CORRESPONDENCE

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Evidence for Dual Pathway for Nitrergic Neuromuscular Transmission in Doubt: Evidence Favors Lack of Role of ICC

Dear Sir:

The evidence provided by Groneberg et al[1] calls to question the central conclusions of "joint mediation of relaxant effect of enteric NO by ICCs and SMCs." Rather, the novel evidence supports the alternate explanation of lack of role of interstitial cells of Cajal (ICC) in transducing nitrergic signal to smooth muscles after electrical field stimulation (EFS). Normal nitrergic relaxation was demonstrated in ICC–GCKO, but relaxation was impaired to nitric oxide (NO) donor DEA-NO in SM-GCKO fundic muscular strips precontracted with 10 μmol/L acetylcholine (ACh).[1] This is direct evidentiary for the lack of role of ICC during nitrergic neuromuscular transmission. These data are in concordance with paradigm-shifting recent evidence of intact slow inhibitory junction potential, the electrophysiologic hallmark of evoked nitrergic neurotransmission, recorded after ICC depletion by tamoxifen treatment of *c-Kit*$^{CreERT2/+}$;*LSL-R26*$^{DTA/+}$mice.[2]

When a 100-fold lower dose of 0.1 μmol/L ACh was used to precontract the strips, there was only slight outward deviation of the relaxation inhibitory curve in ICC-GCKO.[1] Based on this observation of percentage contractions/relaxations (absolute values are not mentioned),[1] an argument is presented of a possible parallel role of ICC in nitrergic neurotransmission, justifying the use of this lower agonist dose as appropriate for generating tetany of the strips. The authors forward the rationale that the lower dose "more closely mimics endogenous levels of evoked ACh release."[1]

Few studies have addressed ACh release in gut neuromuscular strips under basal conditions or EFS.[3] Neurotransmitter release is a non–steady-state phenomenon.[4,5] Suboptimal dose of ACh makes most of the present evidence equivocal.[1] Reliable in vitro and in vivo comparisons of neurotransmission cannot be made.[5] Relaxation inhibition curves depend on agonist doses, making them unreliable to analyze inhibitory neurotransmission.[4] EC_{99} of ACh or U46619 should have been calculated to induce optimal tetany of the strips before quantifying relaxation and, even if the mechanical recordings were challenging, log–log scale should have been used, which are known to generate linear responses with only few data points at a much lower concentration than EC50.[4] The basal tone was increased in SM/ICC-GCKO.[1] It is unclear how normalization was performed.

sGC agonist BAY41-2722, which modifies β1 to effect haem on the β2 subunit, produced residual relaxation in SM-GCKO tissues,[1] raising the possibility of incomplete $sGC_{\beta 1}$ deletion. Nearly 50% residual relaxation observed at 4 Hz EFS in SM-GCKO[1] may be suggestive of incomplete knockdown of sGC, rather than intermediation by a third cell like ICC.[1] Double knockout SM/1CC-GCKO showed 50% relaxation to externally applied DEA-NO and this response was abolished in the presence of sGC inhibitor ODQ,[1] indicating residual sGC enzyme after genomic deletion. Critical missing information is the efficiency of recombinations. Germline-transmitted SMMHC-Cre, which was used to delete sGC in smooth muscles,[1] is known to only marginally transfect in stomach.[6] Control data for gene targeting efficiency, like quantitative Southern for deleted sGC DNA, qRT-PCR for Cre mRNA, and Western blot analysis for sGC specifically in smooth muscles and double knockouts are lacking. The median values of whole gut transit times of all groups nearly overlap,[1] again raising doubts about efficiency of genomic deletion.

SM/ICC-GCKO showed scant ($\sim$10%) relaxation after EFS at 4 Hz.[1] Relaxing responses to higher frequencies of stimulations were not examined in the current study. Earlier studies have utilized EFS frequencies up to 40 Hz to examine enteric nitrergic neurotransmission.[7] The authors hypothesize that "relaxing responses on EFS with frequencies up to 4Hz are based on release of NO." [1] What is intriguing is that slope of the relaxation curve in double knockouts remains unchanged after EFS even in the presence of BAY41-2722.[1] This later curve should have shown a downward shift. Lack of this shift may have resulted from inadequate dose of agonist so that the muscle strip may not have acquired E_{max} at all, and so, failed to show any relaxation. This may also have resulted from inadequate stimulation frequencies. The discrepancy of why DEA-NO but not BAY41-2722 relaxes SM/ICC-GCKO merits resolution in future studies.

The diffusion constant of NO is very high (3300 μ^2/s).[8] Actions of NO are long range and mainly determined by the cell's preprogrammed characteristic response,[8] rather than the widely held notion in the field of enteric neurotransmission of proximity to NO source (nerve varicosity).[1,2] Although several aspects of experimental approaches of this current study[1] may be critiqued, this study utilizing novel, cell-specific genomic deletion provides unique evidence of the lack of role of ICCs in nitrergic mechanical relaxation.[1]

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Conflicts of interest

The author discloses no conflicts.

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Reply. We are grateful for the opportunity to reply to Dr Chaudhury, who disputes the conclusions of our recent publication.[1] In our study, we have shown nitrergic relaxation in the murine fundus to be mediated by a dual pathway involving both interstitial cells of Cajal (ICC) and smooth muscle cells (SMC).

Cre expression is a critical point when working with inducible knockout (KO) mice. However, comparison of the SMMHC-Cre strain cited by Dr Chaudhury[2] to that used in our study[3] is not appropriate. These 2 transgenic strains do not only differ in the construct (SMMHC-Cre vs SMMHC-Cre-ERT2), but also in the genomic location, which critically influences expression. Wirth et al[3] clearly showed SMC-specific Cre expression in the stomach; in addition, we repeated this very critical aspect and confirmed the virtually complete Cre-induced β-gal staining in fundus SMC.[4] In this context, Dr Chaudhury criticizes the lack of a control for the efficiency of gene targeting. We provide this information in the Methods. Also, in a given tissue that contains a multitude of different cells, quantitative Southern blot, qRT-PCR, and so on, would not be adequate for the determination of excision efficiency. Furthermore, one has to keep in mind that inducible Cre-mediated 'KO' technology much rather resembles a 'knockdown' approach. In our paper, we clearly indicate that ICC-specific recombination does not amount to 100%. Nevertheless, even the submaximal deletion of NO–GC clearly results in a functional defect which we use as proof for a promotor-specific deletion.

A fundamental issue when investigating the function of ICC in nitrergic relaxation is the use of pharmacologic versus direct nerve stimulation. As shown in our and many other publications, the pharmacologic approach is powerful for the description of biochemical pathways involved in contraction/relaxation, but one has to be careful with regard to the physiologic interpretation. Therefore, the results obtained from these 2 approaches should not be mixed up, but rather interpreted separately. This has to be kept in mind when discussing a further point of critique, namely that NO from the donor compound DEA-NO relaxes SM/ICC-GCKO fundus but that the GC sensitizer Bay 41-2272 failed to induce an effect in EFS experiments. Relaxation to pharmacologic DEA-NO only occurred at very high concentrations in the ICC/SM-GCKO fundus (nearly 3 orders of magnitude higher than in WT), which can be explained either by a dramatically reduced GC expression, GC expression in yet another cell type, or by unspecific mechanisms of the NO donor. In contrast, the lack of effect of Bay 41-2272 in the EFS experiment is based on the fact that NO-GC in ICC and SMC is absent, which prevents the activation of neuronally released NO.

We apologize for having to correct several aspects of Dr Chaudhury's comment: The isometric force studies including the normalization were performed with IBMX as referenced in the Methods.[1,4] We nowhere state that "basal tone was increased in SM/ICC-GCKO." We used carbachol, not acetylcholine. The compound Bay 41-2272 acts as GC sensitizer; it does not "modify the β1 to affect haem on β2 subunit"; the β2 subunit is not expressed in mice.

In his response, Dr Chaudhury notes that the relaxation after EFS at 4 Hz may be too low to study nitrergic relaxation referring to a study by Kasakov et al.[5] Reference to this study is not appropriate, because it investigates anococcygeus muscle in rabbits (in which the occurrence of ICC is unknown), not fundus smooth muscle in mice. However, Dr Chaudhury is right in stating that the effect of EFS in fact depends on the stimulation frequency; in the murine fundus, frequencies up to 4 Hz have been shown to be basically NO-dependent, whereas at higher frequencies also other inhibitory neurotransmitters (VIP, ATP, etc) may come into play.[6] However, the nitrergic response does not only depend on the stimulation frequency but also on the duration of the stimulus, voltage, tissue size, species, electrode size, and distance to the tissue etc.

We would like to point out that nitrergic relaxation, in addition to ICC, may occur directly through NO-GC in SMC. But we think that our data unequivocally demonstrate the participation of ICC. The most important experiment is shown in Fig. 4 of our publication. Here, we abstained from any pharmacologic administration; the effect of NO is unveiled by the inhibitor ODQ in EF-stimulated tissues. Deletion of NO-GC in ICC or SMC reduced the nitrergic response to about 50%, respectively, and the double deletion abolished the response. These results clearly support the involvement of ICC in nitrergic relaxation.

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