

Regional activation in the rat brain during visceral stimulation detected by *c-fos* expression and fMRI

J. LAZOVIC,* H. F. WRZOS,† Q. X. YANG,* C. M. COLLINS,* M. B. SMITH,* R. NORGRÉN,‡ K. MATYAS‡ & A. OUYANG†

*Department of Radiology, Center for NMR Research, The Pennsylvania State University College of Medicine, Hershey, PA, USA

†Division of Gastroenterology and Hepatology, Department of Medicine, Penn State University College of Medicine, Hershey, PA, USA

‡Department of Neural and Behavioral Sciences, Penn State University College of Medicine, Hershey, PA, USA

Abstract Aim: The aim of the study was to determine and compare the areas of brain activated in response to colorectal distention (CRD) using functional magnetic resonance imaging (fMRI) and *c-fos* protein expression.

Methods: For fMRI study (3.0 T magnet), anaesthetized rats underwent phasic CRD, synchronized with fMRI acquisition. Stimulation consisted of eight cycles of balloon deflation (90 s) and inflation (30 s), at 40, 60 or 80 mmHg of pressure. For *c-fos* study two sets of experiments were performed on anaesthetized rats: comparing (A) brain activation in rats with the inserted colorectal balloon ($n = 5$), to the rats without the balloon ($n = 5$); and (B) rats with inserted balloon ($n = 10$), to the rats with inserted and distended balloon ($n = 10$). The pressure of 80 mmHg was applied for 2 h of 30 s inflation and 90 s deflation, alternating cycles.

Results: Functional MRI revealed significant activation in the amygdala, hypothalamus, thalamus, cerebellum and hippocampus. Significant increase in *c-fos* expression was observed in amygdala and thalamus in the first set of experiments, and hypothalamus and parabrachial nuclei in the second.

Conclusion: The two methods are not interchangeable but appeared to be complementary: fMRI was more sensitive, whereas *c-fos* had much greater resolution.

Keywords brain, *c-fos* expression, colorectal distention, functional magnetic resonance imaging, visceral pain.

INTRODUCTION

Expression of *c-fos* protein, an immediate early gene product, has been widely used to identify many brain structures involved in the central nervous system (CNS) processing of abdominal (visceral) pain in animals.^{1–3} In contrast, studies in humans use non-invasive methods such as PET and functional magnetic resonance imaging (fMRI) to investigate visceral pain processing by CNS.^{4–7} In order to successfully translate findings from animal models to human physiology/pathology, it is necessary to establish whether *c-fos* expression parallels fMRI and/or PET tests results. Visceral pain is the main feature of irritable bowel syndrome (IBS) and other functional bowel disorders (FBD). It is estimated that 10–22% of adults, predominantly women, suffer from IBS or other forms of FBD. The mechanisms and pathways involved in the CNS processing of visceral pain remain poorly understood.^{1,4,5,7–11} Contributing to this are the lack of generally accepted animal model of IBS, the likely multifactorial aetiology of the condition, and the fact that functional disorders of the gut are classified according to the patient symptoms rather than by biological markers.^{8,12,13} Functional MRI, which has been used to examine CNS activation in patients with functional bowel diseases, utilizes the increase in oxygenated blood levels to indirectly reveal areas specifically activated in response to a certain stimulus. At present, reports of patterns of CNS activation in animal studies are limited to *c-fos* expression. Increased *c-fos* expression, in contrast to MRI, is a direct marker of neuronal cell activation, however many non-specific stimuli and stress are able to induce

Address for correspondence

Ann Ouyang, Division of Gastroenterology and Hepatology, Department of Medicine, Penn State University College of Medicine, The Milton S. Hershey Medical Center, Room C5800, MC H039, PO Box 850, Hershey, PA 17033, USA
Tel: +1 717 531 8741; fax: +1 717 531 6770;
e-mail: aouyang@psu.edu

Received: 4 August 2004

Accepted for publication: 20 January 2005

c-fos and may obscure the specificity of the activation. A stimulus of short duration results in increase in oxygenated blood delivery detectable by fMRI in the range of seconds. In contrast, *c-fos* expression requires a stimulus lasting for at least an hour.¹⁴

The aims of this study were to examine brain activation in response to visceral stimuli using fMRI and *c-fos* immunohistochemistry in adult anaesthetized rats, and to compare the results obtained by both methods. We tested the hypothesis that *c-fos* expression and fMRI brain activation will occur in similar brain regions in response to colorectal distention (CRD) in anaesthetized rats.

Some preliminary results have been presented previously.^{15–17}

MATERIALS AND METHODS

Procedure

All procedures were approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine. Male Sprague–Dawley rats (Charles River) weighing 200–450 g were kept in groups of five in stainless steel cages on a 12 : 12 light/dark cycle at 22–25 °C with 60% humidity. Water and rodent pellets were available *AD LIBITUM*.

Anaesthesia

For each experiment, the animals were anaesthetized with chloral hydrate [400 mg kg⁻¹, intraperitoneally (i.p.)]. The rats received additional i.p. injections of chloral hydrate (32 mg) to maintain a level of anaesthesia that eliminated the hind limb pinch-withdrawal reflex. Chloral hydrate was selected because it has an intermediate effect on *c-fos* expression, less than several other anaesthetics used for MRI studies (e.g. halothane, urethane, α -chloralose, pentobarbital^{18,19} and it has been used for fMRI studies in children.²⁰

After the induction of anaesthesia, a 4–5 cm long lubricated, latex balloon was inserted in the rectum and descending colon, 1–2 cm proximal to the anal verge. The tubing leading to the balloon was taped to the base of the tail.

Functional MRI study

After balloon insertion, anaesthetized rats were placed in a stereotaxic frame and in the imaging coil. The balloon was attached to the barostat (Distender; G & J Electronics, Inc., Toronto, Canada) with tygon tubing (5/16" outer diameter and 1/16" wall thickness). The

barostat was interfaced to the MRI system console to synchronize distention and deflation cycles with image acquisition. The fMRI paradigm contained eight inflation and deflation cycles consisting of 90-s baseline period with the balloon deflated, during which time nine images were acquired, followed by 30 s activation or inflation period, during which time three images were obtained, for total 16 min for each of three pressure levels. The desired pressure levels of CRD were achieved by rapidly inflating the balloon controlled by the barostat. The pressure levels studied were 40 mmHg (four rats), 60 mmHg (four rats), and 80 mmHg (nine rats), with some of the rats being subjected to 40, 60 and 80 mmHg (four rats).

To minimize signal loss because of susceptibility artefacts at air–tissue interfaces (sinus cavities), the axial plane was chosen for the fMRI study. Ten axial (0.5 mm thick) slices were positioned relative to the bregma. Anatomical T₂-weighted images (effective TE/TR = 147.2/2700 ms, field of view 3 × 3 cm², matrix size 256 × 256, 8 averages) were acquired in 6 min using the RARE imaging sequence. Functional images were obtained using a T₂*-weighted echo planar imaging sequence (effective TE/TR = 35/1250 ms, 128 × 92 matrix size zero-filled to 128 × 128, 8 averages) 10 s for one image, and with the same slice position, slice thickness, field of view as the anatomical images.

Postprocessing and analysis of fMRI data were performed using the CCHIPS software (Cincinnati Childrens Hospital Image Processing Software/RSI, Boulder, CO, USA).²¹ Image co-registration and motion corrections were achieved with a pyramid co-registration algorithm.²² Signal intensity in different cerebral regions acquired during 'off' cycles and 'on' cycles was cross-correlated with an idealized wave representative of the stimulation paradigm.²³ The cross-correlation coefficient (r) value of the pixels between $r = 0.4$ and $r = 0.7$ is considered statistically significant, and is shown as colour-coded on the image. Coloured areas (pixels) represent the activated regions. Total numbers of activated pixels for brain structures that extended over more than one slice were determined by adding activated pixels per each slice.

c-fos expression study

Anaesthetized rats were kept unrestrained. Two sets of experiments were performed. In the first set, 'A', five rats without balloon insertion, 'control A no balloon', were compared with five rats that underwent insertion of the balloon without inflation. After 2 h, rats from both groups were anaesthetized with Nembutal

(Abbott Laboratories, Chicago, IL, USA) (100 mg kg^{-1} , i.p.) and perfused transcardially with 4% paraformaldehyde. The brains were removed, and processed for *c-fos* protein detection by immunocytochemistry (as below).

In the second set of experiments, 'B', 10 rats with balloon insertion without inflation, 'control B balloon' were compared with 10 rats with balloons that were inserted and inflated using the phasic distention paradigm. The distention paradigm consisted of 2 h of CRD during which the balloon was inflated to the pressure of 80 mmHg (30s) and then deflated (90s), using a computer controlled barostat, the same as used in the fMRI study. At the end of 2 h of either CRD or non-distention, all rats were anaesthetized with Nembutal (100 mg kg^{-1} , i.p.) and perfused transcardially with 4% paraformaldehyde. The brains were removed, and processed for *c-fos* protein detection by immunocytochemistry. The brains were postfixed in 4% paraformaldehyde for 4–24 h at 4°C and cryoprotected in 30% sucrose in phosphate buffer overnight. The brains were sectioned into $50 \mu\text{m}$ coronal sections that were subsequently incubated with rabbit polyclonal anti-*c-fos* antibody (1 mg mL^{-1} , SC 52, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA, USA) and avidin–biotin horseradish peroxidase complex (Vector Labs, Burlingame, CA, USA). Reaction product was shown by using 3,3'-diaminobenzidine (Sigma, St Louis, MO, USA) enhanced by nickel ammonium sulphate and cobalt. The anatomy was confirmed using cresyl violet staining, and control tissue was processed without using anti-*c-fos* antibody.

Quantification of the *c-fos* positive nuclei

Brain sections of interest were digitized from light microscopic images using a Color Digital Camera (Hitachi Instruments, Hitachi High Technologies America, Inc., Schaumburg, IL, USA), and *c-fos* positive neurones were counted (Optimas 6.2, Optimas Corp., Bothell, WA, USA).¹⁴ Brain areas were identified based on the atlas of Paxinos.²⁴ Brain sections with the highest number of *c-fos* positive cells for nuclei of interest [paraventricular nucleus of the hypothalamus (PVN), central nucleus of amygdala (CeA), paraventricular nucleus of thalamus (PVP), parabrachial nucleus (PBN), and area postrema (AP)] were chosen for counting. Cell nuclei were automatically counted as *c-fos* positive if the pixel density reached 200% of the background. The sections were re-counted by hand to include overlapping *c-fos* positive nuclei. Values were presented as mean \pm SEM for the control and experimental rats for the specific nuclei. For statistical analysis the number of positive nuclei in the control and experimental animals were compared using unpaired *t*-tests at the $P \leq 0.05$ as the level of significance.

RESULTS

Functional MRI

No activation in the brain was observed when 40 mmHg balloon pressure was used (Fig. 1A). The total number of activated pixels in response to a distention pressure of 60 mmHg, for each region for each animal, is shown in Table 1, and in response to

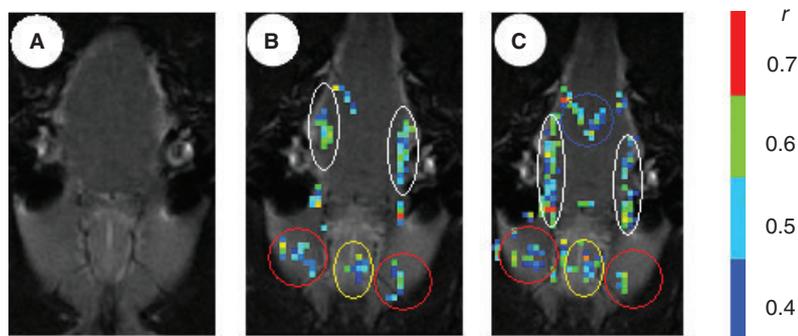


Figure 1 Axial fMRI images of the rat brain with no activation during 40 mmHg CRD (A) and with activated hypothalamus (yellow circle) and the amygdala (red circles) during the rectal balloon stimulation at the pressures of 60 mmHg (B) and 80 mmHg (C), of the same animal. Activation of the nucleus of the solitary tract during the 80 mmHg pressure is also shown (blue circle) and activation of the trigeminal nucleus during 60 and 80 mmHg (white circles). Signal intensity acquired during 'off' cycles and 'on' cycles at corresponding areas is cross-correlated with the stimulation paradigm. When the cross correlation coefficient of particular pixel (r) reaches statistical significance ($0.4 < r < 0.7$), pixel appears as colour-coded on the image. Colored pixels represent the activated regions. The colour scale, shown on the right represents different values of the cross-correlation coefficient.

Table 1 Number of pixels in the rat brain areas activated during CRD at the level of 60 mmHg observed in fMRI

Brain area	Rat no.			
	1	2	3	4
Caudate/putamen	4	17	5	25
Globus pallidus	3			
Hypothalamus	37	20	10	2
Thalamus	15		7	9
Amygdala	40	10	25	12
Hippocampus	9	7	19	5
Piriform cortex				3
Sensory cortex	4	6		
Insular cortex	5			3
Temporal lobe		6		
Retrosplenial cortex	6			
Entorhinal cortex	5	9	4	9
Perirhinal cortex		2		8
Periaqueductal grey	4			
Superior colliculus	7	15		
Parabrachial n.			2	
Trigeminal n.	43	34	33	34
Solitary n.	8	3		
Cerebellum	30	79	28	8

80 mmHg distention stimulus in Table 2. Rats 1–4 in Table 1 and rats 1–4 in Table 2 underwent multiple distentions of 40, 60 and 80 mmHg, while rats 5–9 in Table 2 underwent 80 mmHg distention only. Examples of brain areas activated during 60 mmHg distention and during 80 mmHg distention are shown in Figs 1 and 2, with greater number of pixels activated

during 80 mmHg distention. Different colours shown in Figs 1 and 2 represent different values of the cross-correlation coefficient (see figure legends). All rats undergoing CRD with 60 and 80 mmHg pressure exhibited activation in the amygdala and PVN (Tables 1 and 2; Fig. 1B and C). Additionally, in four of four rats treated with 60 mmHg CRD and eight of the nine rats treated with CRD at 80 mmHg pressure, activation occurred in the hippocampus, cerebellum and trigeminal nerve (Figs 1 and 2; Tables 1 and 2). The thalamus was activated in three of four rats treated with 60 mmHg and seven of nine rats treated with 80 mmHg (Fig. 2; Tables 1 and 2). In the four-five of nine animals subjected to the 80 mmHg, the caudate, putamen, periaqueductal grey, and the insular, retrosplenial, entorhinal, perirhinal, and sensory association cortices were also activated (Tables 1 and 2). The parabrachial nucleus was activated in five of nine animals subjected to 80 mmHg (Table 2). We have compared number of pixels activated during 80 mmHg (for the same brain area) in rats that were subjected to 40, 60 and 80 mmHg (rats 1–4 in Table 1 and rats 1–4 in Table 2) with rats that were subjected to 80 mmHg only (rats 5–9 in Table 2). There was no statistical significance ($P < 0.05$, multiple *t*-tests) in the number of activated pixels for hypothalamus, thalamus, amygdala, cerebellum, Nucleus of the solitary tract (NTS), hippocampus, trigeminal nucleus or any cortical area except for the caudate/putamen where there was significantly more activation ($P < 0.05$) in the rats that were subjected to multiple distention pressures.

Table 2 Number of pixels in the rat brain areas activated during CRD at the level of 80 mmHg observed in fMRI

Brain area	Rat no.								
	1	2	3	4	5	6	7	8	9
Caudate/putamen	8	22	10	25					
Globus pallidus	23	10	5						
Hypothalamus	75	20	19	6	3	16	15	34	35
Thalamus	53		12	17	10	2		7	2
Amygdala	100	19	25	17	8	13	41	52	67
Hippocampus	24	14	25	7	6	4		15	17
Piriform cortex	2			4	5				
Sensory cortex	5	17					2		10
Insular cortex	13			4				4	8
Temporal lobe		7			2				
Retrosplenial cortex	15		3	5	15	6		3	
Entorhinal cortex	6	26	7	8	4	3			7
Perirhinal cortex		20		8					3
Periaqueductal grey	16			3					
Superior colliculus	17	27			22	10			
Parabrachial n.	5	3	3			4		7	
Trigeminal n.	47	35	45	40		31	39	48	4
Solitary n.	15	10	15		18			2	4
Cerebellum	87	91	41	12	57	23		7	4

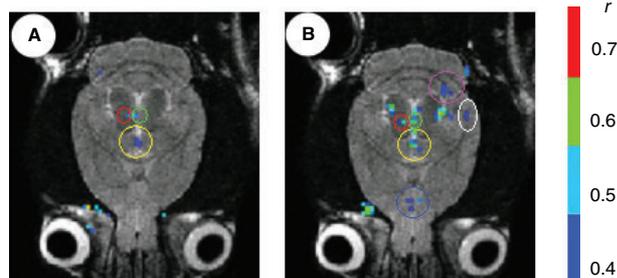


Figure 2 Axial fMRI images of the rat brain with activated thalamus (yellow circle), periaqueductal grey (green circle) and superior colliculus (red circle) during the rectal balloon stimulation at the pressure of 60 mmHg (A) and 80 mmHg (B), of the same animal. During 80 mmHg pressure there was additional activation in the cerebellum (pink circle), hippocampus (white circle) and prelimbic and infralimbic structures (blue circle) for the particular animal (B). The colour scale, shown on the right represents different values of the cross-correlation coefficient.

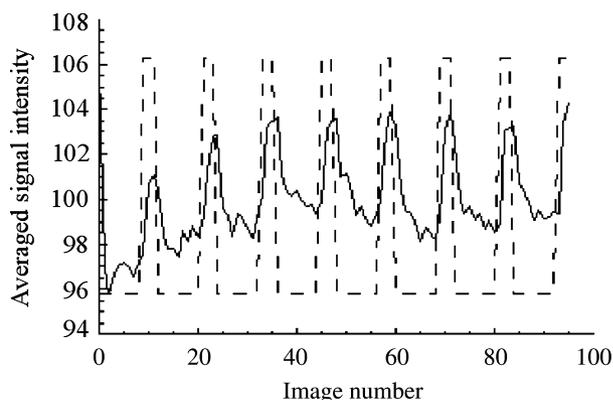


Figure 3 A typical time curve of fMRI signal intensity changes during the rectal balloon stimulation, plotted as the averaged signal intensity of all activated areas. The x-axis represents the image number (96 total number of images) and y-axis represents the averaged signal changes during the rectal balloon stimulation. The balloon was inflated during the images: 10–12, 22–24, 34–36, 46–48, 58–60, 70–72, 82–84 and 94–96 as indicated by the dashed line.

A typical time curve of fMRI signal intensity of all activated areas is shown in Fig. 3. The percent signal changes varied from 4.5 to 6.5% among different rats, which is in accordance with previously published data for 3 T magnetic field strength.²⁵ Following the rectal balloon distention there was no significant difference between signal intensity increase during 60 and 80 mmHg CRD.

c-fos expression

Experiment A: comparison between balloon insertion (without inflation) and no balloon Specific nuclei

Table 3 Number of *c-fos* like-immunoreactivity (LI) in the rat brain areas activated during CRD: control rats without the balloon insertion vs rats with the inserted balloon (no distention)

Brain area	Control A, no balloon	Control B, balloon not distended	P-value (n = 5)
PVN L	240.3 ± 83.9	341.1 ± 56.9	0.3
PVN R	316.7 ± 91.7	383.6 ± 82.6	0.6
CeA L	91.8 ± 29.4	120.7 ± 35.7	0.5
CeA R	63.8 ± 6.3	136 ± 26.8	0.05
PVP	69.2 ± 12.2	169.4 ± 20.2	0.003
PBN L	41.7 ± 9.2	80.6 ± 21.5	0.1
PBN R	34.3 ± 16	79.6 ± 25.2	0.1

examined and counted included the amygdala, PVP, PBN, the PVN and the area postrema (AP). These nuclei were chosen based on our own fMRI data and on published CNS visceral afferent projections.¹ Numbers of activated *c-fos* neurones in rats without balloon inserted, control A, compared with the rats with balloon inserted ($n = 5$) but not inflated, are shown in Table 3. Statistically significant differences were observed in the PVP, 69.2 ± 12.2 vs 169.4 ± 20.2 ($P = 0.003$, $n = 5$); and the central nucleus of amygdala on the right side (CeA R), 63.8 ± 6.3 vs 136 ± 26.8 ($P = 0.05$, $n = 5$). On the left side, at the CeA L, the difference was not statistically significant (91.8 ± 29.4 vs 120.7 ± 35.7 , $P = 0.5$, $n = 5$). There was also no significant difference at the PVN, PBN or AP (Table 3).

Experiment B: comparison of balloon insertion without inflation and balloon insertion with distention Numbers of activated *c-fos* neurones in the rats with an inflated balloon compared with the control rats with the uninflated balloon, control B, are shown in Table 4. Statistically significant differences in *c-fos* expression between control B and experimental rats were observed in the left PBN (PBN L), 93.1 ± 11.8 vs 132.3 ± 14.4 ($P = 0.05$, $n = 10$) and in the right PVN (PVN R) 264.1 ± 66.9 vs 487.2 ± 85.5 ($P = 0.05$, $n = 10$), control B vs experimental, respectively. There were no significant differences at PVN L and PBN R (Table 4). An example showing *c-fos* expression indicating activated neurones in PBN and PVN in control B rats (balloon inserted, not inflated) compared with experimental rats (balloon inserted and inflated) is shown on Fig. 4.

In the amygdala, thalamus, and AP, *c-fos* activation was not significantly different between experimental and control B rats. In the amygdala, intense activation was seen in both control and experimental animals (Table 4). Similar activation was seen in both control B

Table 4 Number of *c-fos* LI in the rat brain areas activated during CRD: control rats with the inserted balloon (no distention) vs distended balloon at the pressure of 80 mmHg

Brain area	Control B, balloon not distended	Experimental distention	<i>P</i> -value (<i>n</i> = 10)
PVN L	250.6 ± 61.7	412.2 ± 66.5	0.09
PVN R	264.1 ± 66.9	487.2 ± 85.5	0.05
CeA L	89.8 ± 24.5	93.6 ± 24.7	0.8
CeA R	93.9 ± 24.6	88.9 ± 20.6	0.9
PVP	110.1 ± 20.1	128.8 ± 18	0.5
PBN L	93.1 ± 11.8	132.3 ± 14.4	0.05
PBN R	72.9 ± 12.2	99.2 ± 9.4	0.1
AP	161.5 ± 28.4	200 ± 32.8	0.4

and experimental rats in the thalamic PVP, supraoptic nucleus, piriform cortex, cortical amygdaloid area, insular cortex, habenula and the thalamic mediodorsal nucleus.

DISCUSSION

Our study compared two different markers of neuronal activation, fMRI and *c-fos* expression, in the brain of rats subjected to the visceral stimulation. The results partially support the initial hypothesis that both methods should detect similar brain nuclei in response to the same stimulus pressure. Nevertheless, certain differences are observed. The stimulus could not be identical because the two techniques require different time course for the marker of activation to be detected and represent different physiological events.

The methods detect different aspects of neuronal activation. In the *c-fos* method, an activated specific early gene, *c-fos*, is expressed as *c-fos* nuclear protein which is detected immunohistochemically and presumably reflects increased somal electrophysiological activity. More than an hour is needed for the protein expression of *c-fos* protein to reach the levels necessary for immunohistochemical detec-

tion,¹⁴ whereas in fMRI, the time course for changes in blood oxygenation level is a matter of seconds. In fMRI, neuronal activity is assessed indirectly as changes in blood oxygenation levels. This activation cannot be specifically assigned to somas or terminal arborizations.

In this study, the two methods detect different areas of the brain activated by CRD. Some of these areas overlap, with more activated areas detected by fMRI. The different duration of studies and therefore, different dose of anaesthesia may contribute to the differences in the area of activation in the brain.

Some of the rats were subjected to one (80 mmHg) and some to multiple levels of pressure (40, 60 and 80 mmHg). It is unlikely that the cortical fMRI response was affected by multiple distension levels because (i) there was no significant difference in the number of pixels activated during 80 mmHg CRD in the cortical areas of the rats that experienced single 80 mmHg stimulus vs those that experienced multiple distention pressures prior to the 80 mmHg stimulus and (ii) significantly more pixels activated in caudate/putamen in rats that underwent multiple distention pressures did not influence the cortical response despite the extensive cortical projections of caudate/putamen.

In our *c-fos* studies, when comparing a control B balloon insertion with experimental distention, significant CNS activation is restricted to the paraventricular nucleus of hypothalamus and parabrachial nuclei. Activation in the thalamic PVP, supraoptic nucleus, piriform cortex, cortical amygdaloid area, insular cortex, habenula, and the thalamic mediodorsal nucleus is present but similar in both control and experimental balloon rats (the results are not shown). It is unclear whether anaesthesia limits areas in which significantly increased activation can be seen.

In our fMRI studies, an increased number of pixels (larger activation area) is consistently observed with

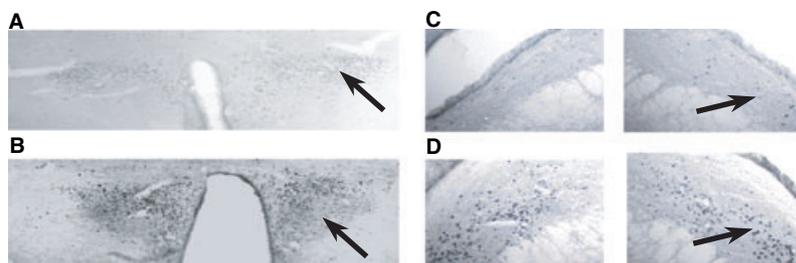


Figure 4 Representative digital micrographs of the brain sections immunolabelled for *c-fos* protein. Examples of the neurons labelled for the *c-fos* are indicated by arrows. Paraventricular nucleus of the hypothalamus (left and right sides) of control animal (A) (100× magnification), and of an experimental animal (B) and parabrachial nucleus (left and right sides) of control animal (C), and of the experimental animal (D) (100× magnification).

increasing distention pressure, suggesting an increase in the number of neurones involved in visceral nociception. This observation is in accordance with the study of Sengupta and Gebhart²⁶ where an increase in mechanoreceptor discharge rate in the gut occurred with an increase in CRD pressure in the anaesthetized rat. 'High-threshold' nociceptive receptors responded to mechanical stimuli (CRD) within the noxious range, while 'low-threshold' receptors encoded the stimulus from innocuous up to the noxious range. When the number of *c-fos* positive nuclei was determined² in response to different pressures of CRD, an increasing number of *c-fos* positive nuclei was observed with increasing pressures (from 10 mmHg to 40 mmHg and 70 mmHg) in NTS, rostral ventrolateral medulla, nucleus cuneiformis (NC), periaqueductal gray and the amygdala.

In this fMRI study, the 40 mmHg pressure did not produce a response, while a response was observed following a stimulus of 60 mmHg and greater. Our results are in agreement with another study,²⁷ in anaesthetized rats, where the threshold for the neuronal response was between 25 and 100 mmHg with a mean pressure threshold of 56 ± 24 mmHg. One explanation for the lack of activation at 40 mmHg is that the 'lower threshold' receptors are inhibited by the chloral hydrate anaesthesia. In general, anaesthesia is considered to reduce number of activated neurones,²⁸ due to depression of neuronal activity and metabolism.²⁹

Although rats are anaesthetized, activation of the amygdala revealed by fMRI might indicate an emotional-affective aspect of visceral stimulation, as extensively demonstrated with somatic stimuli.³⁰ In our *c-fos* studies, control animals that underwent balloon insertion without distention had shown activated neurones in the amygdala, suggesting that the balloon insertion is able to activate the amygdala. Stam *et al.*³ have shown that the alterations in cortico-limbic response to colonic distention do not necessarily require conscious affective responses.

Significant activation of the NTS (approximately 3–5 mm in size depending on a section) was observed when detected using fMRI, whereas the difference between the number of *c-fos*-positive cells found in the central NTS (AP) in experimental rats was not statistically significant compared with control rats. This may be due to the high within-group variance. In contrast, significant activation in the PBN was noted in the *c-fos* studies, but only in few rats by fMRI. The limitations of fMRI spatial resolution may hinder detection of PBN activation in animals brain, because of the need to perform an analysis in the axial plane in

which the cross-sectional area of the PBN is at its smallest. The voxel size used during the echo planar imaging was $234 \times 313 \times 500 \mu\text{m}^3$, which is comparable with the size of the parabrachial nucleus in the axial plane ~ 0.2 – 0.3 mm depending on the section, thus making detection of the PBN nucleus particularly difficult. The resolution of fMRI limits the ability to detect very small nuclei, and is clearly an advantage of the *c-fos* method.

Similar studies performed by others in non-anaesthetized rats identified *c-fos* activation in infralimbic and prelimbic cortices, the mediodorsal thalamic nucleus, the central amygdaloid nucleus, dorsomedial and ventromedial nuclei of the hypothalamus (DMH and VMH respectively), NTS, rostral ventrolateral medulla, NC and periaqueductal gray.^{1,31} We did not observe consistent cortical activation using *c-fos* technique. This probably resulted from the use of chloral hydrate anaesthesia that has been reported to hinder cortical activation.³²

The observed cortical fMRI activation in a small number of animals included insula, retrosplenial, piriform and perirhinal cortex previously identified to play a part in the emotional component of a painful stimulus.³³ A reduced cortical bold response to stimulation under anaesthesia (up to 10-fold) has been observed,³⁴ probably due to decreased metabolism in the cortex, influence on cerebral blood flow and cerebral autoregulation. The number of activated regions differed from rat to rat. A variability in the cortical activation was observed for the somatosensory response³⁵ as well. We have also observed activation of the cerebellum by fMRI in rats. Activation of cerebellum in response to visceral pain has been reported previously in human fMRI studies.^{33,36,37}

Visceral pain is conveyed by the spinal nociceptive viscerosensory neurones located in the laminae I, V and X of the spinal cord to higher brain structures via spino-parabrachio-amygdaloid and hypothalamic pathways.^{38–40} Although not directly involved in conveying the painful stimuli, vagal afferents modulate nociceptive processing in the spinal cord.⁴¹ There is some evidence that PBN along with the spino-parabrachio-amygdaloid pathway play an important role for somatic and visceral pain processing.^{42,43} The PBN projects to the amygdala, the hypothalamus and intralaminar thalamic nuclei, an important relay to the prefrontal cortex. How activation of these afferent pathways becomes perceived as painful or non-painful sensations is not well understood.^{31,44}

These studies demonstrate advantages and disadvantages of two methods of detecting neuronal activation. Functional MRI is non-invasive, and appli-

cable for human studies. Functional MRI studies in animals, however, require use of anaesthesia, which is hindering activation of certain areas. The superior spatial resolution of c-fos over fMRI facilitates detection of activation of very small nuclei and single neurones involved, but is not applicable to humans.

CONCLUSIONS

We conclude that the activation observed by fMRI included brain areas detected by c-fos expression in addition to brain areas that did not stain differentially for c-fos compared with controls. These findings suggest that fMRI is able to detect a larger number of brain areas activated, therefore making it a more sensitive method under the given anaesthesia conditions. Some similarities of regional brain activation in response to gut stimuli do exist when assessed by fMRI and c-fos expression, despite differences in the resolution, methodology and physiology of these methods. The superior resolution of c-fos combined with fMRI sensitivity would be complementary in evaluating whether an animal model shows CNS activation that parallels the altered CNS processing of visceral stimuli in functional disorders such as IBS. The c-fos and fMRI data taken together imply the importance of the spino-parabrachio-amygdaloid pathway in visceral nociception, indicating that the inhibition of the specific steps of the pathway may have potential in the visceral pain management.

ACKNOWLEDGEMENTS

This work was supported by grants from Glaxo-Wellcome and the NIH (DC00240 and MH00653). We would like to acknowledge David Mauger, PhD for his help with the statistical analysis, Lukas Ansel for help in creating the stereotaxic frame and Sam Jundler for constructing the interface for the direct control of the barostat by the MRI spectrometer.

REFERENCES

- 1 Traub RJ, Silva E, Gebhart GF, Solodkin A. Noxious colorectal distention induced c-Fos protein in limbic brain structures in the rat. *Neuroscience* 1996; **215**: 165–8.
- 2 Monnikes H, Ruter J, Konig M *et al*. Differential induction of c-fos expression in brain nuclei by noxious and non-noxious colonic distension: role of afferent C-fibers and 5-HT₃ receptors. *Brain* 2003; **966**: 253–64.
- 3 Stam R, Ekkelenkamp K, Frankhuijzen AC, Bruijnzeel AW, Akkermans LM, Wiegant VM. Long-lasting changes in central nervous system responsivity to colonic distention after stress in rats. *Gastroenterology* 2002; **123**: 1216–25.
- 4 Silverman DH, Munakata JA, Ennes H, Mandelkern MA, Hoh CK, Mayer EA. Regional cerebral activity in normal and pathological perception of visceral pain. *Gastroenterology* 1997; **112**: 64–72.
- 5 Mertz H, Morgan V, Tanner G *et al*. Regional cerebral activation in irritable bowel syndrome and control subjects with painful and nonpainful rectal distention. *Gastroenterology* 2000; **118**: 842–8.
- 6 Naliboff BD, Berman S, Chang L *et al*. Sex-related differences in IBS patients: central processing of visceral stimuli. *Gastroenterology* 2003; **124**: 1738–1747.
- 7 Sidhu H, Kern M, Shaker R. Absence of increasing cortical fMRI activity volume in response to increasing visceral stimulation in IBS patients. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G425–35.
- 8 Mayer EA, Collins SM. Evolving pathophysiologic models of functional gastrointestinal disorders. *Gastroenterology* 2002; **122**: 2032–2048.
- 9 Ness TJ, Gebhart GF. Characterization of neuronal responses to noxious visceral and somatic stimuli in the medial lumbosacral spinal cord of the rat. *J Neurophysiol* 1987; **57**: 1867–92.
- 10 Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudodiffuse reflexes in the rat. *Brain Res* 1988; **450**: 153–169.
- 11 Traub RJ, Herdegen T, Gebhart GF. Differential expression of c-fos and c-jun in two regions of the rat spinal cord following noxious colorectal distention. *Neuroscience* 1993; **160**: 121–5.
- 12 Al-Chaer ED, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 2000; **119**: 1276–1285.
- 13 Camilleri M, Talley NJ. Pathophysiology as a basis for understanding symptom complexes and therapeutic targets. *Neurogastroenterology* 2004; **16**: 135–42.
- 14 Traub RJ, Pechman P, Iadarola MJ, Gebhart GF. Fos-like proteins in the lumbosacral spinal cord following noxious and non-noxious colorectal distention in the rat. *Pain* 1992; **49**: 393–403.
- 15 Wrzos HF, Lazovic-Stojkovic J, Smith MB *et al*. Towards an animal model of Irritable Bowel Syndrome (IBS): Comparison of c-Fos expression and fMRI of the rat brain during noxious visceral stimulation. *Neurogastroenterology* 2002; **14**: 454.
- 16 Wrzos HF, Guillaume M, Norgren R, Smith K, Evey LA, Ouyang A. c-Fos protein expression in the brain of the anesthetized rat induced by colorectal distention. *Gastroenterology* 2001; **120**: A715–6.
- 17 Lazovic-Stojkovic J, Wrzos HF, Yang QX *et al*. Regional fMRI activation and c-Fos expression during visceral pain stimulation in the adult rat brain. In: *Proceedings of the 10th Scientific Meeting ISMRM*. Hawaii: 2002: 400 pp.
- 18 Takayama K, Suzuki T, Miura M. The comparison of effects of various anesthetics on expression of Fos protein in the rat brain. *Neuroscience* 1994; **176**: 59–62.
- 19 Rocha MJ, Herbert H. Effects of anesthetics on Fos protein expression in autonomic brain nuclei related to cardiovascular regulation. *Neuropharmacology* 1997; **36**: 1779–81.

- 20 Altman NR, Bernal B. Brain activation in sedated children: auditory and visual functional MR imaging. *Radiology* 2001; **221**: 56–63.
- 21 Schmithorst VJ, Dardzinski BJ, Holland SK. Simultaneous correction of ghost and geometric distortion artifacts in EPI using a multiecho reference scan. *IEEE Trans Med Imaging* 2001; **20**: 535–9.
- 22 Thevenaz P, Ruttimann UE, Unser M. A pyramid approach to subpixel registration based on intensity. *IEEE* 1998; **7**: 27–41.
- 23 Kern MK, Jaradeh S, Arndorfer RC, Jesmanowicz A, Hyde J, Shaker R. Gender differences in cortical representation of rectal distension in healthy humans. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1512–23.
- 24 Paxinos GWC. *The Rat Brain in Stereotaxic Coordinates*, 2nd edn. New York: Academic Press, 1986.
- 25 Lu H, Mazaheri Y, Zhang R, Jesmanowicz A, Hyde JS. Multishot partial-k-space EPI for high-resolution fMRI demonstrated in a rat whisker barrel stimulation model at 3T. *Magn Reson Med* 2003; **50**: 1215–22.
- 26 Sengupta JN, Gebhart GF. Characterization of mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat. *J Neurophysiol* 1994; **71**: 2046–2060.
- 27 Bernard JF, Huang GF, Besson JM. The parabrachial area: electrophysiological evidence for an involvement in visceral nociceptive processes. *J Neurophysiol* 1994; **71**: 1646–60.
- 28 Ueki M, Mies G, Hossmann KA. Effect of alpha-chloralose, halothane, pentobarbital and nitrous oxide anesthesia on metabolic coupling in somatosensory cortex of rats. *Acta Anaesthesiol Scand* 1992; **36**: 318–322.
- 29 Trulsson ME, Ullissey MJ. Chloral hydrate anesthesia alters cerebral enzymes in the rat. A histochemical study. *Acta Anat (Basel)* 1987; **130**: 319–23.
- 30 Bernard JF, Huang GF, Besson JM. Nucleus centralis of the amygdala and the globus pallidus ventralis: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol* 1992; **68**: 551–69.
- 31 Monnikes H, Ruter J, Konig M *et al.* Differential induction of c-fos expression in brain nuclei by noxious and non-noxious colonic distension: role of afferent C-fibers and 5-HT₃ receptors. *Brain Res* 2003; **966**: 253–64.
- 32 West MO. Anesthetics eliminate somatosensory-evoked discharges of neurons in the somatotopically organized sensorimotor striatum of the rat. *J Neurosci* 1998; **18**: 9055–68.
- 33 Strigo IA, Duncan GH, Boivin M, Bushnell MC. Differentiation of visceral and cutaneous pain in the human brain. *J Neurophysiol* 2003; **89**: 3294–303.
- 34 Lahti KM, Ferris CF, Li F, Sotak CH, King JA. Comparison of evoked cortical activity in conscious and propofol-anesthetized rats using functional MRI. *Magn Reson Med* 1999; **41**: 412–6.
- 35 Keilholz SD, Silva AC, Raman M, Merkle H, Koretsky AP. Functional MRI of the rodent somatosensory pathway using multislice echo planar imaging. *Magn Reson Med* 2004; **52**: 89–99.
- 36 Lotze M, Wietek B, Birbaumer N, Ehrhardt J, Grodd W, Enck P. Cerebral activation during anal and rectal stimulation. *Neuroimage* 2001; **14**: 1027–34.
- 37 Ladabaum U, Minoshima S, Owyang C. Pathobiology of visceral pain: molecular mechanisms and therapeutic implications V. Central nervous system processing of somatic and visceral sensory signals. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: OG1–6.
- 38 Bernard JF, Bester H, Besson JM. Involvement of the spino-parabrachio-amygdaloid and hypothalamic pathways in the autonomic and affective emotional aspects of pain. *Prog Brain Res* 1996; **107**: 243–55.
- 39 Esteves F, Lima D, Coimbra A. Structural types of spinal cord marginal (lamina I) neurons projecting to the nucleus of the tractus solitarius in the rat. *Somatosensory* 1993; **10**: 203–16.
- 40 Hylden JL, Anton F, Nahin RL. Spinal lamina I projection neurons in the rat: collateral innervation of parabrachial area and thalamus. *Neuroscience* 1989; **28**: 27–37.
- 41 Mayer EA, Gebhart GF. Basic and clinical aspects of visceral hyperalgesia. *Gastroenterology* 1994; **107**: 271–93.
- 42 Randich A, Gebhart GF. Vagal afferent modulation of nociception. *Brain Res Brain Res Rev* 1992; **17**: 77–99.
- 43 Cechetto DF, Standaert DG, Saper CB. Spinal and trigeminal dorsal horn projections to the parabrachial nucleus in the rat. *J Comp Neurol* 1985; **240**: 153–60.
- 44 Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. *Science* 1997; **277**: 968–71.