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MAPPING OF PAIN CIRCUITRY IN EARLY POST-NATAL DEVELOPMENT USING MANGANESE-ENHANCED MRI IN RATS

M. M. SPERRY,[a] B. M. KANDEL,[b] S. WEHRLI,[c] K. N. BASS,[d] S. R. DAS,[b] P. S. DHILLON,[b] J. C. GEE[b] AND G. A. BARR[d]*

[a] *Department of Bioengineering, University of Pennsylvania, United States*

[b] *Penn Image Computing and Science Laboratory, Department of Radiology, University of Pennsylvania, United States*

[c] *NMR Core, Children's Hospital of Philadelphia, United States*

[d] *Anesthesiology and Critical Care Medicine, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, United States*

Abstract—Premature or ill full-term infants are subject to a number of noxious procedures as part of their necessary medical care. Although we know that human infants show neural changes in response to such procedures, we know little of the sensory or affective brain circuitry activated by pain. In rodent models, the focus has been on spinal cord and, more recently, midbrain and medulla. The present study assesses activation of brain circuits using manganese-enhanced magnetic resonance imaging (MEMRI). Uptake of manganese, a paramagnetic contrast agent that is transported across active synapses and along axons, was measured in response to a hindpaw injection of dilute formalin in 12-day-old rat pups, the age at which rats begin to show aversion learning and which is roughly the equivalent of full-term human infants. Formalin induced the oft-reported biphasic response at this age and induced a conditioned aversion to cues associated with its injection, thus demonstrating the aversiveness of the stimulation. Morphometric analyses, structural equation modeling and co-expression analysis showed that limbic and sensory paths were activated, the most prominent of which were the prefrontal and anterior cingulate cortices, nucleus accumbens, amygdala, hypothalamus, several brainstem structures, and the cerebellum. Therefore, both sensory and affective circuits, which are activated by pain in the adult, can also be activated by noxious stimulation in 12-day-old rat pups.

Key words: brain, development, MRI, circuitry, nociception.

INTRODUCTION

Approximately 400,000 human infants are admitted to the neonatal intensive care unit (NICU) in the United States every year, and each day in the NICU (Martin et al., 2015) these infants undergo on average 10 or more medically essential but potentially painful procedures (Carbajal et al., 2008). However, the sensory and affective circuits that are activated by pain in infants are not fully understood.

Although there is cortical activation to painful stimuli in human infants as early as 24 weeks of gestational age (Bartocci et al., 2006; Slater et al., 2006a), little is known about pain circuitry, and particularly the function of affective brain regions, in newborns. In a detailed analysis of these circuits using fMRI in full-term newborns receiving a pin prick, 18 of 20 adult brain areas associated with pain were activated in the infant. These brain areas included the primary somatosensory cortices, anterior cingulate cortex (ACC), thalamus, insular cortex, parietal lobe, pallidum, and precuneus cortex. Notably, two brain areas were *not* activated in the infant, the amygdala and orbitofrontal cortex (Goksan et al., 2015). Although these two brain areas are noted in adults for emotional processing and decision-making, this suggests perhaps a divergence in affective pain processing in infants and adults. It should also be noted that some brain regions were activated only in the infant, including the auditory cortices, hippocampus, and caudate, which may be a result of the well-documented developmental reorganization of the cortico-cortical inter-hemispheric pathways (Courtiol and Wilson, 2014).

Despite the aforementioned parallels in infant and adult pain circuitry in full term infants, much about pain-related development, and particularly the affective component of pain, which greatly influences the quality of life (Oluigbo et al., 2012; Elman et al., 2013; Werle et al., 2014), even in children (Weiss et al., 2013), remains unknown. Adverse painful infant experiences can induce long-term changes in cognitive and affective behaviors (Ranger et al., 2014; Vinall and Grunau, 2014). Infant pain, which regulates attachment (Sullivan and Moriceau, 2004), has been hypothesized to play a key role in the development of psychopathologies such as anxiety disorders and depression later in life. Brain function in areas associated with emotional learning, such as the hippocampus and amygdala, appear exceptionally disturbed in people with these pathologies [for review see (Schore, 2002; Teicher, 2002; Connor et al., 2003; Lederhendler, 2003; Teicher et al., 2003; Zeanah et al., 2003)], yet details of how these circuits are affected by pain in infants has not been studied.

*Corresponding author. Fax: +1-267-426-9722.
E-mail address: barrg@email.chop.edu (G. A. Barr).

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In rat pups, odors paired with noxious stimuli become aversive and activate the amygdala after 10 days of age (PN10) but not before (Sullivan et al., 2000; Moriceau and Sullivan, 2006). This transition is mediated by activation of the amygdala dopamine paths between PN8 and PN12 [(Barr et al., 2009); reviewed in (Sullivan and Holman, 2010)]. At PN14, when aversions to noxious stimuli can be learned, but not at PN3 when aversions are not learned, formalin injection to the hindpaw increased levels of Fos protein in the paraventricular and medial nuclei of the thalamus, the paraventricular nucleus of the hypothalamus, and periaqueductal gray (PAG) of the midbrain, suggesting both sensory and stress-related components of pain are present in the second week of life in the rat (Barr, 2011). In the plantar incision model, with and without sciatic nerve blockade, there were long-term alterations in rostroventral medulla (RVM) modulation for the cohort that did not receive sciatic nerve blockade, highlighting the role of supraspinal centers in the development of sensory processing (Walker et al., 2009). However, a more widespread analysis of activated circuits and their interactions at the age at which aversions are first learnable, has not been done.

Although studies using techniques such as fMRI and electrophysiology provide excellent time-resolved data, they do not couple high-resolution spatial details with brain-wide functional information and have rarely been applied to the immature animal. However, fMRI has been used successfully to map maturational changes in the visual and somatosensory systems in the first few weeks of life in the rat and show decreased latency and increased signal to stimulation (Colonnese et al., 2008; Chan et al., 2010). Fos expression permits visualization of brain activation in immature animals (Wiedenmayer and Barr, 2001; Barr, 2011) yet Fos labels active cell bodies and does not identify active circuits. Chemical analyses, such as magnetic resonance spectroscopy, are generally limited to data collection from a single brain voxel and identify hippocampal changes accompanying neurobehavioral changes in adults who experienced infant maternal deprivation (Hui et al., 2011). To bridge the gap between processes at the cellular and genomic levels and function of brain circuitry globally, we examined the neural response to a noxious stimulus (hindpaw injection of dilute formalin) at the level of specific brain nuclei and cell populations within the brain using manganese-enhanced MRI (MEMRI). MEMRI incorporates a functional contrast agent approach to provide highly resolved functional and structural information and is a calcium analog that enters cells through voltage gated calcium channels, is packaged into vesicles, and transported down the axon (Lee and Koretsky, 2004). Transport transsynaptically is a function of activity. For example, $MnCl_2$ injected into the eye of seeing mice shows activation of thalamic and cortical structures whereas the same injection in congenitally blind mice restricts transmission of this tracer to the optic tract (Bearer et al., 2007). Therefore labeling is dependent on neuronal signaling (Kikuta et al., 2015). As a result of this mechanism, MEMRI is a powerful tool to map functionally active circuits that change with behavior, pharmacology, and physiology (Pautler, 2006; Saar et al., 2015; Szulc et al., 2015; Ulyanova et al., 2016). Further, MEMRI can be easily performed in conscious animals by activity-dependent distribution of manganese contrast in awake rats for longitudinal studies as with fMRI but with better resolution, and subsequently *ex vivo* MRI of the brain. To our knowledge there are no data using MEMRI to map functional circuits for behavior in the immature animal; however, MEMRI has successfully mapped both the postnatal development and plasticity of the visual system in neonatal rats (Chan et al., 2012).

The goal of this study was to provide a detailed map of altered brain function induced by pain in the infant rodent at an age at which aversions are first learned, testing hypothesized pain circuit models, to establish a point of departure for subsequent mechanistic and long-term follow-up studies.

EXPERIMENTAL PROCEDURES

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Long-Evans hooded rats from Harlan Laboratories (now ENVIGO), born in our AAALAC approved CHOP animal facility, were housed under AAALAC-compliant conditions, with a 12-h light/dark cycle under otherwise standard conditions and abundant nesting materials. Females and males were bred by introducing the male into the home cage of the female. After 5 days, they were separated. Female cages were monitored twice daily and the day of birth labeled day 0.

Treatment & behavior testing

A male and female twelve-day-old (PN12) rat pup ($N = 38$) were randomly selected from a litter, and one pup of each sex from a single litter was used in each condition. In the morning of the lights-on cycle, pups were injected systemically with manganese chloride [$MnCl_2$; 75 mg/kg Sigma #244589; s.c. in bicine buffer, pH $\sim$ 5.9; (Silva et al., 2004)] or the vehicle control. We observed the pups for approximately 60 min after injection and again at 7 and 24 h. Although we did not formally record behavior, we noted no unusual behaviors.

Twenty-four hours after the $MnCl_2$ injection, we injected dilute formalin (2% intra-plantar; 1 μl/g body weight; $N = 19$) into the right hindpaw to induce a nociceptive response that lasted about 45–60 min. The matched littermate control was not injected so as to avoid any noxious input ($N = 19$). Thus, the nociceptive signal is the needle insertion and the formalin injection. Twenty-four hours after the $MnCl_2$ or vehicle injection, formalin was injected. Contrast enhancement reaches equilibrium at 24 h (Malheiros et al., 2015) and MEMRI enhancement is maximized at that time (Wadghiri et al., 2004). All animals were treated as identically as possible during the 24 h, and each pair of formalin injected and controls were littermates and returned to the dam. Thus

the difference between experimental and control subjects was the formalin injection.

We assessed the behavioral response to formalin injection in a separate test chamber, using a 5 point nociceptive scoring system (Barr, 1998). Those data were analyzed by a two-way ANOVA (treatment × time) with repeated measures for the time variable ($N = 11$ $MnCl_2$; $N = 17$ vehicle). In a separate cohort of animals, the formalin injection ($N = 14$) was paired with a distinct odor (orange) present to assess whether an aversion could be conditioned in response to formalin injection. Controls were placed in the odor-adulterated chamber but with no injection ($N = 13$). Four hours later pups were tested for preference/aversion to odor in a three-chamber test as previously described for infant rats (Barr and Rossi, 1992; Barr et al., 1994).

$MnCl_2$ pups were perfused 7 h after the formalin injection with saline followed by 4% paraformaldehyde and the brains carefully removed under a dissecting microscope to avoid damage. Because we were not conducting longitudinal studies, repeatedly imaging the same animal, we chose to image perfused brains because it allowed us long scanning times (e.g. 7–8 h) providing detailed images. Specific comparisons of perfusion methods showed no loss of signal in aldehyde perfused brains compared to microwave fixation (Liu et al., 2013) and functional studies of neural (Bonny et al., 2008) and non-neural (Lamprianou et al., 2011) tissue show no loss of sensitivity over several days after sacrifice of the animal.

Scanning procedures

Prior to scanning, the fixed brains were immersed in PBS for 1 h and then placed in a glass tube containing Fomblin, which provided a good susceptibility match for the tissue, a black background in the images, and prevented the sample from drying during scanning. We performed T1-weighted 3D spin echo images to visualize the manganese uptake (Bruker Biospin) on a 9.4 T Bruker Avance DMX spectrometer equipped with a microimaging accessory. The Micro2.5 gradient set, with actively shielded gradients of 2.5 G/cm/A (i.e. 100 G/cm at 40 A), was used with the microimaging probe. Acquisition times were 7–8 h in length to achieve a good signal to noise ratio at a resolution of 75-μm isotropic voxels with the following parameters: TR 30 ms, TE 3.28 ms, flip angle 45o, 25 NEX. The raw data were apodized by a 25% trapezoidal window to reduce noise, then reconstructed using the Paravision™ (Bruker Inc.) software and saved for further analysis and identification of the regions. Example images are shown in Fig. 1. Scans of brain in which $MnCl_2$ was not injected showed no signal and were uniformly gray (Fig. 1).

Image processing

We used the suite of open source Advanced Normalization Tools (ANTs) developed and provided by the Penn Image Computing and Science Laboratory (http://www.picsl.upenn.edu/ANTS/). First, a template image was constructed from the control specimens to provide a common stereotaxic space to which both control and experimental subjects were spatially normalized (Avants et al., 2008, 2009). Subsequently, due to intensity fluctuations inherent to MR techniques, the intensity range of each brain scan was normalized by the mean of the visual cortex region, a region that is not known to be affected by pain stimuli. Normalization to particular brain regions or background noise signal is commonly employed to account for such fluctuations in both human and non–human animal studies (Pautler et al., 2003; Aoki et al., 2004; Chan et al., 2014)

Morphometric analysis

We performed voxel-wise statistical tests over the whole brain and within specific regions to determine group differences using the randomize function in the FMRIB Software Library (FSL, http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL). Threshold-free cluster enhancement (TFCE) was applied across the brain images of matched littermate pairs (paired *t*-test, *p*-level of 0.05, uncorrected for multiple comparisons) to evaluate brain activity resulting from the formalin stimulus. Group differences in brain activity were tested using two *t*-contrast designs: (Formalin − Control) and (Control − Formalin), to evaluate increased and decreased brain signaling due to formalin-treatment. Changes were evaluated both in anatomical brain regions, as well as in specific nuclei, where appropriate.

Segmentation

We developed and applied a PN12 regional atlas to segment image volumes into anatomical brain regions and specific nuclei. The atlas was developed with ITK-SNAP (http://www.itksnap.org/pmwiki/pmwiki.php) using manual slice-by-slice delineation and included 25 bilateral regions, chosen based on previous literature review, with a focus on pain-related nuclei (Yushkevich et al., 2006).

Structural equation modeling (SEM)

To understand more deeply the brain circuits that are implicated in pain responses at day 12, we used SEM, a statistical technique that defines causal relationships between independent variables (predictors) and dependent variables (outcomes). This method has been used successfully to map circuits in the visual system (McIntosh and Gonzalez-Lima, 1994; Kohler et al., 1998; McIntosh et al., 1998; Wohr and Schwarting, 2013), limbic system (Jenkins et al., 2003; Stein et al., 2007; Poirier et al., 2008), frontal cortical areas (Patel et al., 2003), and in a variety of behaviorally defined tasks (McIntosh and Gonzalez-Lima, 1994; Poirier et al., 2008). We created multiple structural equation models based on the literature and included a wide number of circuits for ‘‘sensory” and ‘‘affective” pain. We started with these hypothesized models, which define many known, anatomically correct, structures and interconnections thought to be involved in nociception. From this we hypothesized several simple subcircuits with 3–6 regions of interest

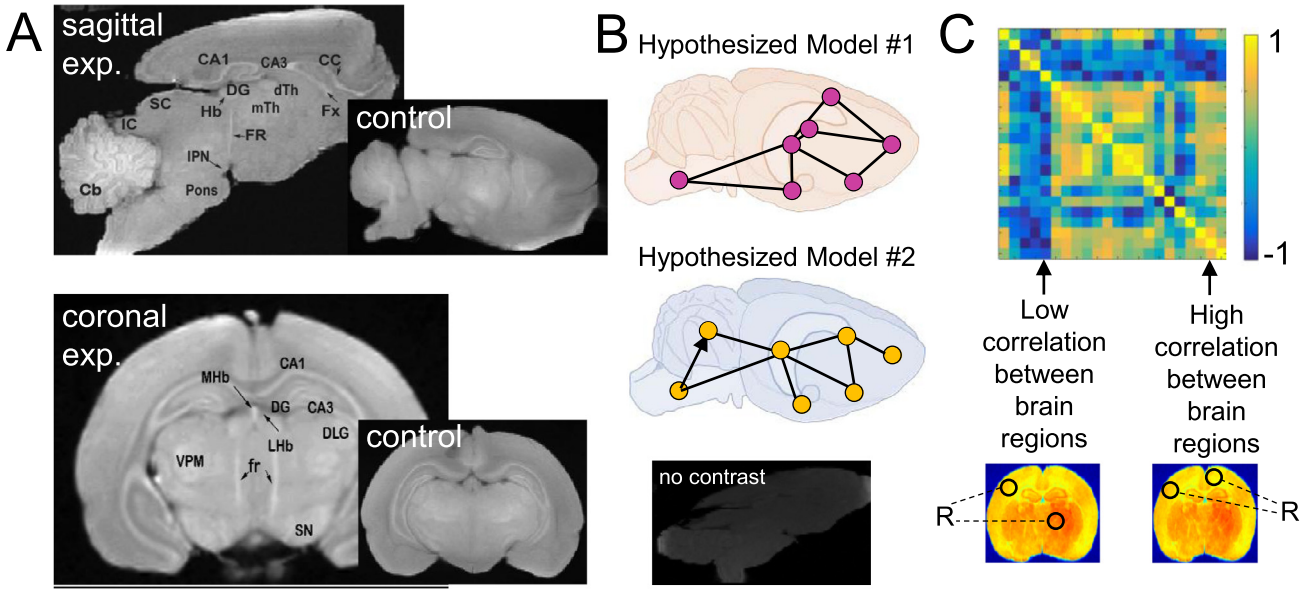

Fig. 1. (A) The P12 rat brain atlas was fit to individual brain scans and these definitions allowed for measurement of regional mean intensity values, which were used as inputs into both (B) structural equation models and (C) co-activation networks. The networks for the control and formalin cohorts were constructed by calculating the correlation coefficient, R, between each brain region. The difference network between the two states, ΔR, was subsequently calculated by taking the absolute difference between $R_{formalin}$ and $R_{control}$ networks.

(ROI's) and interconnections (Fig. 1B). We assessed the strength of each model's fit to the MEMRI data in R [R Foundation for Statistical Computing; (Fox et al., 2014; Team, 2014)], defining a strong fit as one in which the comparative fit indices (CFIs) were greater than 0.90 and the standardized root mean square of the residuals (SRMRs) was less than 0.08.

Co-activation networks

Although SEM allows for mapping of anatomical brain regions that are physically connected and communicating, it was also of interest to investigate brain regions that are functionally coupled during the pain experience. Although the MEMRI technique does not provide time series data, as one would have when using techniques such as blood–oxygen level dependent (BOLD) MRI, investigation of co-activating regions during an experience (in this instance, nociception), can provide additional insight into regional synchronization. Similar studies of PET metabolic and cortical thickness networks described these datasets as covariance patterns (He et al., 2007; Di et al., 2012). Undirected co-activation matrices were constructed for both the formalin-treated and control groups through calculation of the mean of each anatomically defined brain region for all subjects and subsequent computation of the correlation coefficient between the vector of subject means for each pair of brain areas (Fig. 1C). In the final networks, all regions in the network were connected and weighted by the Pearson correlation coefficient (R) of average voxel intensity between defined anatomical brain regions (Matlab, Mathworks). To investigate changes in connectivity between the control and formalin-treated groups, absolute difference matrices were constructed (ΔR) and subsequently thresholded at various levels ($\Delta R > 0.7$–0.9) to identify correlations that exhibit the greatest change in response to formalin stimulation. We used these methods of analysis for networks averaged over the left and right sides of the brain.

RESULTS

Animal behavior

All rat pups exhibited normal post-formalin injection behaviors, including flexion of the limb, shaking the limb, and licking of the injected paw. In addition, the characteristic biphasic response is present in both cohorts, with a high nociception score at early time points, followed by a dropin score, and finally a return to high scores at approximately 15 min after injection. Quantification of these behaviors using nociceptive scoring showed significant differences between pups injected with the contrast agent $MnCl_2$ versus a vehicle injection at a single time point (Fig. 2A). Pups treated with formalin showed a conditioned odor aversion test (treatment by side interaction; $p < 0.02$), with the formalin-treated group demonstrating significantly greater aversion compared to the no treatment cohort (Fig. 2B). This aversion is not learned at 3-days of age (separate cohort of animals; data not shown) which is consistent with the literature showing limited aversion learning before PN10 [e.g. (Landers and Sullivan, 2012); reviewed by (Tallot et al., 2016)].

Regional brain analyses

Morphometric analyses revealed increased activation of sensory structures, including the somatosensory cortex, cerebellum, and medulla in the formalin-treated cohort

(Figs. 3 and 4). Activation is notably lateralized to the left side of the brain in sensory regions, contralateral to the right hindpaw injection. In addition to increased activation of sensory components, limbic, midbrain, and brainstem structures and the cerebellum were also differentially activated in response to formalin exposure. Within the limbic system, we found activation of the dorsal hypothalamus, cortical amygdala, ventromedial hypothalamus, as well as the mammillary bodies. In the brainstem, formalin injection activated the superior colliculus, ventral tegmental area, and the pontine reticular nucleus.

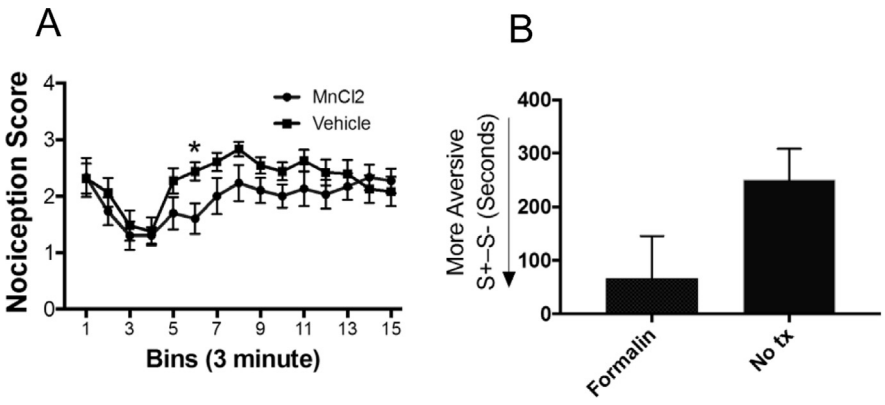

Fig. 2. Behavioral measures demonstrate that (A) $MnCl_2$ produced reduced nociception scores (two-way ANOVA, main effect of treatment $F(1,24) = 7.49$, $p = 0.011$; Interaction bin X treatment: $F(14,336) = 2.01$; $p = 0.017$; Tukey posthoc: $p < 0.01$ at bin 6; denoted by *); (B) at P12 formalin produced a conditioned odor aversion (Interaction side × treatment: $F(1,44) = 6.20$; $p = 0.02$).

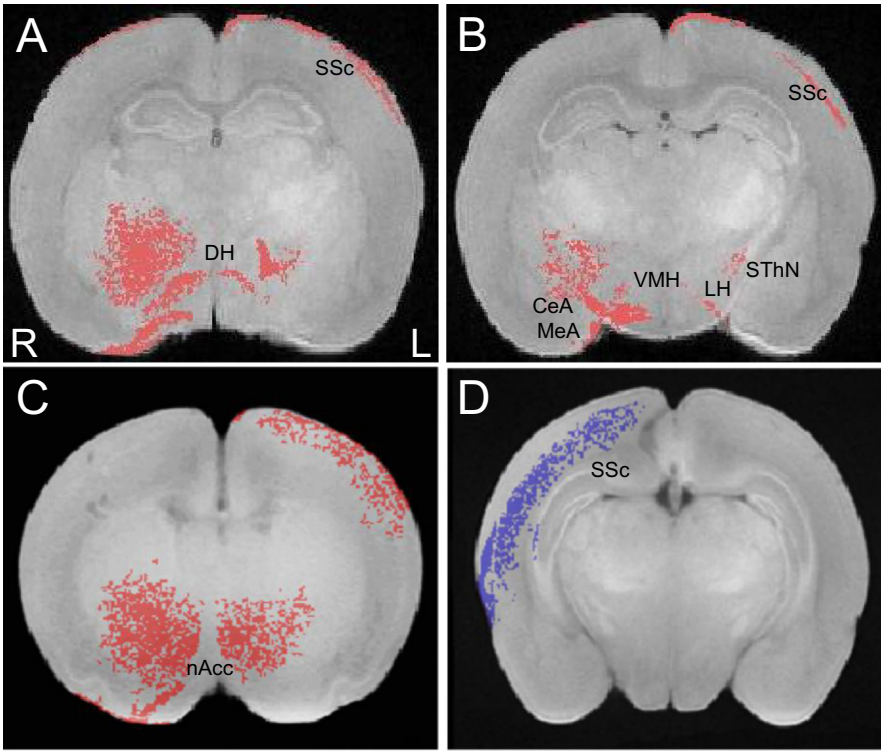

Fig. 3. The P12 brain is differentially activated (red shading; formalin-injected group > control) by a nociceptive stimulus in several regions, including (A) the dorsal hypothalamus (DH) and somatosensory cortex (SSc), (B) the central (CeA) and medial (MeA) amygdala, the ventromedial (VMH) and lateral (LH) hypothalamus, and the subthalamic nucleus (SThN), (C) the nucleus accumbens (nAcc), and (D) the somatosensory cortex ipsilateral to injury which is less active following nociceptive stimuli compared to control (blue shading; control > formalin-injected group).

Pain circuitry

SEM of the brain's response to formalin provided evidence for three principal models of nociceptive pain circuitry at P12. The first model (Fig. 5A), which highlights medial and limbic structures, fits strongly to the formalin-treated cohort (CFI = 1.00, SRMR = 0.065) but not to the control group (CFI = 0.791, SRMR = 0.195). The second model (Fig. 5B), is also focused on medial and limbic structures, yet provides evidence for involvement of the cerebellum in nociceptive pain circuits. The formalin-treated cohort demonstrated a significant fit to this proposed model (Experimental CFI = 0.919, SRMR = 0.060), while the control group did not (Control CFI = 0.873, SRMR = 0.040). A third proposed model (Fig. 5C), is a purely sensory circuit: the sensory cortex, lateral thalamus, and medulla. In this model, both the controls and formalin-treated cohorts exhibit CFI > 0.9, yet the CFI for the formalin group is higher (CFI = 1.00) in comparison to the controls (CFI = 0.923). However, the control group has a high amount of residual error, and therefore this model is a significant fit only for the formalin group.

Co-activation networks

Analysis of co-activation networks using techniques from graph theory provided an opportunity to look at *global* brain activity, as opposed to specific sub-circuits. The difference network, defined by the absolute value of $\Delta R = R_{formalin} - R_{control}$, highlights the correlations that undergo the most change in response to formalin exposure (Fig. 6; $\Delta R > 0.9$). The nucleus accumbens was a key hub within this network, showing strong correlations with several limbic, hypothalamic and cortical structures including the amygdala (basolateral and central subnuclei), caudate/

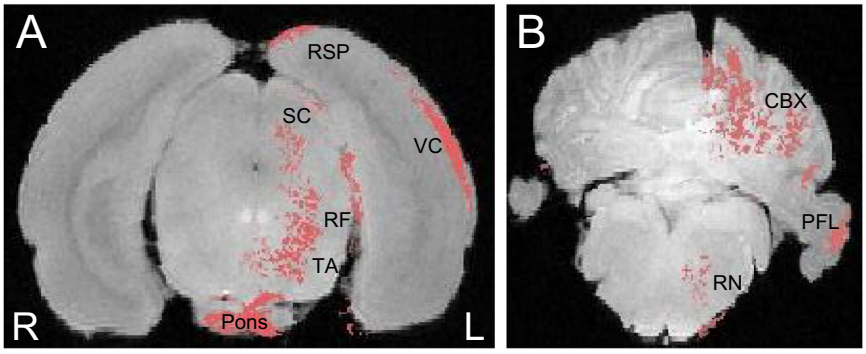

Fig. 4. Differential activation (formalin injection > control) is also present in the cerebellum and brainstem regions following formalin injection, including (A) the superior colliculus (SC), reticular formation (RF), tegmental area (TA), retrosplenial area (RSP), visual cortex (VC), and pons, and (B) cerebellar cortex (CBX), paraflocculus (PFL), and red nucleus (RN).

putamen, cerebellum, hippocampus, and insula. There was also a strong change in the medulla – ventral hypothalamus connection between controls and formalin-treated pups. The results show a connected limbic network that is consistent with the "affective" SEM networks that may mediate the aversiveness of the formalin-induced pain.

DISCUSSION

Summary of results

In the present study, MEMRI provided a highly resolved mapping of activity within the whole brain. Inflammatory pain following intraplantar formalin induced a conditioned aversion, with evidence for functional maturity of both affective and sensory pain circuitry at 12 days of age. The noxious stimulus activated a variety of limbic and related structures including the amygdala, nucleus accumbens, anterior cingulate, insula and prefrontal cortices, hypothalamus, and medial thalamus. Connected to these networks were the cerebellum and periaqueductal gray. The somatosensory cortex and other structures that may mediate this aversive state were laterally activated. These data are consistent with both human work showing activation of somatosensory cortex in premature to full-term infants following a painful event (Bartocci et al., 2006; Slater et al., 2006a). The results here expand upon prior work that used Fos as a marker of activity and that had shown activity of the paraventricular and medial dorsal nuclei of the thalamus, the paraventricular nucleus of the hypothalamus, and periaqueductal gray at 14 days of age but not at 3 days of age (Barr, 2011). There are parallels between MEMRI results as found here and those in adults. Systemic $MnCl_2$ injection enhanced MRI signaling in the agranular insular cortex, auditory cortex, primary somatosensory cortex of the hind limb following noxious electrical hindpaw stimulation in adult rats, suggesting activation of both affective and sensory circuitry (Cha et al., 2016). To assess specific thalamic circuitry, $MnCl_2$ was directly injected into right medial thalamus which labeled the ACC, cingulate cortex, retrosplenial cortex, ventral medial caudate-putamen, nucleus accumbens, and amygdala after prolonged noxious electrical stimulation of the forepaw, again both affective and sensory paths. Most but not all of this activation was attenuated by systemic morphine (Yang et al., 2011). Thus, similar to immature P12 rats, MEMRI in adult rats has shown differential activation of overlapping paths, suggesting that these structures are critical components of both immature and mature pain circuitry.

Sensitive periods

During early development, there is a sensitive period for pain exposure such that if there is an injury that is more than momentary, long-term adult pain processing is altered. Infant pain changes adult sensitivity to pain, including sensory thresholds. For example, preterm infants who had been subjected to repeated painful procedures in the NICU have a later dampened response to pain, whereas full-term neonates who have a surgical procedure, such as circumcision, have a heightened pain response (Taddio and Katz, 2005). In an animal model, inflammatory injury, lasting at least 24 h, to a limb in rats or mice prior to PN8 produces consistently altered pain responses in the adult: there is hypoalgesia testing for baseline reflex changes and a strong hyperalgesia upon re-injury in the adult (Ruda et al., 2000; Ren et al., 2004; Walker et al., 2009). After PN8, similar infant injuries have no long-term consequences on nociceptive thresholds (Ruda et al., 2000; Ren et al., 2004). But if the affective components develop at a later age than the sensory components, it is possible that the sensitive period for long-lasting changes in the affective component of pain occurs at a different age. If true, injuries that alter the sensory component of pain (e.g. thermal and mechanical thresholds) only at young ages, may alter the affective components of pain at later ages. To our knowledge this has not been tested

Utility of MEMRI in sub-region analysis

MEMRI is a unique imaging tool for simultaneously probing structural and functional aspects of brain connectivity. As alluded to earlier, it has several advantages over fMRI, including higher spatial resolution. This is an important consideration when investigating specific circuits and cell populations. $MnCl_2$, a calcium analog, moves into activated neurons through activity dependent trans-synaptic transport. For example, intra-ocular injection of $MnCl_2$ into the eye of normal mice labels the thalamus and more distal structures in the visual path, whereas injection into the eye of genetically blind mice does not label paths beyond the optic tract (Bearer et al., 2007). This unique form of *functional* imaging provides a clearer picture of activated circuits compared to reliance on analogs for brain function, such as blood flow (BOLD fMRI) or glucose

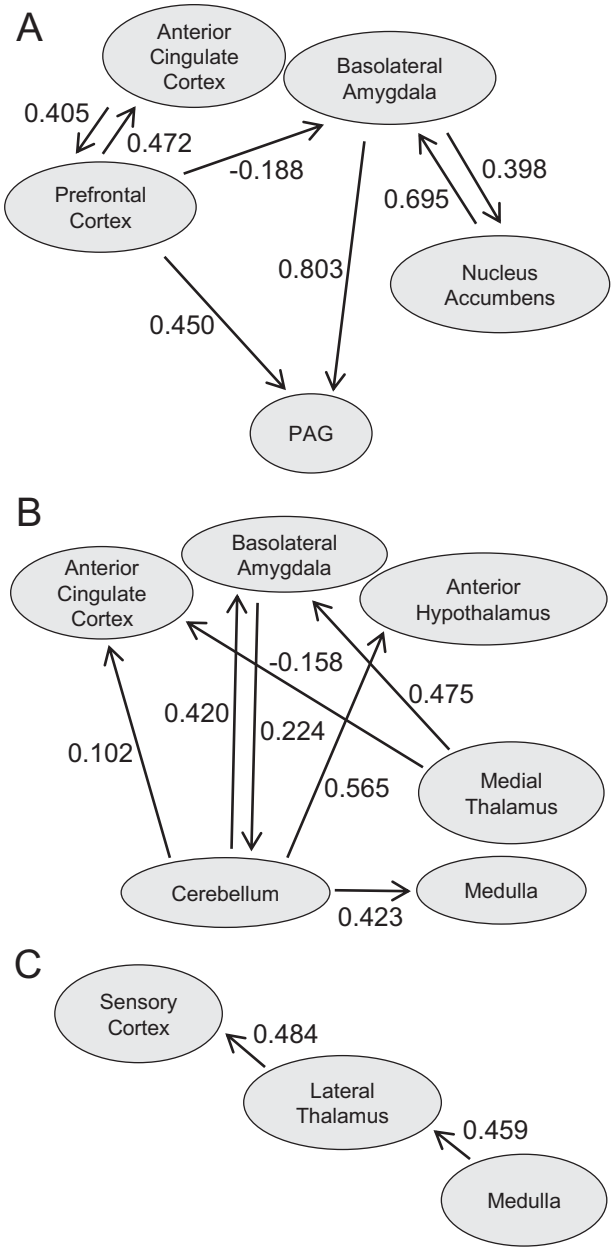

Fig. 5. Structural equation models exhibiting strong fit to MEMRI distributions in the formalin-treated cohort. These three circuits provide evidence for (A) interaction between affective components (B) interactions between affective regions and sensory structures, such as the medulla, and (C) a sensory-only pathway. Arrow labels indicate the regression coefficient between two components in the model.

uptake (2DG or FDG PET; see Table 1). However, MEMRI produces a single snapshot of cumulative brain function and does not provide time course information of brain fluctuations in real time. Additionally, MEMRI, fMRI, and cFos imaging are unable to provide information about excitatory versus inhibitory signals, and therefore other techniques, such as voltage sensitive dyes, are needed to investigate this question (Canepari et al., 2010). Despite these drawbacks, MEMRI is a tool that may provide detailed information on brain activity and can be an added tool to characterize brain circuits in future studies.

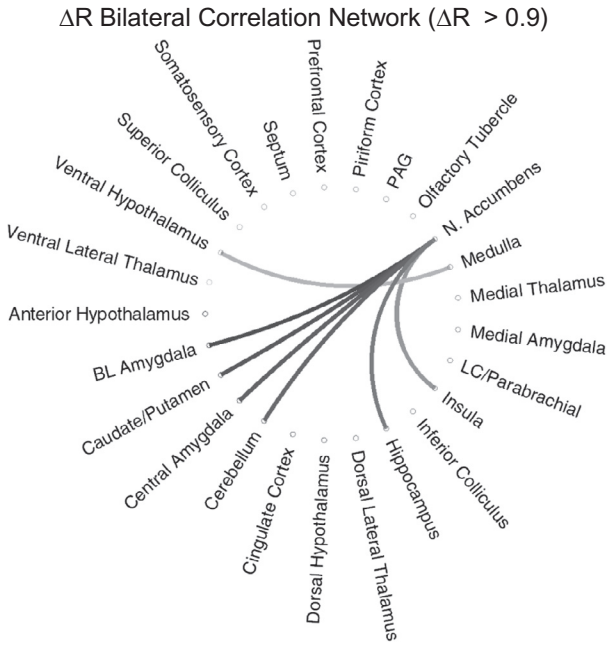

Fig. 6. Correlation network in the brain defined by the absolute difference between formalin-treated and control groups ($\Delta R > 0.9$).

Brain activity and lateralization

Most human studies of the neural circuitry of pain have focused on cortical activation to acute pain (heel sticks, needle sticks, venipuncture) in premature to full term infants (Bartocci et al., 2006; Slater et al., 2006b; Verriotis et al., 2015). In these studies, cerebral hemodynamics were increased in response to noxious stimuli. Despite different protocols, these studies showed cortical activation by 25–26 weeks gestation. In human infants, pain induces a bilateral cortical response that was stronger contralateral to the hand venipuncture or heel stick as early as 25 weeks of gestation and increased with age. (Bartocci et al., 2006). These responses were increasingly lateralized with age, compared to initial bilateral activity. Likewise, we found lateralization throughout sensory-motor structures, including sensory cortex, midbrain and cerebellum. A more symmetrical response was seen in the hypothalamic regions and the nucleus accumbens

Despite clear nociceptive reflexes, using pain facial expressions, or cortical activation at birth, to probe the extent of subcortical involvement in pain processing of infants has been difficult. Recently, functional MRI provided evidence that the pain circuitry in full-term infants is quite similar to that of adults (Goksan et al., 2015). Newborns receiving a pinprick activated 18 of the 20 adult brain areas using the same stimulus. These regions included the primary somatosensory cortices, ACC, thalamus, insular cortex, parietal lobe, pallidum, and precuneus cortex but not the amygdala and orbitofrontal cortex. The amygdala likewise is not activated in very young rat pups (< PN10) but is in older infants when tested alone, but not when tested with the mother (Sullivan et al., 2000; Moriceau and Sullivan, 2006; Barr et al., 2009). We tested pups alone in the present work

Table 1. Tools to investigate brain activity

	MEMRI	2DG	FOS/CREB
Mode of label	• Contrast enhanced after uptake into voltage dependent Ca^{2+} channels • Axonal transport by microtubules	• Glucose uptake dependent	• Nuclear Transcription factors
Advantage	• Excellent resolution • Activity dependent • Labels trans-synaptic activation, especially with direct injection • Repeated *in vivo* imaging possible	• Activity dependent • Crosses BBB	• Cellular resolution • No external agents required
Disadvantage	• Possible neurotoxicity at high dose • Limited ability to cross BBB in adult animals	• Limited resolution • Diffusible • Labels astrocytes and neurons • Dependent on hemodynamics • Does not label transsynaptic paths	• Labels only nuclei in soma • Does not always label classic pathways • Low throughput-labor intensive

and thus saw widespread activation of the limbic system including the amygdala. Given that previous studies have shown that the amygdala is quiescent when pups are tested with their mother, whether that suppression occurs more widely throughout the brain is an important unanswered question.

Pain circuitry at P12

Hypothetical models were constructed using the findings from the morphometric analyses and the prior literature and these models showed significant fits to the MEMRI datasets in the formalin-treated cohort. These models included sensory and affective brain regions. Models 1 and 2 (Fig. 5A, B) provide an outline of medial and limbic structures, including affective components such as the anterior cingulate-frontal cortices, and midbrain structures that mediate descending inhibition. The nucleus accumbens, which interacts strongly with the basolateral amygdala in Model 1, is shown further to be a key connection in the global network ($\Delta R > 0.9$ in the correlation network analyses). Model 2 draws connections between the limbic system and the sensory system, with the inclusion of the cerebellum and the medulla. The cerebellum is of particular interest, often neglected in studies of pain circuitry, as discussed recently (Diano et al., 2015), but is important given that cerebellar volume at 8 years of age is reduced in children with a history of neonatal pain (Ranger et al., 2015). In the global brain network, the cerebellar connections present in this network change between the control and formalin-treated cohorts at a level of $\Delta R > 0.6$ (data not shown). Model 3 (Fig. 6C) specifically outlines the sensory pathway at P12. Unlike the co-activation networks, the sensory structures are not prominently represented in the ΔR correlations.

Co-activation of components within the central nervous system is widely regarded as a means to understand the function of the whole brain (Crossley et al., 2013), specific sub-circuits (Rao et al., 2015), and groups of neurons (Muldoon et al., 2012). In addition to investigating hypothesized sub-circuits using a structural modeling approach, studying the brain as a global co-activation network allowed for analysis of relationships previously undefined in the literature. At the $\Delta R > 0.90$ value, the nAcc was a hub connecting to insula, basolateral and central amygdala, cerebellum and hippocampus, generally confirming the results of the SEM analyses. In the co-activation network, the medulla and ventral hypothalamus were highly co-activated, a result not found in the SEM data.

CONCLUSIONS

Infant rats begin to learn aversions around PN10 and we show here that they avoid cues associated with an inflammatory injury. The formalin injection activated circuits thought to mediate the sensory aspects of injury (medulla, lateral thalamus and sensory cortex) and the affective aspects of injury (prefrontal cortex, ACC, basolateral and central amygdala, medial thalamus and hypothalamus), with the nucleus accumbens serving as a hub with high correlations of activity with many of these structures. Thus, because affective circuits as well as sensory circuits were activated by injury at this early age, injury at young ages may have long term effects on adult affective circuits and function as well as sensory circuits. Finally, these data show the utility of MEMRI methods for evaluating circuitry mediating behavior during early development.

AUTHOR CONTRIBUTIONS

Studies were conceived and planned by GAB and JCG. Data was collected by SW and KNB. Data was processed and analyzed by MMS, BMK, SRD, and PRD. GAB and MMS wrote the paper. All authors discussed the results and commented on the manuscript.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest

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