Evidence for engagement of the nucleus of the solitary tract in processing intestinal chemonociceptive input irrespective of conscious pain response in healthy humans

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Abstract

Neuroimaging studies have revealed important pathomechanisms related to disorders of brain–gut interactions, such as irritable bowel syndrome and functional dyspepsia. More detailed investigations aimed at neural processing in the brainstem, including the key relay station of the nucleus of the solitary tract (NTS), have hitherto been hampered by technical shortcomings. To ascertain these processes in more detail, we used multiecho multiband 7T functional magnetic resonance imaging and a novel translational experimental model based on a nutrient-derived intestinal chemonociceptive stimulus. In a randomized cross-over fashion, subjects received duodenal infusion of capsaicin (the pungent principle in red peppers) and placebo (saline). During infusion, functional magnetic resonance imaging data and concomitant symptom ratings were acquired. Of 26 healthy female volunteers included, 18 were included in the final analysis. Significantly increased brain activation over time during capsaicin infusion, as compared with placebo, was observed in brain regions implicated in pain processing, in particular the NTS. Brain activation in the thalamus, cingulate cortex, and insula was more pronounced in subjects who reported abdominal pain (visual analogue scale > 10 mm), as compared with subjects who experienced no pain. On the contrary, activations at the level of the NTS were independent of subjective pain ratings. The current experimental paradigm therefore allowed us to demonstrate activation of the principal relay station for visceral afferents in the brainstem, the NTS, which was engaged irrespective of the conscious pain response. These findings contribute to understanding the fundamental mechanism necessary for developing novel therapies aimed at correcting disturbances in visceral afferent pain processing.

Keywords: Brain imaging, Nucleus of the solitary tract, Visceral pain, Gut–brain axis

1. Introduction

Neuroimaging studies have proven to be invaluable for exploring the distinct properties of visceral sensory processing. Specifically, processing of afferent input from the gastrointestinal tract has received increasing attention as a key mechanism in disorders of brain–gut interaction (DBGI). These include highly prevalent conditions such as functional dyspepsia (FD) and irritable bowel syndrome (IBS), both characterized by visceral pain as a cardinal

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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http://dx.doi.org/10.1097/j.pain.0000000000002538
symptom. The perception of visceral pain requires integration of interoceptive signals with emotional–cognitive inputs in the brain, which occurs in a nonlinear fashion. Previous studies focusing on brain–gut interactions in the context of visceral pain have primarily used mechanical stimuli, such as balloon distension. These studies have uncovered key brain regions including the perigenual anterior cingulate cortex, anterior midcingulate cortex, anterior insula, thalamus, secondary somatosensory cortex, and amygdala. More recently, the relevance of these regions across different types of visceral pain (esophageal, gastric, and rectal distension) has been corroborated in analyses from functional magnetic resonance imaging (fMRI) studies investigating various types of somatic and visceral pain. In addition to the ability to discriminate visceral from somatic pain, distinct neural features were shown across different types of visceral pain. The specificity of such features is intriguing and calls for more in-depth understanding from different visceral models.

We therefore consider possible knowledge gaps regarding studying visceral nociception using neuroimaging. First, ecological validity could be improved by looking beyond mechanical stimulation and including chemical stimuli, which in daily life originate from dietary intake. In addition, administering stimuli to areas of the gastrointestinal tract that are accessible through a minimally invasive manner, such as the esophagus and rectum, may be less relevant within the general context of DBGI pathophysiology. The duodenum, in particular, has been implicated as a key region in this respect but until now has remained unexplored in visceral pain neuroimaging studies. Finally, previous experiments performed in the early 2000s have identified key primary afferent processing regions in the brainstem, which purportedly include the nucleus of the solitary tract (NTS). These studies have formed the cornerstone of early brainstem research in visceral pain processing but have been limited in spatial resolution and spatial specificity by the technology available at that time. Technological advancements from the past 2 decades have vastly improved the localization accuracy of the brainstem MRI signal.

We therefore used multiecho multiband 7T fMRI, specifically designed to study brain regions, in particular the brainstem, that exist but have not been able to capture properly because of technical limitations of standard whole-brain 1.5 and 3T fMRI. In addition, we used a novel translational experimental model based on a nutrient-derived intestinal chemoinceptive stimulus, reflective of the clinical phenomenon of meal-induced exacerbation of pain in DBGIL. We hypothesized that the NTS, a key relay station for visceral inputs, would show increased activity during chemostimulation of the duodenum. In addition, we hypothesized that the aforementioned brain regions known to be responsive to visceral pain would be engaged and that the blood oxygenation level–dependent (BOLD) response in these regions would correlate with subjective pain ratings obtained during scanning.

2. Methods

2.1. Subjects

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center/University of Maastricht and registered on clinicaltrials.gov (NCT02551029). Subjects gave written informed consent and received a monetary compensation for participation. Only women were included, given that most of the patients with DBGI are female, as well as to select a homogeneous population. Subjects aged between 18 and 65 years and with a body mass index between 18 and 30 kg/m² were considered eligible. Subjects did not have any significant medical history, did not meet the Rome IV criteria for IBS or FD, and were all right-handed.

2.2. Sample size

The current study was the first to investigate NTS engagement during a duodenal chemoinceptive stimulus using multiecho multiband 7T fMRI, yielding a high number of repeated measurements. Both multiecho acquisition (which enables multiecho independent component analysis) and repeated measurements (resulting in reduced within-subject variance) are associated with increased statistical power. Therefore, a sample size of 21 subjects was deemed sufficient for the current study during conceptualization. This sample size is in line with earlier brain imaging studies, which provided statistical evidence using small collectives, that is, between 12 and 21 participants.

2.3. Experimental design

The study was performed in a randomized cross-over fashion with the administration of a solution of the transient receptor potential vanilloid 1 (TRPV1) agonist capsaicin or saline in the duodenum in 2 separate scanning sessions. We have previously successfully implemented this model outside the scanner environment to induce pain and other upper gastrointestinal sensations. The test days were separated by a wash-out period of at least 1 week. At the start of each scanning visit, a nasoduodenal tube (Bengmark, Flocare; Zoetermeer, the Netherlands) was positioned under fluoroscopic guidance. In all subjects, fMRI scanning was first commenced without the administration of any substance. After a baseline scanning period of 6 minutes, the duodenal infusion of 1 of 2 substances (capsaicin or saline) was started automatically, as the infusion pump (EmpowerMR Injector System; Bracco Injeneering SA, Switzerland) was programmed with a fixed delay. Web-based randomization software (http://www.randomization.com) determined the order of the substances administered. Randomization was counterbalanced to limit confounding sequence effects. Subjects were blinded to the substance being administered. Duodenal infusion lasted 20 minutes for both capsaicin and saline. Postinfusion functional scanning continued for 10 minutes. The total duration of each functional run was 36 minutes and was completed without interruption in every subject. During scanning, subjects completed visual analogue scale (VAS) every 3 minutes for 3 symptoms: abdominal pain, abdominal discomfort, and abdominal burning. This resulted in a total of 13 VAS measurement time points (each including 3 symptoms). An MRI-compatible joystick was used to gather VAS scores on a continuous scale, with at one end 0 mm, meaning that the respective symptom was not present, and at the other end 100 mm, which translated to the symptom being unbearable. VAS scales were back-projected onto a screen visible to the subject by a mirror while in the scanner. Each scale was visible for a fixed duration of 6 seconds to allocate each VAS score to the corresponding fMRI images.

All subjects received a dose of 1.2 mg of capsaicin, which is slightly less than the 1.5 mg previously used, as we needed the necessary adjustments for flow velocity considering the setting of scanner experiments. This dose corresponds to the daily intake of capsaicin in Europe. The capsaicin solution was administered at a rate of 6 mL/minute (concentration 0.01 mg/mL), yielding a total administered volume of 120 mL. Physiological NaCl 0.9% (B. Braun) was used for saline infusion. Given that the capsaicin was dissolved in alcohol, the same amount of alcohol (0.46 mL 96%)
was added to the final saline solution to rule out any (brain activity) effect related to alcohol administration. The same flow rate was used for saline as for capsaicin infusion.

### 2.4. Magnetic resonance imaging and physiological data collection

Blood oxygenation level–dependent fMRI data were collected on a Siemens Magnetom 7T scanner (Siemens Healthineers, Erlangen, Germany) using a 32-channel Nova Medical head coil. Functional MRI data were acquired with a multiecho EPI (ME-fMRI) sequence.27,28

Each fMRI scan consisted of 1350 volumes per echo time, yielding 4050 images in total. Concurrent with MRI scanning, pulse rate and respiration signals were collected with the use of a pulse oximeter (left index finger) and respiratory belt, respectively.

### 2.5. Magnetic resonance imaging data analysis

Preprocessing was performed using a combination of Statistical Parametric Mapping (SPM12, Wellcome Centre for Human Neuroimaging, UCL, London, UK), CONN toolbox (19.b),41 the Oxford Centre for Functional MRI of the Brain (FMRIB) Software Library (FSL; v.6.0), and TE Dependent ANAlysis (tedana). For details, please see supplementary methods (available at http://links.lww.com/PAIN/B537).

Data were analyzed with SPM12 using a previously developed pharmacological MRI analysis method.42 As a part of the first-level analyses, the 225 volumes acquired during the preinfusion period were used as baseline for each condition (capsaicin vs saline). No significant differences were found when comparing baseline scans between conditions (data not shown). All remaining volumes were then equally divided into 45 time bins of 25 volumes (40 seconds per time bin). Signal averages for each time bin were compared with the baseline average per condition using regression analysis. Signal changes were subsequently subtracted between conditions, resulting in 1 t-contrast per time bin comparing the brain response between capsaicin and saline relative to preinfusion baseline. A high-pass filter of 2160 s (number of volumes × TR) was used to minimize the influence of very low frequency noise in the BOLD signal. A second-level voxel-wise analysis was performed on the t-contrasts from the first-level analysis. For this purpose, a repeated-measures analysis of variance model was applied to compare the difference in signal change (relative to baseline) between capsaicin and saline over time bins at the group level, with the condition-by-time interaction effect being the effect of interest. The resulting statistical parametric map was thresholded at the voxel level at $P < 0.05$ with family-wise error correction. A predefined mask encompassing pain-responsive regions was used, as obtained from the Neurosynth meta-analytic registry (https://neurosynth.org/analyses/terms/pain). The Neurosynth pain mask is based on z scores from a 2-way analysis of variance testing of 516 studies for the presence of a nonzero association between term “pain” and voxel activation using a false discovery rate criterion of 0.01.

To aid interpretability, a cluster extent threshold of 10 voxels was used for all analyses. Additional region-of-interest and laterality analysis was performed for the NTS (see supplementary methods, available at http://links.lww.com/PAIN/B537).

### 2.6. Subjective responses and their relation to the blood oxygenation level–dependent signal

Visual analogue scale scores were calculated as differences compared with baseline ($\Delta$VAS). Given the high number of zero values in the subjective responses, the distribution of the $\Delta$VAS scores was strongly right-skewed. A Wilcoxon signed-rank test (proc univariate, SAS 9.4 [SAS Institute, Cary, NC]) was therefore used for comparing mean $\Delta$VAS scores.

Correlational analyses between fMRI BOLD data and VAS scores were deemed unfeasible because of the nonnormal distribution. A response criterion was therefore introduced. Groups were defined based on a response criterion for abdominal pain ratings during capsaicin infusion. This criterion was set at a maximum VAS score of 10 mm, allowing to differentiate subjects who only perceived supraliminal nonpainful sensation at most (“nonresponders”) from subjects who perceived at least minimal to moderate abdominal pain (“responders”).

### 2.7. Photoplethysmographic signal processing and analysis

Photoplethysmographic (PPG) data were used to compute pulse rate variability (PRV). Pulse rate variability, analogous to heart rate variability, is considered a parameter for cardiac parasympathetic activity.3,30 Specifically, the root mean square of successive difference (RMSSD) is one of the widely used and well-validated measures of heart (and pulse) rate variability.25,26 Although the RMSSD is generally calculated from electrocardiogram data, it can also be calculated accurately from PPG data in healthy subjects at rest.35 Marginal linear mixed models (SAS proc mixed) were used to compare RMSSD change from baseline between conditions using SAS 9.4 (SAS Institute, Cary, NC). For more details, see the supplementary methods, available at http://links.lww.com/PAIN/B537.

All authors had access to the study data and reviewed and approved the final article.

### 3. Results

#### 3.1. Study subjects

Twenty-six participants were included in the study. Subjects’ mean age was 25 years ($\pm$ 4.4), with a mean body mass index of 22.7 kg/m² ($\pm$ 1.9). Two subjects were excluded because of failure to position the nasoduodenal tube within the duodenum, 2 subjects were excluded based on excessive movement during scanning (confirmed with the ART toolbox after visual quality control, >20% of volumes with frame-wise displacement $>0.9$ mm), 1 for inadequate fMRI data quality (significant ghosting effects suspected to be related to a dreadlock hairstyle), 1 subject requested termination of scanning because of urinary urgency, and 2 subjects dropped out for logistic reasons (inability to plan second scanning visit). This resulted in a study population of 18 subjects that was used for data analysis.

#### 3.2. Subjective symptom reporting

The intensity of symptoms elicited varied across individuals (Fig. 1 and Supplementary Figure 1, available at http://links.lww.com/PAIN/B537), in line with our previous observations.38 The group mean abdominal pain score was 9.8 mm (on a 100 mm VAS, $\pm$ 2.4 mm, range 0-25.2 mm, Wilcoxon signed-rank test, $W = 248$, $P = 0.001$). Based on the responder criterion, 8 participants were abdominal pain responders and 10 participants nonresponders.
Visual analogue scale scores for abdominal discomfort during and after capsaicin infusion were significantly higher as compared with VAS scores during and after saline infusion, with a mean of 11.5 mm for capsaicin (SD 19.5 mm, range 0-80.9 mm) and 4.5 mm for saline (SD 10.5 mm, range 0-48.0 mm, Wilcoxon signed-rank test, W = 244, P = 0.004). The mean VAS for abdominal burning was 6.6 mm for capsaicin (SD 15.6 mm, range 0-73.8 mm) and 2.3 mm for saline (SD 8.6 mm, range 0-54.0 mm, Wilcoxon signed-rank test, W = 221.5, P = 0.026). For all symptoms, VAS scores were significantly higher during the infusion period as compared with the postinfusion period (data not shown).

Abdominal pain and discomfort demonstrated a strong correlation (Spearman r = 0.74, P < 0.001), whereas correlation between abdominal pain and burning was more modest (Spearman r = 0.54, P < 0.001).

3.3. Blood oxygenation level–dependent response
Capsaicin significantly increased the BOLD signal (condition-by-time interaction effect) in most regions included in our mask consisting of predefined regions of interest, including the right subgenual anterior cingulate cortex, bilateral anterior midcingulate cortex, bilateral insula (across all subregions), left postcentral gyrus, right supramarginal gyrus, bilateral Rolandic operculum, left superior temporal gyrus, right putamen, right amygdala, bilateral ventral thalamus, left cerebellum (regions 4, 5, and 8), nucleus cuneiformis, and posterior region of the medulla oblongata (Fig. 2 and Supplementary Table 1, available at http://links.lww.com/PAIN/B537). The latter was of particular interest given the primary hypothesis of NTS activation.

3.4. Brainstem responses
To aid differentiation of active brainstem nuclei, analyses were repeated with unsmoothed data, as shown in Figure 2. We observed several distinct clusters in the medulla oblongata. A bilateral activation was seen in the upper medulla (Fig. 3A and B), whereas the lower medulla comprised 1 posteriorly centered cluster (Fig. 3C). A high-resolution brainstem atlas was used for identification of active brainstem clusters. A—primarily right sided—portion of the typical V shape of the NTS can be recognized in Figure 3, with the most caudal part displayed in Figure 3C. The latter represents the commissural nucleus, which corresponds to the NTS subregion that preferentially receives projections specifically from visceral TRPV1-positive vagal fibers. In addition, the axial slice shown in Figure 3B and E encompasses a larger right-sided cluster, which includes purported nucleus ambiguus (Namb) and spinal trigeminal nucleus (SpV). Finally, 1 smaller cluster could be seen just posterior to the left NTS, which includes purported dorsal motor nucleus of the vagus nerve (DMNX) (Figure 3E). Given the presence of second-order projections from the NTS to the SpV, Namb, and DMNX, we believe that the activations of these regions are downstream from the primary NTS activation. Namb and DMNX are both premotor nuclei implicated in the generation of autonomic response patterns evoked by physiological and various sensory stimuli.
3.5. Differences in blood oxygenation level–dependent response according to perceptive responses

We subsequently compared BOLD responses between groups according to the perceptive responses, i.e., responders vs nonresponders. Results are shown in Figure 4 and Supplementary Table 3, available at http://links.lww.com/PAIN/B537. Blood oxygenation level–dependent responses particularly in the ventral thalamus, pregenual anterior cingulate cortex, and anterior insula were more pronounced in responders compared with nonresponders. Interestingly, we observed an increased BOLD response at the location of the NTS (y coordinate $-44$, z coordinates between $-52$ and $-58$) both in responders and nonresponders. The fact that no difference was seen between responders and nonresponders was confirmed by an additional region-of-interest analysis (Supplementary Table 3, available at http://links.lww.com/PAIN/B537).

We further examined differences in BOLD response between responders and nonresponders at the level of the thalamus, an important relay station to the cortex downstream from the NTS, using high-quality parcellations that included 16 functional zones (Supplementary Table 4, available at http://links.lww.com/PAIN/B537). According to the perceived pain response, significant differences in BOLD response were found for the mediodorsal nucleus, ventral lateral nucleus, and intralaminar nuclei of the thalamus. In addition, to ascertain the potential role for other subcortical sensory relay stations more rostral to the NTS, we also examined, a posteriori, activation in the parabrachial complex and locus coeruleus in responders and nonresponders, but this provided no evidence for their involvement because ROI analysis did not demonstrate significant clusters (Supplementary Table 3, available at http://links.lww.com/PAIN/B537). Similarly, we investigated further the activation of the periaqueductal gray matter (PAG), which was included in the pain mask. For the PAG, we did observe an active cluster just above the extent threshold (i.e., 10 voxels) but in responders alone (Supplementary Table 4, available at http://links.lww.com/PAIN/B537). When comparing responders with nonresponders, however, we did not observe a significant difference.

3.6. Autonomic outflow: heart rate variability

We finally ascertained the effects of capsaicin infusion on autonomic outflow, as a functional peripheral readout of the intervention, using PRV as calculated from the PPG signal of the pulse wave. We observed a significant increase in the root mean square of successive beat-to-beat difference (RMSSD), which is a marker that reflects on cardiac parasympathetic activity, during capsaicin infusion. Such changes were not observed during or after saline infusion, resulting in a significant difference between conditions (marginal linear mixed model, main effect of condition $F(1,12) = 5.49, P = 0.037$). Moreover, the increase in RMSSD after capsaicin was driven by the abdominal pain responder group, resulting in a significant difference between responders and nonresponders.
and nonresponders (marginal linear mixed model, condition-by-responder interaction effect $F(1,11) = 5.49, P = 0.039$) (Fig. 5).

4. Discussion

Brainstem nuclei are assumed to play a key role in the processing of visceral afferent inputs, although investigating their function in more detail has hitherto been hampered by technical challenges. Here, by combining dedicated multiecho multiband 7T fMRI with a nutrient-derived intestinal chemonociceptive stimulus in healthy individuals, we demonstrate activation of the principal relay station for vagal afferents, the NTS, which was engaged irrespective of the conscious pain response.

Complex statistical analytic methods and computational algorithms aside, advances in the field of neuroimaging in relation to DBGIs have more recently been limited by the lack of relevant technical developments that allow more specific identification of brainstem nuclei. These challenges are related to the small size of many brainstem structures, MRI signal distortion due to physiological noise (generated by chest motion and the propagation of cardiac pulse pressure waves), and magnetic susceptibility–induced distortions because of its proximity to air-filled cavities. Given the length of the paradigm in the current study and the near whole-brain coverage, an EPI image resolution of 2.2 mm isotropic was deemed the maximum, despite the high-power field strength. This was considered sufficient for NTS imaging because of the added benefits of multiecho acquisition and ultra-high field strength, providing increased signal-to-noise and contrast-to-noise ratios. The latter is corroborated by a recent 7T fMRI study mentioned previously in which NTS engagement was reported after applying a 2-mm Gaussian smoothing kernel (using single echo EPI), resulting in a target resolution similar to our unsmoothed data.

In addition, we here applied a duodenal chemonociceptive stimulus that has hitherto not been used within the context of neuroimaging. Given the observed NTS activation, the question arises which conduit was used to transmit the afferent input from the intestinal mucosa to the brainstem. The upper GI tract receives dual innervation, vagal and thoracolumbar spinal, where the former is believed to transmit chemical and the latter more nociceptive stimuli. The NTS has been hypothesized to receive input from both systems. Considering the—at least on average—relatively low intensity of the pain perceived after the stimulus administered, one would assume the principal involvement of the vagal pathway. This proposition is supported by the fact that cardiac parasympathetic activity, as reflected in cardiac parasympathetic activity, was indeed the pain responders who were engaged by the RMSDD, increases over the course of the intervention, whereas a decreased parasympathetic activity would be expected of a more salient, spinally mediated acute painful stimulus. However, it was indeed the pain responders who demonstrated this increase in RMSDD in particular, which would be contrary to this commonly accepted view. On the other hand, previous studies have failed to show a relationship between perceived pain intensity and changes in heart rate variability, which we believe is reflective of the complexity of neural mechanisms, integrating perceptual, interoceptive, and cognitive elements, which are involved in the control of autonomic outflow.

An important observation we made here was that the degree of BOLD response to capsaicin was related to the subjects’ perceptive response, ie, more pronounced in responders vs nonresponders. This observation is in line with the postulate that there is a direct relationship between visceral stimulus salience (subliminal, liminal, or supraliminal) and the extent of brain activation, although this phenomenon has hitherto only been described for cortical responses, but not for the brainstem. Interestingly, no differences in BOLD activation were observed between responders and nonresponders on the level of the NTS, and the differences only became apparent when looking at the thalamus (and more rostral areas). It remains unknown which...
levels of the neuraxis support the differences in thalamic activation that are related to perceptive responses. The PAG showed activation in a small cluster in responders alone, with no significant difference between responders and nonresponders, rendering the exact involvement of PAG uncertain. In addition, we did not identify activation in any other sensory relay nuclei, such as the locus coeruleus or the parabrachial nucleus, albeit these analyses were performed a posteriori and might have been limited in extent. Nevertheless, we speculate that monosynaptic projections from the NTS directly to the thalamus may have substantial influence in determining whether the nociceptive signal will result in painful perception. As for the origin of the thalamic input, we investigated the activation of the different thalamic subnuclei. Those showing the most pronounced activation, ie, the mediodorsal nucleus, ventral lateral nucleus, and intralaminar nuclei, have all been implicated to receive input from the vagus nerve, with the mediodorsal nucleus projecting interoceptive information to the insula and orbital frontal cortex. Therefore, we believe that this observation supports the postulate that differences in perceptive responses primarily originate from vagal pathways as opposed to spinal pathways because the latter would be expected to yield a more pronounced BOLD response in the ventral posterolateral nucleus.

More detailed understanding of the processing of afferent input through the vagus nerve derives its relevance from the fact that the vagus is the major bidirectional highway of brain–gut connection. The afferent branch of the vagus nerve has therefore been the focus of multiple studies examining its effects on brain–gut communication and more broadly the microbiota–gut–brain axis, in both health and disease states. Therefore, although this study was performed in healthy volunteers, findings can potentially carry important clinical implications. First, they allow fundamental insights necessary for the development of novel therapeutic strategies aimed at the correction of disturbed visceral afferent pain processing. One of these recent developments include the use of transcutaneous stimulation of the auricular branch of the vagus nerve. Initial studies have provided evidence for its efficacy when using subliminal electrical stimuli to the auricular vagus for the treatment of functional abdominal pain and IBS in adolescents. The authors assumed that this
therapy acts by virtue of NTS stimulation, and heart rate variability was able to predict clinical responses,\textsuperscript{14} although the exact mechanisms of action admittedly remain to be elucidated. Second, the experimental capsaicin pain model and ultra-high field imaging technology applied here also allow their future application for investigating underlying pathomechanisms in conditions related to altered visceroperception beyond FD and IBS, such as other DBGIs, ie, reflux hypersensitivity, functional heartburn or chronic nausea, and vomiting syndrome, but more broadly also eating disorders, including avoidant-restrictive food intake disorder.

Although this study was limited by its sample size, which could imply that findings are idiosyncratic to this particular experimental context, rather than truly reflecting the mental operations under study,\textsuperscript{15} the cortical activations correspond to those described in previous studies examining visceral pain.\textsuperscript{36} In addition, the magnitude of alterations in both brain activation and autonomic outflow was clearly delineated according to the individuals’ pain perception, suggesting that these differences indeed reflect a distinct biological response. Although we eventually included 18 subject in the final analysis instead of the 21 originally planned, a recent study using 7T brainstem imaging to show activation of the NTS in 16 subjects that became available at the time we had completed data collection, corroborated the adequacy of our sample size.\textsuperscript{33} In addition, we acknowledge that localization of the NTS as based on an atlas that is not provided in a normalized (common) brain space, such as Montreal Neurological Institute, is difficult. We therefore complemented our analysis of the NTS by using a hand-drawn NTS ROI based on high-resolution brainstem scans from a subset of subjects, which confirmed the medullary clusters to be part of the NTS. In addition, a recent study using vagus nerve stimulation at 7T fMRI reports similar coordinates of the NTS in the y and z direction, further corroborating our findings.\textsuperscript{32} Furthermore, the use of a predefined mask as opposed to whole-brain analysis means that the results primarily provide high sensitivity but lower specificity by excluding regions that are not of interest.

Another limitation is the overall low pain ratings, making distribution not normal and cutoffs therefore somewhat arbitrary by necessity. This might emphasize the importance of a wider range of other visceral symptoms and aversive sensations, such as nausea and early satiation, which were not assessed in detail here but make this type of investigation more relevant also to adjacent fields and clinical conditions, as mentioned above.

5. Conclusion

In this study, we exploited the benefits of multiecho multiband 7T fMRI to identify central processing of visceral afferent inputs to the brainstem arising from the duodenal region. Using advanced imaging and a novel stimulation paradigm, we were able to identify activation of the brainstem, corresponding to the key anatomical area of the NTS, assumed to result from vagal afferent excitation. At this level of the gut–brain axis, nociceptive processing seems to operate regardless of the perceptive responses. The differentiation according to the perceived pain seems to occur more downstream along the afferent gut–brain axis. The results presented here can further prompt future work on the exploration of fundamental mechanisms related to how pain emerges from nociception as well as new therapeutic approaches to treating visceral pain conditions, for instance, by targeting specific areas within the gut–brain axis to downregulate disturbed visceroception and hyperalgesia.

\begin{figure}
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\caption{Mean RMSSD (along with standard error of the mean) plotted as change from baseline at the whole group level (A) and separately for responders (B) and nonresponders (C) as identified by the abdominal pain response criterion. Post hoc paired t-tests were used to identify specific time windows at which there were significant differences between conditions. Significant windows were highlighted with asterisks (*$P<0.05$, **$P<0.008$ [adjusted for multiple comparisons]).}
\end{figure}

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Conflict of interest statement
D. Keszthelyi has received grants from Will Pharma, Allergan, Grunenthal, ZonMw, MLDS, and UEG, outside of submitted work. Ad A. M. Mascele has received grants from Will Pharma, Allergan, Grunenthal, ZonMw, Pentax Europe GmbH, and Dutch Cancer Society, outside of submitted work. S. Eisenbruch has received grants from the German Research Foundation (Deutsche Forschungsgemeinschaft), outside of submitted work. The authors state no conflicting interests.

Acknowledgements
The authors thank Patrick Dupont, Jie Wu, and Imke Masuy for their assistance in the automation of data processing and analysis. The authors thank the Scannexus support team for their assistance during data acquisition. The authors acknowledge the assistance of Job van den Hurk for creating MATLAB-based scripts for VAS presentation. This study was in part funded by grants from the Brains Unlimited Pioneer Fund and ‘Stichting Sint Annadaf’ Foundation.

Author contributions: A. B. Beckers, D. Daniel Keszthelyi, Z. Z. R. M. Weerts, and L. van Oudenhove were responsible for the study concept and design. A. B. Beckers was responsible for acquisition of the data. A. B. Beckers and L. van Oudenhove analysed the MRI data. A. Gholamrezaei analysed the physiological log data. A. B. Beckers, L. van Oudenhove, and D. Keszthelyi wrote the manuscript. H. I. L. Jacobs, N. Priovoulos, B. A. Poser, and D. Ivanov provided technical support regarding MRI sequences. Z. Z. R. M. Weerts, H. I. L. Jacobs, N. Priovoulos, B. A. Poser, D. Ivanov, A. Gholamrezaei, Q. Aziz, S. Elsenbruch, and Ad A. M. Mascele were responsible for critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript submitted for publication, and all persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

Data transparency statement: The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. All custom code used for data processing and analysis is available in the following GitHub repository: https://github.com/BramBeckers/CapsaicinProject.

Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B537.

Supplemental video content
Video content associated with this article can be found online at http://links.lww.com/PAIN/B538.

Article history:
Received 23 March 2021
Received in revised form 20 October 2021
Accepted 25 October 2021
Available online 15 November 2021

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