statistical test. For quantification of neurons, we used unpaired t-test, after testing for the normality of our data. Since our data is normally distributed, we used unpaired t-test. We also assessed the data using non-parametric t-test, but the significance remained in the same order of magnitude range, i.e. the same number of “stars” per comparison. Details on data distribution testing are now added into the MM in the appropriate section.*  
  
2. The control group of samples used for each comparison is also another flaw of this work. Indeed, it is not clear what was the sample(s) that the authors used to compare with the deltaCt method the wt, wtGdnfhyper or the deltaCre animals in term of gene expression in the different tissues. This is crucial to the idea that GNDF regulates several genes including those of nitrergic signalling. As an example, in Figure 1A, which should represent GDNF expression variability in healthy human gut (from expression databases) it is reported the log2FC (fold change). Since these are all healthy, which is the comparison group? This is an unclear key aspect since it generates all subsequent data.

*ANSWER: We appreciate the reviewer's question and apologize for not opening these important details in sufficient degree. These issues are now better explained in the relevant MM sections as further detailed below. Point-by-point answer: When Gdnf hypermorphs animals were analyzed control group was litter matched wild type animals.* *When cHyper x Del-Cre animals were analyzed Control group was litter matched –Cre (Cre negative) x cHyper animals and +Cre (Cre positive) x wt animals. This is information is now included into materials and methods under the Animals section. Regarding Figure 1A, we would like to clarify that our intention was not to compare two distinct groups; rather, our objective was to analyze the diversity of GDNF expression levels observed in healthy human individual colon tissues. To achieve this, we indeed utilized publicly available databases, specifically deriving data from the GEPIA online tool based on the TCGA database. The y-axis in Figure 1A, represents the TPM (transcript per million) values in log2 scale. TPM normalizes the read counts by transcript length and the total number of mapped reads in the individual sample, allowing for comparisons of gene expression levels between different individual samples. The more formal explanation is:*

*where denotes reads mapped to the transcript , and is the transcript length.*

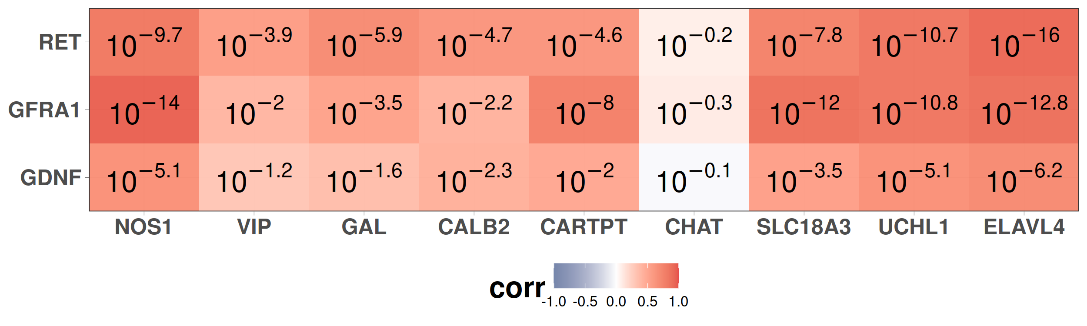
*The Figure 1A illustrates a roughly 5-fold variation between individuals in GDNF expression levels across samples from normal human colon. This 5-fold difference is derived from the ratio between the maximum and minimum values of GDNF expression. The maximum GDNF expression level corresponds to because the y-axis is on a log2 scale, where the maximum value is 2.6. Hence, the GDNF level in non-log2 scale (or TPM) is . Conversely, the minimum TPM value on the log2 scale is 0.3, resulting in a minimum TPM level of . Therefore, the fold change is calculated as Details about this has been now included into Figure 1 legends and into the MM.*

3. It should be stated how the authors retrieved these values, the comparison group used, or if the database is made by RNA sequencing data, they should show the TPM, such as for example in GTex (Genotype/Tissue database).

*ANSWER: We agree and have now included a more thorough explanation as detailed above. To summarize, The Cancer Genome Atlas (TCGA) is a landmark for cancer genomics studies that includes gene expression from cancerous and normal samples. To obtain and analyse gene expression levels data from the TCGA database, we utilized the GEPIA online tool, which integrates RNA sequencing data from large-scale projects like TCGA. In GEPIA, gene expression levels are in the TPM values in log2 scale. In GEPIA, we have selected the "COAD" from only TCGA normal (not GTEx) to access samples derived from healthy colon tissue, ensuring relevance to our study's objectives. So, the gene levels used in Figure 1A, B, and C are TPM values in log2 scale. As noted above we have now included more details into Figure 1 legend and into the MM.*  
  
4. Moreover, it seems to this referee quite naïve not to assess RET expression in healthy tissues (while looking only to GDNFRalpha1 receptor), since the actual signaling requires RET activation…Ret expression appears only in some parts of the paper (Figure 6), but it would be important to see if it occurs in all experiments.

*ANSWER: We thank the reviewer for highlighting the importance of assessing RET expression in healthy tissues, particularly in the context of GDNFRalpha1 receptor signalling. We recognize the significance of RET activation in mediating GDNF signalling pathways and agree that its inclusion in our analysis would provide valuable insights into the mechanisms underlying our observations.*

*To accommodate this suggestion, we have updated Figure 1B to incorporate correlations between RET and relevant the nitrergic neuron markers. Specifically, we have included correlation coefficients and corresponding visual representations of these relationships within the heat-map (see image below). Also, Ret expression in adult mouse is included now in Figure 1I.*



5. Data shown in the supplementary Figure 2 regarding the miRNA expression in the duodenum, ileum and colon cast doubts on the accuracy of the study. As reported in the figure, the expression value is less than the error of the Ct from a real-time experiment, therefore it is not expected to be significant. Again, it is not clear for the "relative expression" which is the control group.

*ANSWER: miR expression data is relative to snoRNA202, validated and widely used housekeeping RNA to measure miR expression levels. To further assess this point, we have now repeated the experiment with new TaqMan probes and instead of pooling samples from 8 embryos we have analysed duodenum, ileum and colon in several individual embryos. The new data is presented in new Figure 2B. Analysis in individual embryos confirmed lower expression of miR-9 and miR-133b in the colon at E13,5. However, analysis with new TaqMan probes in individual embryos did not confirm initial observation in pooled samples of miR-96 reduced expression in the colon. We thank the reviewer for the rigour. Due to relatively high cost of TaqMan miR assay, we repeated the runs for downregulated in the colon miRs (miR-9, miR-133b and miR-96) and for miRs with putative strongest effect on Gdnf mRNA and/or with strongest seed sequence conservation(Kumar et al PLOS GEN PMID: 26681446 ) and with the highest expression levels in E13.5 gut based on our previous runs on pooled RNA, thus for miR-30a, mir-33a, miR-129, miR-204, new Figure 2B and S Figure 2).*

*To further clarify, on Figure 2B relative expression is shown, giving duodenum the value of 1. More extensive detail on MM is now also given in Figure legend 2B and in Supplementary Figure legend 2A. We also note that we have now replaced Figure 2C with new data on Gdnf mRNA from individual embryos which has more datapoints and stronger statistical power as compared to the data from pooled samples on GDNF protein with n=2. Gdnf mRNA expression is given relative to housekeeper Gapdh expression, the commonly used reference mRNA. As expected for 3’UTR replacement and as demonstrated in vitro for miR-9 and miR-133b in repressing Gdnf mRNA expression (Kumar et al), in the colon Gdnf expression is comparable between genotypes (new Figure 2C).*

6. Figure 1K: HuC/D staining is very poor, no DAPI or nuclei staining is present. Moreover, are the images representative of the increase of HuC/D neurons in the wthyper? I would rather think there are no differences at all…Please provide better quality images.

*ANSWER:*We apologise for not including a more representative photo in the initial submission. New HUD+ IHC images have now been provided to better illustrate the increase in ENS ganglia size in wt/hyper animals relative to the wt (new Figure 1L). We feel that addition of DAPI might not enhance the illustrative power of the figure since all, not only the ENS nuclei would provide the signal. Important quantitative data is shown on graphs on Figure 1M, O and P.   
  
7. Figure 2B and Supplementary Figure 2: the data are presented with histograms and not as scatter plot as other real-time PCR image. Please modify according to the CMGH guidelines.

*ANSWER:**We have now replaced the Figure 2B and Supplementary Figure 2 as noted above.*

8. In methods, please change rpm with x g, since the latter are universal values, the first depend on the centrifuge used for the analysis.

*ANSWER:**Values have been updated from rpm to g.*