1. Introduction

Symptom-related anxiety and maladaptive coping (e.g., catastrophizing, worst-case assumption of pain outcomes) play a prominent role in the pathophysiology of persistent pain disorders, including irritable bowel syndrome (IBS) [8,28,67]. Importantly, expectation of pain established through associative learning can lead to affective responses similar to that evoked by acute pain, as well as coping responses that modulate subsequent pain perception. It has been hypothesized that while the effective coping responses in healthy people involve activation of a cortico-limbic-pontine inhibitory network that inhibits pain perception, failure of this inhibitory mechanism may contribute to hyperalgesia in persistent pain disorders [5,35,56,62,71].

In light of the critical involvement of erroneous outcome predictions in chronic pain disorders, an increasing number of human brain imaging studies have focused on understanding the neural substrates of pain expectation. Many of the general brain regions activated during acute pain are also activated in expectation.
of pain [11,21,40,49,51,72], including the anterior insula (aINS), dorsolateral prefrontal cortex (PFC), and anterior midcingulate cortex (MCC).

In contrast to the rapidly growing human brain imaging research on pain expectation, there has been limited animal research in this field. Lei et al. [31] studied brain c-fos expression in rats during recall of formalin-conditioned place avoidance, and showed that similar brain regions expressed c-fos as those during acute formalin treatment. Our group has previously applied an autoradiographic blood flow mapping method to the colorectal distension (CRD) model of visceral pain [68,69]. Acute CRD induced activation in the aINS, anterior cingulate cortex (ACC), PFC (prelimbic area, Prl), and amygdala, in close agreement with human findings. Such functional brain mapping in rodent models of pain can serve as a translational tool to bridge preclinical and clinical pain research [19]. To map functional brain activation in expectation of visceral pain, we applied blood flow mapping to the step-down passive avoidance (PA) model using CRD as the aversive conditioning stimulus.

PA (inhibitory avoidance) is a well-studied model of aversive learning, having both Pavlovian and operant components (reviewed in [32,37,61]). Converging evidence has implicated the basolateral amygdala and dorsal hippocampus in the acquisition, consolidation, and expression of PA. The step-down version of PA has been successfully applied to validate the aversive nature of several distension-based visceral pain models [42,44,45,57]. Whereas animal research has focused mostly on the learning and memory aspect of PA, not enough attention has been given to the affective component associated with expectation of aversive stimuli. We hypothesize that during the retrieval phase of PA, in addition to regions implicated in PA expression, brain regions implicated in the processing of the affective component of visceral pain also show increased activation. The study provides further validation for using the functional brain mapping method to delineate central mechanisms underlying the affective responses associated with pain expectation. Such platform can be used to validate animal models of functional pain disorders at the brain level, and to assess drug responses in these models.

2. Methods

2.1. Animals

Twenty-two adult male Wistar rats were randomized into 2 groups: conditioned and control (n = 11 per group). Rats were received from the vendor (Harlan Sprague Dawley, Indianapolis, IN, USA) 1 week prior to experimentation and were individually housed in the vivarium on a 12-hour light/12 hour dark cycle with free access to water and rodent chow. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Southern California and are in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.2. Surgical procedures

Animals were anesthetized (isoflurane 2% in 70% oxygen, 30% nitrous oxide). The right external jugular vein was cannulated with a 5-Fr Silastic catheter advanced into the superior vena cava. A port at the distal end of the catheter was tunneled subcutaneously and externalized dorsally in the region rostral to the scapula. Animals were allowed to recover for 6 days before training started. The catheter was flushed every other day postoperatively to ensure patency (0.3 mL of 0.9% saline, followed by 0.1 mL of saline with 20 U/mL heparin).

2.3. Step-down PA training

Fig. 1 shows the experimental setup and timeline. Animals were habituated to a training arena (wall height = 40 cm, diameter = 60 cm) for 15 minutes the day before training (day 0). Over the next 2 days (day 1 and day 2), animals were trained for 18 trials/day. Each animal was instrumented with a colorectal balloon. Briefly, under light isoflurane anesthesia (1.5% isoflurane × 2 minutes), a flexible latex balloon (length: 6 cm) was inserted intraperitoneally such that its end was 1 cm proximal to the anus. The balloon was connected to a barostat (Distender Series II, G and J Electronics Inc., Toronto, Canada) through a piece of Tygon tubing (R-3603, Saint-Gobain Performance Plastics, Akron, OH, USA), which was fixed to the base of the tail with adhesive tape and covered by a stainless steel spring for protection against animal biting. The animal was allowed to recover for 30 minutes in a transit cage, the floor of which was covered with bedding from the animal's home cage. At the beginning of each training trial, the rat was put on an elevated platform (W × L × H: 13 × 13 × 10 cm) placed next to the wall in the training arena. Step-down latencies were recorded using a stopwatch to the nearest second as the time taken for the animal to step-down by putting both forepaws on the arena floor. Upon stepping down, a conditioned rat received a 60-mm Hg, 20-second CRD delivered through the barostat, whereas the balloon of a control rat remained uninflated. Animals were then returned to the transit cage. If the animal remained on the platform for 120 seconds (cutoff time), it was returned to the transit cage, and step-down latency was recorded as 120 seconds. Each trial lasted 3 minutes, including the time spent in the transit cage. The protocol has been adapted from studies using step-down PA to validate animal models of visceral pain [42,44,45,57]. Whereas in previous studies, retrieval was assessed within the same training session, we modified the protocol so that we could assess functional brain activation during PA retrieval 1 day after training and in the absence of a balloon. The objective was to avoid possible confounding factors such as short-term stress associated with balloon insertion and CRD, and sensitization to the inserted balloon in the conditioned rats. The modifications included placing the platform next to the arena wall during training and recall, rather than in the center of the arena, and increasing the training trials to 18 per day for 2 days. The final protocol reflected a balanced approach to achieving a robust behavioral end point, while limiting the intensity of training to avoid excessive stress.

2.4. Retrieval of PA and cerebral perfusion

On day 3, PA behavior was tested in the absence of a colorectal balloon. A piece of Silastic tubing was filled with radiotracers [14C]-iodoantipyrine (125 μCi/kg in 300 μL of 0.9% saline, American Radiolabeled Chemicals, St. Louis, MO, USA). The radiotracer-filled tubing was then connected to the animal's cannula on one end, and to a syringe filled with euthanasia agent (pentobarbital 75 mg/mL, 3 M potassium chloride) on the other. The animal was allowed to rest for 15 minutes in the transit cage before the retrieval trials. On the first trial, the animal was placed on the platform to record the step-down latency. The animal was put back into the transit cage immediately after stepping down, or after 120 seconds if it remained on the platform. On the second trial, 45 seconds after the animal was put on the platform, radiotracer was infused at 2.25 mL/min by a motorized pump, followed immediately by euthanasia, which resulted in cardiac arrest within approximately 10 seconds, a precipitous fall of arterial blood pressure, termination of brain perfusion, and death. This 10-second time window provided the temporal resolution for the mapping of regional cerebral blood flow-related tissue radioactivity (rCBF).
2.5. Brain slicing and autoradiography

Brains were rapidly removed, flash frozen in dry ice/methylbutane (approximately −55 °C) and embedded in Optimal Cutting Temperature compound (Sakura Fintek Inc., Torrance, CA, USA). Brains were subsequently sectioned on a cryostat (HM550 Series, Microm International GmbH, Walldorf, Germany) at −20 °C into 20-μm-thick coronal slices, with an interslice sampling space of 300 μm. Slices were heat-dried on glass slides and exposed for 2 weeks at room temperature to Ektascan Diagnostic Film (Eastman Kodak Co., Rochester, NY, USA). Images of brain sections were then digitized using an 8-bit gray scale.

2.6. Functional brain mapping data analysis

rCBF-related tissue radioactivity was quantified by autoradiography and analyzed on a whole-brain basis using SPM (version SPM5, Welcombe Centre for Neuroimaging, University College London, London, UK). Recently, we and others have developed and validated an adaptation of SPM for use in rodent brain autoradiographs [12,20,30,43]. In preparation for the SPM analysis, a 3-dimensional reconstruction of each animal’s brain was conducted using 57 serial coronal sections (starting at approximately bregma +4.5 mm) with a voxel size of 40 × 300 × 40 μm. Adjacent sections were aligned both manually and using TurboReg, an automated pixel-based registration algorithm implemented in ImageJ (http://rsbweb.nih.gov/ij/). This algorithm registered each section sequentially to the previous section using a nonwarping geometric model that included rotations and translations (rigid-body transformation) and nearest-neighbor interpolation. Global mean of voxel optical density was computed for each brain, and proportional scaling was performed to normalize optical density across brains. One artifact-free brain was selected as reference. All brains were spatially normalized to the reference brain. Spatial normalization consisted of applying a 12-parameter affine transformation followed by a nonlinear spatial normalization using 3-dimensional discrete cosine transforms. All normalized brains were averaged to create the final rat brain template. Each original 3-dimensional reconstructed brain was then spatially normalized to the template. Normalized brains were smoothed with a Gaussian kernel (FWHM = 3 × voxel dimension in the coronal plane). A nonbiased, voxel-by-voxel analysis of regional brain activation was performed in SPM. Voxels for each brain failing to reach a specified threshold (70% of the mean voxel value) were masked out to eliminate the background and ventricular spaces without masking gray or white matter. We implemented a Student t test at each voxel, testing the null hypothesis that there was no effect of conditioning. Threshold for significance was set at P < .05 at the voxel level and an extent threshold of 100 contiguous voxels. This combination reflected a balanced approach to control both type I and type II errors. The minimum cluster criterion was applied to avoid basing our results on significance at a single or small number of suprathreshold voxels. Brain regions were identified according to a rat brain atlas [47].

2.7. Functional connectivity analysis

To understand organization of the underlying brain network, we performed functional connectivity analysis. Interregional correlation-based functional connectivity analysis has been applied on rodent brain imaging data to understand brain network activity underlying the resting state [46,55,73] and extinction of conditioned fear [4] among other behaviors (reviewed in [6]). More recently, graph theoretical analysis has been adapted to the study of functional and structural brain networks (reviewed in [7]). This method is particularly useful for visualizing overall network structure and identifying network hubs. We applied these methods to understand how brain regions interact at the network level during retrieval of visceral pain-conditioned PA.

We performed functional connectivity analysis based on interregional correlation of rCBF. Region of interest (ROI) was functionally defined as a set of voxels of a brain area showing significant increases in rCBF in conditioned, as compared with control rats. Anatomical ROIs were first drawn manually in MRicro (version 1.40, http://cnl.web.arizona.edu/micro.htm) over the template brain according to the rat brain atlas. A functional ROI was created by combining the anatomical ROI with the SPM clusters (contrast: conditioned—control, P < .05, extent threshold >100 contiguous voxels) by logical conjunction. Mean optical density of each ROI was calculated for each animal using the Marsbar toolbox for SPM (version 0.42, http://marsbar.sourceforge.net/).

An interregional correlation matrix was calculated across animals for each group in Matlab (version 6.5.1. The MathWorks, Inc., Natick, MA, USA). The matrices were visualized as heat maps with Pearson’s correlation coefficients color-coded. Statistical significance of between-group difference of a correlation coefficient was evaluated using the Fisher’s Z-transform test (P < .05) [15].

Graph theoretical analysis was performed on networks defined by the above correlation matrices in the Pajek software (version...
Fig. 2. Acquisition and retrieval of step-down passive avoidance. After 2 days of training, the conditioned rats learned to refrain from stepping down to avoid the 60-mm Hg CRD, showing greater step-down latencies than control subjects (day 1, F(1,20) = 1.6, P = .22; day 2, F(1,20) = 6.8, P = .017, mixed-model analysis of variance). The learned PA behavior was retrieved on day 3 in the absence of the colorectal balloon. Conditioned rats showed significantly greater step-down latencies on the first trial (37 ± 6 seconds in conditioned rats vs 15 ± 4 seconds in control subjects, P = .01, Student t test), as well as on the second trial (36 ± 4 seconds in conditioned rats vs 21 ± 5 seconds in control subjects, P = .03, Wilcoxon rank test). The presence of a balloon during training combined with repeated exposure to the platform may have caused habituation. This may account for the increase in step-down latency in the control rats during training, as well as the drop in step-down latency in both groups on day 3 (without the balloon) as compared with trial 1, day 2 (with the balloon). Step-down latency on day 3 was likely a more accurate measurement of contextual recall of PA, without the nonspecific effect of an inserted balloon.

3. Results

3.1. Conditioned rats acquired PA behavior

Brain areas showing significant differences in rCBF between the conditioned and the control group are depicted in Fig. 3 and summarized in Table 1. Compared with control subjects, conditioned animals showed increased rCBF bilaterally in a wide range of areas, including medial PFC subregions (ventral cingulate, Cg2; right dorsolateral cingulate, Cg1; retrosplenial, RS, equivalent to posterior cingulate in primates; Prl), AIINS, nucleus accumbens (NAcc), amygdala (including basolateral amygdala, BLA; amygdalopiriform transition area, APIr; cortical amygdaloïd nuclei, PClO, PMCo), and dorsomedial periaqueductal gray (DMPAG). In addition, increased rCBF was noted bilaterally in primary motor cortex (M1), primary and secondary somatosensory cortices (S1, S2), as well as the anterior dorsolateral caudate putamen (adCPu), lateral caudate putamen (ICPus),

2.03, http://vlado.fmf.uni-lj.si/pub/networks/Pajek/) [9]. Each ROI was represented by a vertex (node) in a graph, and 2 vertices with significant correlation (positive or negative) were linked with an edge. A Kamada–Kawai algorithm [24] was implemented to arrange the graph such that strongly connected regions were placed closer to each other, whereas weakly connected regions were placed further apart. Absolute values of correlation coefficients were used for the strength of connection.

To identify hubs of the networks, we calculated 4 centrality metrics in Pajek: betweenness, degree, closeness, and k-core [9,18]. Edges were converted to binary format for centrality calculation. The betweenness centrality of a vertex is defined as the fraction of shortest paths connecting any pair of other vertices that go through this vertex. Betweenness evaluates the importance of a vertex in connecting different parts of a network. A vertex with high betweenness is thus crucial to efficient communication. The degree of a vertex is defined as the number of edges linking it to the rest of the network. Intuitively, vertices with higher degrees are more extensively connected and more central in the network organization. The closeness centrality of a vertex is defined as the reciprocal of the average distance from the vertex to all the other vertices and is computed as the number of other vertices divided by the sum of shortest paths from this vertex to all others. Vertices with higher closeness can reach other parts of the network faster through the paths and are considered more central to the network. A k-core is a measure of modularity and indicates a maximal subnetwork in which every vertex has a degree greater or equal to k. It identifies clusters of vertices that are tightly connected to each other. To derive k-core of a graph, vertices with a degree lower than k are recursively removed until none remain. Each vertex is assigned a k-core number, defined as the highest k-core that contains the vertex. Vertices ranked in the top 25th percentile (top 8 of 30 ROIs) in a centrality measurement were considered hubs in the network.

2.8. Statistical analysis of step-down latency

A mixed model analysis of variance was used to assess statistical significance between control and conditioned rats in day 1 and day 2 training. The Student t test and Wilcoxon rank test were used for statistical comparison of the first and second retrieval trials on day 3, respectively. The nonparametric Wilcoxon rank test was used because distribution of step-down latencies for the second trial was not normal due to the 45-second cutoff time. P < .05 was considered statistically significant.
dorsal hippocampus (dHPC), lateral septum (LS), and the cerebellum (vermis, CbVermis; hemisphere, CbHemis). White matter tracts, including the forceps minor of the corpus callosum (fmi) and external capsule (ec) bilaterally, and left anterior commissure (AC), also showed increased rCBF in conditioned rats. Significant decreases in rCBF were noted in the conditioned rats in the inferior colliculus (IC) and pontine nuclei (Pn) bilaterally, and in the entorhinal cortex (Ent) in the right hemisphere.

Shaded cells in Table 1 depict regions that also showed changes in rCBF in response to acute CRD as we previously reported [68]. Regions showing activation in both studies included the Cg1, PrL, aINS, S1, S2, M1, and adCPu, with differences noted in the extent of activation in these regions. Important differences in regional brain activation were also noted between these studies. In response to acute CRD, but not retrieval of CRD-conditioned PA, broader cortical areas, including the auditory and visual areas, as well as the central and lateral nuclei of the amygdala, showed activation. In contrast, the BLA, dHPC, NAcc, DMPAG, and cerebellum showed activation only in the current study.

3.3. Functional connectivity of brain networks in the control rats

An interregional correlation matrix of rCBF was constructed for the control group and visualized as a heat map in Fig. 4A. Significant correlations (P < .05) were interpreted as functional connections and marked with white dots. The matrix is symmetric.
significant correlations. Certain similarities in the functional connectivity pattern between the 2 groups were noted, including the infrastructural positive correlations in the cortex, as well as positive correlations between the cortex and striatum, and negative correlations between the cortex and amygdala. Meanwhile, there were important group differences (Fig. 5). In the cortex, M1 and PrL/PFC showed more connectivity with other cortical areas, whereas RS showed less connectivity in the conditioned group. Strong positive connections were seen between the amygdala and cerebellar hemispheres. The amygdala was negatively connected to PrL/PFC in the conditioned group, whereas in the control subjects the amygdala was negatively connected to RS. In addition, M1, S1, PrL/PFC, Cg2, RS, alNS, adCPu, dHPC, amygdala and the cerebellar hemisphere showed positive cross-hemisphere correlation.

Graph analysis revealed a cortical cluster, with RS seemingly removed from the core (Fig. 4D, red vertices). The amygdala and cerebellum in conditioned animals formed a separate cluster, and were negatively connected to the cortical cluster (PrL, PFC, Cg1, Cg2). The NAcc, PrL/PFC, and alNS were shown to be crucial to the network structure, with the highest betweenness centrality. In addition, cingulate cortex (Cg1, Cg2), adCPu, and amygdala were also identified as network hubs by graph analysis (Table 2).

4. Discussion

Our main findings were: (1) During the retrieval of visceral pain-conditioned PA, conditioned rats showed activation in the prelifuic area of the prefrontal, anterior insular, and anterior cingulate cortices—areas previously shown to be activated during acute nocuous visceral stimulation [17,68]. (2) Conditioned rats also showed activation in the basolateral amygdala, dorsal hippocampus, and nucleus accumbens—regions implicated in memory recall of PA. (3) In the control group, connectivity analysis revealed a corticostriatal core, which connected negatively to the amygdala, mainly through the retrosplenial cortex. (4) In the conditioned group, by contrast, a modified corticostriatal core connected negatively to the amygdala through the prefrontal area of the medial prefrontal cortex, which, together with the nucleus accumbens and anterior insula, emerged as network hubs. Whereas the brain circuits underlying PA memory recall and affective responses associated with expected pain are likely intertwined, and the current protocol does not dissociate these circuits, we discuss our findings in 2 separate sections to reflect our interpretation of the data based on the literature.

4.1. Expectation of visceral pain: comparison to human brain imaging literature

The findings of similar activation of key brain regions during expected pain as during acute noxious CRD [68] are in agreement with human brain imaging findings implicating homologous regions (alNS, anterior MCC, dorsolateral PFC) in central processing of actual and expected visceral pain [5,41,72]. The insula, the primary interoceptive cortex, is the most commonly reported brain region activated by acute visceral noxious stimulation in humans [34,60]. Based on a meta-analysis of human imaging studies, it was concluded that the anterior basal insula shows dense connectivity to all the amygdala and limbic areas, whereas the anterior dorsal insula is more closely associated with frontal association areas [27]. This pattern of functional connectivity in humans correlates with reported anatomical connectivity in the monkey [2]. Using a classical conditioning paradigm, Yaguez et al. showed activation of anterior insula during expectation of esophageal distension in healthy subjects [72]. In contrast, Berman et al. showed in healthy

Table 1

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Left</th>
<th>Right</th>
</tr>
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<tbody>
<tr>
<td>Cingulate, dorsal (Cg1)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cingulate, ventral (Cg2)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Insular, anterior (AI)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motor, primary (M1)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Premotor (PrL, homologous to human premotor cortex, PFC)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Retrosplenial (RS, homologous to human posterior cingulate, PCC)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Somatosensory, primary (S1)</td>
<td>+</td>
<td>+**</td>
</tr>
<tr>
<td>Somatosensory, secondary (S2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Entorhinal (Ent)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Significant increases or decreases in regional cerebral blood flow are indicated with ‘+’ and ‘−’, respectively, for the left and right hemispheres. Significance is shown at the voxel level (P < 0.05) with extent threshold of 100 contiguous voxels. Shaded cells depict regions that showed changes in rCBF in response to acute colorectal distension (CRD) as we previously reported, with the difference that amygdalar activations during acute CRD were noted in the central and lateral nuclei, and changes in the lateral CPu and PAG showed a decrease rather than increase in rCBF [68].

**Significant at the voxel level P < 0.001.

Table 2

Table 4C shows the interregional correlation matrix for the conditioned group. There were fewer connections in the conditioned group than in the control subjects, 25 positive and 16 negative significances. Certain similarities in the functional connectivity pattern between the 2 groups were noted, including the infrastructural positive correlations in the cortex, as well as positive correlations between the cortex and striatum, and negative correlations between the cortex and amygdala. Meanwhile, there were important group differences (Fig. 5). In the cortex, M1 and PrL/PFC showed more connectivity with other cortical areas, whereas RS showed less connectivity in the conditioned group. Strong positive connections were seen between the amygdala and cerebellar hemispheres. The amygdala was negatively connected to PrL/PFC in the conditioned group, whereas in the control subjects the amygdala was negatively connected to RS. In addition, M1, S1, PrL/PFC, Cg2, RS, alNS, adCPu, dHPC, amygdala and the cerebellar hemisphere showed positive cross-hemisphere correlation.

Graph analysis revealed a cortical cluster, with RS seemingly removed from the core (Fig. 4D, red vertices). The amygdala and cerebellum in conditioned animals formed a separate cluster, and were negatively connected to the cortical cluster (PrL, PFC, Cg1, Cg2). The NAcc, PrL/PFC, and alNS were shown to be crucial to the network structure, with the highest betweenness centrality. In addition, cingulate cortex (Cg1, Cg2), adCPu, and amygdala were also identified as network hubs by graph analysis (Table 2).

4. Discussion

Our main findings were: (1) During the retrieval of visceral pain-conditioned PA, conditioned rats showed activation in the prefrontal area of the prefrontal, anterior insular, and anterior cingulate cortices—areas previously shown to be activated during acute nocuous visceral stimulation [17,68]. (2) Conditioned rats also showed activation in the basolateral amygdala, dorsal hippocampus, and nucleus accumbens—regions implicated in memory recall of PA. (3) In the control group, connectivity analysis revealed a corticostriatal core, which connected negatively to the amygdala, mainly through the retrosplenial cortex. (4) In the conditioned group, by contrast, a modified corticostriatal core connected negatively to the amygdala through the prefrontal area of the medial prefrontal cortex, which, together with the nucleus accumbens and anterior insula, emerged as network hubs. Whereas the brain circuits underlying PA memory recall and affective responses associated with expected pain are likely intertwined, and the current protocol does not dissociate these circuits, we discuss our findings in 2 separate sections to reflect our interpretation of the data based on the literature.

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subjects reduced activation of the anterior insula during a cued anticipatory period preceding rectal distension [5]. The observation of bidirectional modulation of anterior insula suggests different anticipatory and coping responses.

Subregions of the cingulate cortex have frequently been implicated in the affective and motivational response to pain [35,65], including the rostral ACC and anterior MCC. Homology in cingulate regions between primate and rodent is an evolving subject [66]. The Cg1 area in the rat is considered homologous to ACC and dorsal aspects of MCC in primates, whereas the Cg2 area in the rat is homologous to ventral aspects of MCC in primates, and the RS in the rat is homologous to posterior cingulate cortex (PCC) in primates. In humans, the anterior MCC is activated during acute visceral pain, and slightly more rostral aspects of anterior MCC in expectation of visceral pain [54,72]. This is consistent with the activation of Cg1 during acute CRD [68] and activation of Cg1 and Cg2 in the conditioned rats in the current study. Cingulate lesions in animals cause severe deficits in avoidance behavior to noxious somatic stimuli [23,26]. Interestingly, Gao et al. [16] reported that lesion to the Cg1 region selectively impairs formalin-induced, but not foot shock-induced, place avoidance learning, suggesting that this region specifically mediates pain-related negative affect.

We noted an almost identical activation of the PrL/PFC in conditioned rats and in rats receiving acute CRD [68]. The exact homology between rodents and primates in regions of the PFC remains unresolved [63]. The PrL in the rat is generally considered part of
bilateral lesions of deep cerebellar nuclei impair the learning of glosus system responses [74]. Steinmetz et al. [58] showed that and motor learning [59], as well as in mediating autonomic neural. The cerebellum plays a critical role in classical conditioning of decision making [3,10]. spones [10], whereas dorsal striatum mediates important aspects and spatial memory tasks [13]. The nucleus accumbens may play well-established role in contextual Pavlovian fear conditioning textural information required for the retrieval, consistent with its function of which is not well understood, but which has been pro-

Functional activation of the basolateral amygdala is consistent with the notion that this structure plays a critical role in the encoding and storage of emotional memory [14]. Increases in rCBF were also noted in the nearby amygdalopiriform transition area, the function of which is not well understood, but which has been pro-

medial PFC, with features of dorsolateral PFC [63,64] and ACC [52,66]. Activation of dorsolateral PFC, ventrolateral PFC, and medial PFC in response to visceral pain have been reported in humans (reviewed by [34]), and activation of these regions has been implicated in corticolimbic inhibition [33,48,70].

4.2. Functional brain activation associated with the expression of PA

PA is an extensively studied model of aversive learning. A majority of studies have used electric foot shock as the aversive stimulus in either a step-down or a step-through design. Of particular relevance to our study, key brain regions critically involved in the retrieval of PA have been reported to include the basolateral amygdala, dorsal hippocampus, and striatum, including the nucleus accumbens [32]. Our results provide the first blood flow mapping evidence implicating all of these regions in PA retrieval. Functional activation of the basolateral amygdala is consistent with the notion that this structure plays a critical role in the encoding and storage of emotional memory [14]. Increases in rCBF were also noted in the nearby amygdalopiriform transition area, the function of which is not well understood, but which has been pro-

Correlational and graph theoretical analysis revealed a number of findings not immediately apparent from the regional analysis in SPM. In control rats, a corticostriatal core was revealed that demonstrated strong positive correlations between midline cortical regions, including Cg1/ACC, Cg2/anterior MCC, PrL/PFC, RS/ PCC, as well as with NAcc and adCPu. Within this corticostriatal core, Cg2 and RS were negatively correlated with the amygdala, whereas the PrL/PFC was negatively correlated with the cerebel-

Hubs of functional brain network were identified as region of interests (ROIs) ranked in the top 25% in four measurements of centrality. Refer to Methods for detailed descriptions of the measurements. Abbreviations are as indicated in table 1. ROIs in the right or left hemisphere are denoted with suffixes “R” and “L”, respectively. Note there are a few instances of tied ROIs in the degree and k-core rankings.

### Table 2

<table>
<thead>
<tr>
<th>Rank</th>
<th>Control rats</th>
<th>Betweenness</th>
<th>Degree</th>
<th>Closeness</th>
<th>k-Core</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ChHemis_L</td>
<td>Cg2_L</td>
<td>Cg2_L</td>
<td>adCPu_R, NAcc_R, Cg2_L, Cg2_R RS_L, RS_R</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>RS_R</td>
<td>Cg2_R</td>
<td>Cg2_R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cg2_L</td>
<td>PrL_L</td>
<td>Prl_L</td>
<td></td>
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</tr>
<tr>
<td>4</td>
<td>PrL_L</td>
<td>adCPu_R</td>
<td>Cg1_L</td>
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<tr>
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<td>dHPC_R</td>
<td>Cg1_R</td>
<td>RS_L</td>
<td>adCPu_L, Cg1_R PrL_L, PrL_R</td>
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</tr>
<tr>
<td>6</td>
<td>PrL_R</td>
<td></td>
<td></td>
<td>NAcc_L</td>
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<td>LS</td>
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</tr>
<tr>
<td>8</td>
<td>Cg2_R</td>
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**Conditioned rats**

<table>
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<th>Rank</th>
<th>Betweenness</th>
<th>Degree</th>
<th>Closeness</th>
<th>k-Core</th>
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<td>AMYG_R</td>
<td>PrL_R</td>
<td>adCPu_L AMYG_L, AMYG_R Cg1_R Cg2_R PrL_L, PrL_R</td>
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<tr>
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<td>PrL_R</td>
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<td>NAcc_L</td>
</tr>
<tr>
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<td>dINS_L</td>
<td></td>
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<td>AMYG_R</td>
</tr>
<tr>
<td>4</td>
<td>S1_L</td>
<td>AMYG_L</td>
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<td>6</td>
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<tr>
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<td>AMYG_L</td>
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<tr>
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<td>M1_L</td>
<td>PrL_L</td>
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</table>

Hubs of the functional brain network.
the amygdala and cerebellar hemispheres; and (4) a significant positive cross-hemisphere correlation in aINS, and a positive correlation between aINS and NAcc.

Graph theoretical analysis underscored these findings. In conditioned animals, the PrL/PFC emerged as a clear hub, which connected both locally (as a provincial hub) and to other modules (as a connector hub). Locally, the PrL/PFC served as a hub within the corticostriatal core. The RS/PCC was notably dissociated from this core, in contrast to its inclusion in the core in the control animals. The PFC-centric cluster was negatively correlated to the amygdala, which functioned as a connector hub to a smaller module comprised of itself and the cerebellar hemispheres. The negative connection between the PFC and amygdala is consistent with the notion of reciprocal inhibitory modulation between these regions in emotion and pain processing [22,36,38]. Of note, recent work by Labus et al. examining brain responses to aversive visceral stimuli in IBS patients showed that rostral ACC exerts a significant negative influence on the amygdala in male subjects during expectation of visceral pain [29]. In addition, our data revealed that the PFC-centric cluster connected via the NAcc (a connector hub) to a smaller module comprised of the NAcc, aINS, and motor and somatosensory areas.

In conclusion, we established an animal model to study brain mechanisms underlying the affective responses associated with visceral pain expectation. Important specificity issues remain to be addressed because brain circuits underlying pain processing of different modalities as well as other emotional processing often overlap significantly. Reverse translation from human brain imaging findings may help interpretation of animal imaging data. Homologous findings at the circuit level between the rodent and human functional brain imaging suggests that neuroimaging may provide a means for bidirectional translation between preclinical and clinical pain research, with implications for the future identification of novel therapeutic targets in the modulatory network of visceral pain processing.

Conflict of interest statement

The authors declare no conflicts of interest.

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