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Antenatal Maternal Stress Alters Functional Brain Responses In Adult Offspring During Conditioned Fear

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Abstract

Antenatal maternal stress has been shown in rodent models and in humans to result in altered behavioral and neuroendocrine responses, yet little is known about its effects on functional brain activation. Pregnant female rats received a daily foot-shock stress or sham-stress two days after testing plug-positive and continuing for the duration of their pregnancy. Adult male offspring (age 14 weeks) with and without prior maternal stress (MS) were exposed to an auditory fear conditioning (CF) paradigm. Cerebral blood flow (CBF) was assessed during recall of the tone cue in the nonsedated, nontethered animal using the ¹⁴C-iodoantipyrine method, in which the tracer was administered intravenously by remote activation of an implantable minipump. Regional CBF distribution was examined by autoradiography and analyzed by statistical parametric mapping in the three-dimensionally reconstructed brains. Presence of fear memory was confirmed by behavioral immobility ('freezing'). Corticosterone plasma levels during the CF paradigm were measured by ELISA in a separate group of rats. Antenatal MS exposure altered functional brain responses to the fear conditioned cue in adult offspring. Rats with prior MS exposure compared to those without demonstrated heightened fear responsivity, exaggerated and prolonged corticosterone release, increased functional cerebral activation of limbic/paralimbic regions (amygdala, ventral hippocampus, insula, ventral striatum, nucleus accumbens), the locus coeruleus, and white matter, and deactivation of medial prefrontal cortical regions. Dysregulation of corticolimbic circuits may represent risk factors in the future development of anxiety disorders and associated alterations in emotional regulation.

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Keywords

prenatal; stress; brain mapping; cerebral blood flow; amygdala; fear

1. INTRODUCTION

Maternal psychological stress during pregnancy has been implicated as a risk factor for the development of affective disorders and schizophrenia in exposed offspring, as well as for decrements in their cognitive and language abilities (Koenig et al., 2002, Kofman, 2002, Weinstock, 2008, Laplante et al., 2008). Antenatal maternal stress (MS) has been shown to elicit changes in brain structure (Kawamura et al., 2006, McClure et al., 2004, Murmu et al., 2006, Salm et al., 2004, Wiggins and Gottesfeld, 1986, Zhu et al., 2004), brain neurochemistry (Adrover et al., 2007, Son et al., 2007, Van den Hove et al., 2006, Barros et al., 2006), stress hormone release (Kapoor et al., 2006) and behavior (Bowman et al., 2004, Lemaire et al., 2000, Louvart et al., 2005, Sternberg and Ridgway, 2003, Takahashi et al., 1992). The effects early life stress has on adult brain functional activation are just beginning to be examined. Prior work examining the effects of MS on functional brain activation during an acute stress challenge have been limited to a few studies documenting alterations in c-fos expression in isolated brain areas (hypothalamus, locus coeruleus) (Del Cerro et al., 2010, Fujita et al., 2010, Humm et al., 1995, Viltart et al., 2006). Using a rodent model, our study is the first to examine functional brain activation using whole brain perfusion mapping during a stress challenge in adult rats with or without a prior history of MS.

2. RESULTS

2.1. Antenatal maternal stress heightens adult psychophysiological responses

Effects of MS were examined in a classic auditory fear conditioning paradigm. During the baseline, prior to receiving tone/footshock pairings, MS and no maternal stress (NMS) rats were actively engaged in exploratory behavior in the training chamber with no significant group difference in anxiety-like responses (percent 'freezing', range 0.0–26.0%, Fig. 1A). During the CF training phase, animals with prior exposure to MS showed greater anxiety-like behavior compared to NMS animals (freezing response during minutes 4–15; 'MS', $F_{1,36} = 13.2$, $P < 0.001$). Surprisingly, the effects of MS were most apparent in animals that had not received footshocks (CON: MS vs. NMS, freezing $63.4 \pm 13.0\%$ vs. $32.2 \pm 12.2\%$, mean $\pm$ SD, Fig. 1B). This increased response may be attributed to the tone, which itself may have been interpreted as an unfamiliar, fearful stimulus. A ceiling effect was noted for rats exposed to the footshock (CF: MS vs. NMS, freezing $66.5 \pm 14.9\%$ vs. $56.1 \pm 16.0\%$, Fig. 1A), with no significant group differences between MS/CF and NMS/CF, except during the initial freezing responses to the footshocks ('Time $\times$ CF $\times$ MS', $F_{14,23} = 2.6$, $P < 0.05$). Similarly, histograms plotting the number of 30-second intervals displaying freezing behavior during training showed a significant separation in the MS and NMS animals that had not received the footshocks (MS/CON vs. NMS/CON, $P < 0.05$), with a nonsignificant trend in the CF animals (MS/CF vs. NMS/CF) (Figs. 1A–B, right panels).

Twenty-four hours later during reexposure to the tone cue (recall), CF-trained animals had significantly elevated freezing behavior compared to controls ('CF', $F_{1,36} = 58.9$, $P < 0.0005$). Comparison of MS/CF to NMS/CF animals showed statistically significant differences in freezing between these groups ('MS', $F_{1,36} = 10.8$, $P < 0.002$, CF: MS vs. NMS, $99.3 \pm 1.4\%$ vs. $88.0 \pm 12.4\%$, CON: MS vs. NMS, $65.9 \pm 12.2\%$ vs. $44.0 \pm 13.0\%$, Figs. 1D–E, left panels). NMS/CF animals gradually increased their freezing response to near maximal levels (~97%) over two minutes, whereas MS/CF animals were 100% motionless starting with the first 30 seconds of tone playback, remaining so throughout the entire recall. Histograms also showed

a significant separation in the MS and NMS populations during fear conditioned recall (MS/CF vs. NMS/CF, $P < 0.05$; Figs 1D–E, right panels).

Blood corticosterone (BC) results paralleled those noted in the behavioral scoring. Prior to CF training, BC levels of NMS and MS rats were both within ranges of previously reported basal values (24–56 ng/mL and 32–65 ng/mL, respectively) (Cordero et al., 1998). After the first tone/foot-shock pairing, BC levels increased to 68 ± 15 ng/mL in NMS/CF rats, while levels in MS/CF animals dramatically increased to 235 ± 64 ng/mL ('CF', $F_{1,28} = 7.1$, $P < 0.01$) (Fig. 1C). Significant differences in BC levels between MS/CF and NMS/CF rats persisted during the duration of tone exposure (MS/CF 230–280 ng/mL, NMS/CF 68–107 ng/mL), with continued elevation even 120 minutes after the last tone-shock pairing for MS/CF rats (142 ± 58 ng/mL) ('MS', $F_{1,28} = 9.9$, $P < 0.004$). As noted in the behavioral measures, exposure to 'tone alone' elicited greater BC levels in MS/CON than in NMS/CON animals (MS/CON 172 ± 54 ng/mL, NMS/CON 57 ± 20 ng/mL), suggesting that the auditory stimulus was perceived as being more stressful by animals with prior MS exposure. Twenty-four hours later during recall, significant effects were noted for MS and CF ('MS', $F_{1,28} = 10.8$, $P < 0.003$, 'CF', $F_{1,28} = 19.6$, $P < 0.0005$). MS/CF rats again demonstrated elevations in BC levels compared to NMS/CF rats (MS/CF 136 ± 19 ng/mL and NMS/CF 79 ± 13 ng/mL) (Fig. 1F).

2.2. Functional brain activation is reorganized by antenatal maternal stress

Group subcortical differences in the distribution of regional cerebral blood flow (rCBF) are shown as color-coded statistical parametric maps superimposed on the brain coronal and transverse slices (Fig. 2). Lists of cortical and subcortical regions of interest for which group differences were significant for the SPM analysis are shown in Table 1, with full results available in a supplementary table online.

2.2.1. Amygdala—Exposure to the conditioned tone (MS: CF vs. CON and NMS: CF vs. CON) resulted in a significant increase in rCBF in the lateral nucleus of the amygdala, a region considered the primary sensory interface for unimodal, sensory processes associated with auditory stimuli in the acquisition and expression of CF. Significant decreases in rCBF were noted in anterior portions of the basolateral and basomedial amygdala, a sensory interface for multimodal, complex, configural, conditioned stimuli (Pare et al., 2004, Yaniv et al., 2004). Regional CBF was decreased in the amygdala's central nucleus (CE) for the NMS/CF versus NMS/CON comparison. Deactivation of the CE may have been the result of the fact that rCBF measurements were obtained during the later stages of recall (after 2 min. continuous tone exposure). In human subjects during CF exposure, it has been suggested that learning-related activation occurs only during early acquisition, whereas deactivation is seen at later stages of retention and extinction (Buchel et al., 1998, LaBar et al., 1998), possibly due to inhibitory modulation arising from the medial prefrontal cortex (Quirk et al., 2003). Maternal stress accentuated functional activation of the amygdala during exposure to the conditioned tone (CF: MS vs. NMS), as well as during exposure to the tone in the absence of prior conditioning (CON: MS vs. NMS). Significant increases in rCBF noted in MS rats compared to NMS rats were broadly expressed in the lateral amygdala and in the medial, basal and central nuclei. Because the time of injection of the perfusion tracer (2 min.) occurred immediately following the point of maximal behavioral differences between MS and NMS rats (0–60 sec), it is likely that changes in amygdalar perfusion and freezing behavior offer different sensitivities and/or temporal profiles in detecting anxiety-like behavior.

2.2.2. Hippocampal Formation—Rats conditioned to the tone cue compared to controls (NMS: CF vs. CON and MS: CF vs. CON) showed significant increases of rCBF in the posterior ventral HPC (CA1), the fimbria, and ventral subiculum, while a decrease was

noted in the dorsal HPC (CA1), dentate gyrus and the dorsal subiculum. MS compared to NMS animals showed significantly greater increases of rCBF in the fimbria and significantly greater decreases of rCBF in the ventral posterior HPC (CF: MS vs. NMS), as well as the dorsal posterior HPC (CA1, dentate gyrus) and dorsal subiculum (CF: MS vs. NMS and CON: MS vs. NMS).

2.2.3. Prefrontal Cortex—The conditioned tone cue elicited greater deactivation in the dorsal structures and greater activation in the ventral structures of the medial prefrontal cortex. A decrease in rCBF was elicited in the dorsal mid-cingulate (Cg1) and posterior cingulate (retrosplenial cortex, RS) in MS and NMS rats in response to the conditioned tone compared to controls (MS: CF vs. CON and NMS: CF vs. CON). This decrease was significantly greater in MS animals and was noted both during exposure to the conditioned tone (CF: MS vs. NMS), as well as during exposure to the tone in the absence of prior conditioning (CON: MS vs. NMS). Prelimbic cortex was activated in response to the fear conditioned tone in NMS rats (NMS: CF vs. CON) but not in MS rats. Infralimbic cortex showed increases in rCBF in response to the fear conditioned tone in both MS and NMS animals (MS: CF vs. CON and NMS: CF vs. CON), as well as in response to unconditioned tone itself in MS compared to NMS control animals (CON: MS vs. NMS).

2.2.4. Other—MS compared to NMS exposure was associated with increased rCBF in the insula, the ventral striatum, the locus coeruleus/subcoeruleus region, nucleus accumbens, as well as broad activation of deep cerebral white matter, and deactivation of the medial dorsal nucleus of the thalamus (CF: MS vs. NMS and CON: MS vs. NMS). Increases in relative regional CBF within the white matter tracks of MS compared to NMS rats were confirmed by user defined regions-of-interest (ROI's). Group mean Z-scores were greater (less negative) in MS than in NMS rats for all ROI's, with statistical significance ($P < 0.05$) achieved in 15 of the 20 regions. There were no statistical differences between the MS and NMS groups in nontransformed CBF calculated globally across the brain.

3. DISCUSSION

The current study demonstrated for the first time significant differences in functional brain activation of adult offspring from dams exposed to psychological stress during pregnancy. Offspring demonstrated amygdala hyperresponsivity to fear conditioned stimuli and decreased responsivity of medial dorsal prefrontal cortex -- a region previously implicated in top-down control of the amygdala (Bissiere et al., 2008, Petrovic et al., 2004). Consistent with prior work showing that MS rats demonstrate increased emotionality, defensive behavior and anxiety (Chapillon et al., 2002), our study showed that MS compared to NMS animals showed greater fear behavior during CF training and CF recall. The effects of MS were apparent even in animals that had not received footshocks, suggesting that MS animals were sufficiently 'sensitized' to allow the tone by itself to elicit a partial fear response. While there were no significant group differences in basal BC levels, rats with a prior history of MS increased their BC during CF training to a level that was 2–3-fold higher than that of rats without a prior history of MS. These elevations in BC were again observed during fear conditioned recall 24 hours later. Overall our corticosterone results corroborate prior reports that MS results in a dysregulated HPA axis (Weinstock, 2008).

Fear conditioned animals compared to controls demonstrated increased rCBF in the lateral amygdala. Exposure to MS accentuated changes in rCBF in the amygdala, where increases appeared broadly in the lateral, central, medial, and basolateral/basomedial nuclei. These findings are consistent with prior neuroanatomic work reporting enlargement of the amygdala of adult rats previously exposed in-utero to MS (Salm et al., 2004). Maternal stress compared to NMS was also associated with increased rCBF in the insula, entorhinal/

perirhinal regions, ventromedial striatum, as well as infralimbic cortex. Such changes in limbic and paralimbic regions were noted not only in CF-exposed animals but also in controls, suggesting that the auditory stimulus, even in the absence of fear conditioning, was perceived as more emotionally stressful by animals with prior MS exposure. In addition, MS exposed rats compared to NMS rats, showed significant decreases in rCBF in the anterior and mid-cingulate (aCg1, mCg1) and retrosplenial cortex (posterior cingulate). Prior work has shown that the anterior cingulate, in particular, demonstrates changes in spine density and dendritic complexity in offspring of mothers exposed to stress during pregnancy (Murmu et al., 2006). Furthermore, the anterior cingulate is thought to exert an inhibitory effect on the amygdala (Petrovic et al., 2004, Bissiere et al., 2008), while the retrosplenial region in the rat, apart from a role in sensorimotor integration, is felt to play a role also in processing of emotional memories, though its role in auditory fear conditioning remains debated (Keene and Bucci, 2008, Lukoyanov and Lukoyanova, 2006).

Our results are consistent with the idea proposed by Phillips et al. (Phillips et al., 2003) of the existence of a ventral and a dorsal stream of emotional cognition. The ventral stream, consisting of the amygdala, insula, ventral striatum, and ventral regions of the prefrontal cortex, is posited to appraise emotional behavior and produce an affective state. The dorsal stream, consisting of dorsal regions of the anterior cingulate gyrus and prefrontal cortex, as well as the hippocampus, exerts a modulatory influence on the ventral stream. Our study shows clear effects of MS in these two neural systems, with a predominant ventral activation and dorsal deactivation in MS exposed animals during CF recall. Hyperresponsivity of the amygdala in association with decreased activation and decreased inhibitory input of the rostral, anterior cingulate and hippocampus, has been reported in a number of psychiatric disorders, including posttraumatic stress disorder and depression (Rauch et al., 2006, Stein et al., 2007, Bluhm et al., 2009). Bluhm et al. have suggested a link between a history of childhood abuse and alterations in the default network, specifically in connectivity of the posterior cingulate/precuneus to the amygdala and the hippocampus/parahippocampal gyrus (Bluhm et al., 2009). In these studies, it is proposed that in the absence of a normal inhibitory input, activity in the amygdala remains unchecked, leading to heightened emotionality and continued maintenance of learned aversive responses. Consistent with this earlier work on the effects of postnatal stress, our results demonstrate the importance of antenatal stress in determining the functional response of a prefrontal network in the modulation of limbic structures during the processing of fearful stimuli.

Maternal stress compared to NMS exposure also was associated with increased rCBF in the locus coeruleus/subcoeruleus (LC/SubC). The LC/SubC is the major source of noradrenergic innervation to almost every region in the brain including limbic and brain stem autonomic structures (Berridge and Waterhouse, 2003). These neurons have been implicated in a variety of functions including regulation of attentional states and autonomic processes. Relevant to the current study is past work that suggests a role for the LC/SubC in defensive-like, immobile posturing, as well as the startle response (Adams and Geyer, 1981, Tsuruoka et al., 2010). We speculate that increased functional activation in the LC/SubC associated with prior MS may reflect an increased attentional state, as well as greater autonomic activation in the offspring as has been previously reported (Igosheva et al., 2004).

Fear conditioning increased rCBF response in the ventral HPC, while decreases were noted in the dorsal HPC. These findings are consistent with the known dorsoventral disparity in the functional organization of the hippocampus in which the ventral sector processes dominantly information related to the affective and homeostatic state of the animal, and the dorsal HPC performs primarily cognitive functions (Segal et al., 2010). Prior exposure to MS decreased CBF response dominantly in the dorsal, posterior HPC (CA1, dentate gyrus). Though implications of this remain to be determined, these findings are consistent with

reports suggesting effects of MS on the HPC, including inhibition of neurogenesis in the dentate gyrus, learning deficits (Lemaire et al., 2000, Odagiri et al., 2008), as well as altered c-fos expression (Viltart et al., 2006). A final notable observation was the broad increases in rCBF noted in the deep white matter of animals exposed to MS, which may provide a functional correlate to structural white matter changes observed in response to antenatal stress (Wiggins and Gottesfeld, 1986) or early postnatal stress (Jackowski et al., 2008, Paul et al., 2008, Sanchez et al., 1998).

Limitations

Debate continues whether problems associated with early life stress arise during early maturation of neural circuits, and/or if such stress exposure establishes vulnerability for progressive circuit dysfunction in the face of future stress challenges. While excess maternal stress hormones may alter fetal development, as well as placental perfusion (Antonow-Schlorke et al., 2003, Avishai-Eliner et al., 2002), maternal care is also significantly diminished after MS, which can be deleterious to pups (Smith et al., 2004). Decreased postnatal care has been reported to result in altered behavior of adult offspring (Seckl, 2008), as well as permanent decreases in GC receptor density in the hippocampus and prefrontal cortex, with resultant attenuated feedback sensitivity and increases in the stress-hormone response (Liu et al., 1997). The possible neurotoxic effects of such excessive responses (Conrad, 2008) may suppress cell proliferation in the developing brain (Kawamura et al., 2006) and may modulate hippocampal volume in later life (Buss et al., 2007). Our study did not allow us to distinguish between effects on brain function derived from the in-utero effects of elevated maternal corticosterone and the effects of altered postnatal maternal care. Future work may wish to address this with cross-fostering studies.

Cerebral blood flow as a proxy measure for neuronal activity operates under several assumptions (Keri and Gulyas, 2003). Unresolved issues include the role of excitatory compared to inhibitory neurotransmitters in altering brain perfusion and metabolism, and the fact that hemodynamic changes may be driven by subthreshold synaptic activity. In addition, there are the limits of proxy measures such as CBF in detecting changes in spatial and temporal neural processing, in which the overall energy demands may remain unchanged. Finally, it is important to note that while there is significant homology between rats and humans at the level of limbic circuits (Uylings et al., 2003, Vertes, 2006), there are likely major differences at the cortical level, particularly in prefrontal areas.

Conclusion

This study demonstrated clear differences in functional brain activation in animals previously exposed to antenatal MS. Maternal stress elicited a dysregulation of corticolimbic circuits and a heightened fear response that was apparent in the adult offspring. Stress hormone levels that were normal at baseline were increased in MS compared to NMS rats in response to a threat-related stimulus. These findings point to the potential importance of MS in determining how traumatic events are processed in adult life, the longevity of associated symptoms, and the risk of developing brain-based alterations in function.

4. EXPERIMENTAL PROCEDURE

Generating male offspring exposed to antenatal maternal stress

Experiments were performed under approval of the Institutional Animal Care and Use Committee (USC). Fourteen pregnant Wistar dams were obtained from the vendor (Harlan Sprague-Dawley, Indianapolis, IN) one day after testing plug-positive; this was established as embryonic day 1. Dams were individually housed under standard vivaria conditions (7 a.m.–7 p.m. lights on, 12-hour light cycle, standard rodent laboratory chow). Daily,

inescapable, scrambled foot-shocks (80×1.0 mA, 1s duration, 30–120s variable intershock interval) were delivered to the dams two days after testing plug-positive and continuing for the duration of their pregnancy. Control dams were similarly exposed in the absence of footshock (Takahashi et al., 1992). Footshocks were delivered in the morning (8 a.m. – 12 noon) over a period of 95–105 minutes in a 30 × 30 × 30 cm stainless steel Plexiglas cage through a floor of stainless steel rods of 2-mm diameter and 8-mm separation. The chamber was illuminated with the indirect ambient fluorescent light from a ceiling panel and was subjected to background ambient sound level of 57 dB.

After 20 days of gestation, pups (male, female) were born and left undisturbed with their mothers. There was no significant difference in litter size or sex of the pups based on MS exposure. Upon weaning on postnatal day 21, male offspring exposed either to MS or no maternal stress (NMS) were separately placed in social groups of 4, and housed until 14 weeks of age under standard vivaria conditions. At 14 weeks, animals were aseptically implanted with external jugular vein catheters (3.5 Fr. Silastic) with the tip advanced into the superior vena cava. A subgroup of these (MS: n=14, NMS: n=18) was retained for serial analysis of blood corticosterone (BC) levels during fear conditioning. Remaining rats (MS: n=25, NMS: n=17) were implanted with a subcutaneous minipump for functional brain mapping. This self-contained minipump developed by our group, allows for bolus administration of radiotracers by remote activation for functional neuroimaging applications in freely moving, nontethered animals (Holschneider et al., 2002, Givrad et al., 2010).

Conditioned Fear Response

One week after surgery, rats were exposed to a classic auditory conditioned fear (CF) paradigm (10 a.m.–1 p.m.) (Fanselow, 1980, Holschneider et al., 2006). Rats were habituated to the experimental room for 40 minutes, and then placed in a Plexiglas/stainless steel box (30×30×30 cm³, indirect ambient fluorescent light, 57dB background sound level) with a floor of stainless steel rods. Training consisted of a 3-minute baseline, followed by exposure to eight pairings of a tone (30s, 72dB, 1000Hz/8000Hz continuous alternating sequences of 250msec pulses), each ending with a final footshock (1mA, 1s) followed by a 1-minute silent interval. Controls (CON) were exposed only to the tone. Recall was tested 24 hours later in a novel context (a cylindrical, dimly lit, Plexiglas cage with a flat floor). Following a quiescent two-minute baseline, rats were reexposed continuously over two minutes to the auditory cue. Behaviors were recorded on tape by a ceiling-mounted camera.

Functional neuroimaging during fear conditioned recall

Twenty-four hours after the training session, animals were immobilized for 5 minutes in a rodent restrainer (Decapicone, Braintree Scientific, Braintree, MA) while the radiotracer ([¹⁴C]-iodoantipyrine, 100μCi/kg in 300μl of 0.9% saline, ARC, St. Louis, MO) was loaded into the pump through a percutaneous port. At this time, a euthanasia agent was also loaded (1ml of 50mg/ml pentobarbital, 3 M KCl). Animals were returned to a transport cage, where they rested undisturbed for 40 minutes. Thereafter, recall was tested as noted above in the conditioned rats (MS/CF, NMS/CF) and control animals (MS/CON, NMS/CON). After two minutes of continuous tone exposure, an infrared pulse from a ceiling-mounted LED transcutaneously activated an optical sensor in the implanted minipump. This triggered intravenous release of the radiolabelled perfusion tracer into the animal's circulation (Sakurada et al., 1978), followed immediately by injection of the euthanasia solution, while the sounding of the tone continued. This resulted in cardiac arrest within ~5–8 seconds, a precipitous fall of arterial blood pressure, termination of brain perfusion, and death (Holschneider et al., 2002). Brains were rapidly removed over 3–4 minutes, flash-frozen in dry ice/methylbutane, and cryosectioned (fifty-seven 20μm slices starting 3.9mm anterior to bregma, 300μm interslice distance).

Sections were heat-dried on glass slides and exposed for 2 weeks to Ektascan Diagnostic Film (Eastman Kodak, Rochester, NY, USA) in spring-loaded x-ray cassettes along with 16 radioactive ^{14}C standards (Amersham Biosciences, Piscataway, NJ). Autoradiographs were placed on a voltage stabilized light box with diffuser plate (Northern Lights Illuminator, InterFocus Ltd), imaged with a Retiga 4000R charge-coupled device monochrome camera (Qimaging, Surrey, BC, Canada), digitized on an 8-bit gray scale using Qcapture Pro 5.1 (Qimaging) on a microcomputer. Cerebral blood flow related tissue radioactivity (CBF-TR) was measured by the classic [^{14}C]-iodoantipyrine method (Goldman and Sapirstein, 1973, Patlak et al., 1984, Sakurada et al., 1978). In this method, there is a strict linear proportionality between tissue radioactivity and CBF when the data is captured within a brief interval (~10 sec.) after the tracer injection (Jones et al., 1991, Van Uiter and Levy, 1978). Perfusion mapping using autoradiographic methods (Nguyen et al., 2004), fills a gap in the current armamentarium of imaging tools in that it can deliver a three dimensional assessment of functional activation of the awake, nonrestrained animal, with a temporal resolution of ~5–10 sec. and spatial resolution of 100 microns.

Stress Hormone Assay

In separate groups of rats undergoing CF training (MS/CF, NMS/CON), as well as in controls (MS/CON, NMS/CON), 300 μL of venous blood was removed at times 0, 4 and 12 minutes via a jugular vein catheter connected to an overhead tether (10 a.m. – 1 p.m.). Samples were also obtained 120 minutes thereafter in the homecage. Twenty-four hours later during recall, blood was again sampled at times 0 and 4 minutes. Corticosterone levels were determined in plasma by an Enzyme-Linked ImmunoSorbent Assay kit (ELISA) using a rabbit polyclonal antibody-coated microplate, corticosterone enzyme conjugate (HRP) and K-Blue substrate according to the manufacturer's protocol (Neogen Corp, Lexington, KY #402810).

Data and Statistical Analyses

Behavioral analysis—The animal's 'freezing' response served as the behavioral measure of fear conditioned memory. Freezing was defined as the absence of all visible movements of the body and vibrissae, aside from respiratory movement. Behaviors were coded continuously in a blinded fashion using the Observer 5.0, a software package for behavioral analysis (Noldus Information Technology, Leesburg, VA). Group averages of the percentage of time spent freezing was counted for each 30-second interval across the training period (baseline: minutes 0–3, training: minutes 3–15) and recall period (baseline: minutes 0–2, recall minutes 2–4). Group differences were evaluated with a repeated measures analysis of variance (ANOVA, $P < 0.05$), using "MS" and "CF" as between-subject factors and "time" as a within-subjects factor. Post-hoc Student's t-tests (two-tailed, $P < 0.05$) were used to examine differences for each 30-second interval. Finally, total counts for freezing within each decile (0–10%, 10–20%, ... 90–100%) were separately displayed in a histogram for each group. Significant differences were examined with an ANOVA with multiple pairwise comparisons with Bonferonni adjusted p-values ($P < 0.05$).

Functional Brain Mapping

3D brain reconstruction & spatial normalization: Fifty-seven serial coronal sections were digitized as noted above. Adjacent sections were aligned both manually and using TurboReg, (<http://bigwww.epfl.ch/thevenaz/turboreg/>) using a non-warping geometric model that includes rotations and translations (rigid-body transformation), and uses nearest-neighbor interpolation (Thevenaz et al., 1998). The aligned sections were imported as an image stack using ImageJ (NIH, Bethesda, MD – <http://rsb.info.nih.gov/ij/>) with final voxel dimensions of $0.072 \times 0.072 \times 0.3 \text{ mm}^3$.

SPM Analysis: Statistical Parametric Mapping (SPM) (<http://www.fil.ion.ucl.ac.uk/spm/>, SPM5) (Friston, 1995, Friston et al., 1990) was developed for analysis of imaging data in humans and has been adapted by us for use in rat brain autoradiographs (Nguyen et al., 2004) and confirmed by others (Dubois et al., 2008, Lee et al., 2005). After 3D reconstruction, one ‘artifact free’ brain was selected as reference and smoothed with a Gaussian kernel (FWHM=3 × voxel dimension). Brains from both groups were spatially normalized to the smoothed reference brain. Following spatial normalization, normalized images were averaged to create a mean image, which was then smoothed to create the final brain template. Each original three dimensional reconstructed brain was then spatially normalized into the standard space defined by the template. Background and ventricular spaces were masked using a 70% threshold as confirmed by visual inspection of the mask. Global differences in the absolute amount of radiotracer delivered to each animal were adjusted by scaling the voxel intensities so that the mean intensity for each brain was the same. Using SPM, we implemented a Student’s t test (unpaired) at each voxel, testing the null hypothesis that there was no effect of group, that is, motor-induced cerebral-activation was tested by comparing the treadmill group with the quiescent group. Maps of positive and negative t were separately analyzed. We chose to set a significance threshold $P < 0.05$ (uncorrected for multiple comparisons) for individual voxels within clusters of contiguous voxels, and a minimum cluster size of 100 contiguous voxels (extent threshold). We then evaluated the significance corrected for multiple comparisons ($P < 0.005$) of individual voxels, clusters of contiguous voxels exceeding the threshold, and number of clusters detected in the entire SPM. Brain regions were identified using an anatomic atlas of the rat brain (Paxinos and Watson, 2007).

Findings in the white matter were reconfirmed by region of interest (ROI) analysis for 20 circular ROI’s placed manually in each animal onto the external capsule and corpus callosum of each hemisphere in five digitized coronal brain sections of each animal (bregma +2.4mm, +2.1mm, 1.8mm, 0.0mm, −0.3mm). Z scores were calculated for each ROI as: $z \text{ score}_i = (TR_i - \text{mean})/SD$, where $z \text{ score}_i$ was the standard normal deviate of tissue radioactivity at location i, and TR_i was the tissue radioactivity of location i (Hays, 1973). The mean and standard deviation (SD) were defined in a given animal as the average and SD of 878 ROI’s sampled across the brain of each rat using a standardized grid (Holschneider et al., 2008). Significant group differences between MS and NMS animals were examined for each region across all slices by student T-test ($P < 0.05$). Group differences in the nontransformed tissue radioactivity were also examined globally across the brain.

Stress Hormone Response—Corticosterone levels were analyzed for the training and recall periods with a repeated measures analysis of variance (ANOVA, $P < 0.05$), using ‘S’ and ‘CF’ as between-subject factors and ‘time’ as a within-subjects factor. Post-hoc Student’s t-tests (two-tailed, $P < 0.05$) were used to examine differences for each time interval.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRAINING **Figure 1.**

Psychophysiological responses of adult male rats, with previous exposure to maternal stress (MS) or no maternal stress (NMS) during a conditioned fear (CF) paradigm. **TRAINING** (Top): Behavioral responses of (A) NMS/CF (n=9) and MS/CF (n=14) rats during CF training. (B) NMS/CON (n=8) and MS/CON (n=11) controls exposed to tone without footshock. The left panel depicts percent behavioral immobility (Freeze) over time, with each point representing the group average ($\pm$ SEM) over 30 sec. The right panel depicts a histogram plotting the number of 30 sec. intervals displaying a specified percent of freezing behavior. Also shown are blood corticosterone levels during (C) CF training for NMS/CF (n=8), NMS/CON (n=10), MS/CF (n=7), and MS/CON (n=7) rats. **RECALL** (Bottom): (D)

Behavioral results and (E) corticosterone results during recall, i.e. reexposure to the tone alone 24 hours after CF training. The horizontal arrow indicates the time and duration of the tone exposure. The vertical dashed bar represents the time of radiotracer infusion. * Comparison of the specified group average with each of the comparison groups, $P < 0.05$.

Figure 2.

Changes in regional cerebral blood flow related tissue radioactivity in rats during recall to a conditioned tone 24-hours after CF training. Evaluation is made of the effects of MS and CF on functional brain activation. Depicted is a selection of representative coronal slices (anterior-posterior coordinates relative to the bregma), a transverse and a sagittal brain section. Color-coded overlays show statistically significant positive (red) and negative (blue) differences ($P < 0.05$ at the voxel level, cluster > 100 contiguous voxels). NMS/CF = No Maternal Stress / Conditioned Fear (tone + footshock), $n=9$; NMS/CON = No Maternal Stress / Control (tone only), $n=8$; MS/CF = Maternal Stress / Conditioned Fear, $n=14$; and MS/CON = Maternal Stress / Control, $n=11$. Color bar represents statistical differences (P -

values) as determined by the statistical parametric mapping (SPM). The bilateral nature of many of the significant changes adds to the significance of the findings, something not accounted for by the SPM analysis. Line drawings have been adapted from those from the Paxinos and Watson (2007) rat brain atlas (Paxinos and Watson, 2007). CORTEX: Au, auditory; aCG1, anterior dorsal cingulate, Cg2, ventral cingulate; I, insula; IL, infralimbic; M1, primary motor; Pir, piriform; PrL, prelimbic; PtA, parietal association area; RS, retrosplenial; S1BF, primary somatosensory barrel field; S1FL, primary somatosensory forelimb area; V1, V2, primary, secondary visual. SUBCORTEX: aca, anterior commissure; ACo, anterior cortical amygdala; AV, anterior ventral thalamic n.; BL, BM, basolateral, basomedial amygdala; Ce, central n. of the amygdala; cp, cerebral peduncle; CPu, caudate putamen; dcw, deep cerebral white matter; DG, dentate gyrus; ec, external capsule; IC, inferior colliculus; La, lateral amygdala; LC, locus coeruleus n.; LSI, lateral septal intermediate n.; LV, lateral ventricle; MD, medial dorsal thalamic n.; MS, medial septal n.; PH, posterior hypothalamus; Pn, Pons; Re, reuiens thalamic n.; RLi, rostral linear n. of the raphe; Sim, simple lobule; SubC, subcoeruleus; vmCPu, ventral medial caudate putamen; vHPC, ventral hippocampus; VA, ventral anterior thalamic n.; VPM, ventral posteromedial thalamic n.; VS, ventral subiculum; 5, trigeminal n.

Table 1

Summary of statistically significant differences in functional brain activation during recall to conditioned tone 24-hours after CF training in prefrontal, limbic and paralimbic regions.

	MS	NMS	CF	CON
	CF vs. CON	CF vs. CON	MS vs. NMS	MS vs. NMS
CORTEX				
Cingulate: anterior dorsal (aCg1, 'perigenual')	↓	—	↓* _c	↓*
mid-cingulate, dorsal (mCg1)	↓* _c	↓	↓* _c	↓*
ventral (Cg2)	—	↑	—	—
Entorhinal, perirhinal (Ent, PR)	—	↑*	↑(R)	↑
Infralimbic (IL)	↑	↑	—	↑* _c
Insula (I)	↑* _c	—	↑* _c	↑*
Prelimbic (PrL)	—	↑* _c	—	—
Retrosplenial (RS)	↓* _c	↓	↓*	↓*
SUBCORTEX				
Amygdala: basolateral, basomedial, medial n. (BL, BM, Me)	↓	↓	↑* _c	↑(L) ^c
central n. (Ce)	—	↓	↑* _c	↑
lateral n. (La)	↑	↑*	↑	↑*
anterior area, cortical anterior, cortical posterior (AA, ACo, PLCo)	↓* _c	↓	—	—
Colliculi: inferior (IC)	↓	—	↓*	—
Hippocampal Formation: fimbria of hippocampus (fi)	↑	↑	↑* _c	↑* _c
hippocampus anterior dorsal (CA1-3, dentate gyrus, DG)	—	↓*	—	↓
hippocampus dorsal, posterior (CA1; dentate gyrus, DG)	↓* _c	↓*	↓*	↓* _c
hippocampus ventral, posterior (CA1; dentate gyrus, DG)	↑*	↑*	↓*	—
subiculum, dorsal (DS)	↓ _c	↓* _c	↓*	↓*
subiculum, ventral (VS)	↑*	↑* _c	↓	—
Locus coeruleus, Subcoeruleus n. (LC, SubC)	↑*	—	↑* _c	↑* _c
Nucleus accumbens (Acb)	—	—	↑* _c	↑* _c
Striatum: central, lateral (CPu)	↓	↓*	↓	↓* _c
ventromedial (vmCPu)	↑	↑* _c	↑	↑*
Thalamus: lateral dorsal dorsomedial, lateral posterior mediodorsal (LDDM, LPMR)	—	↓*	↓	↓* _c
medial dorsal, anterior ventral, ventral anterior (MD, AV, VA)	↓* _c	—	↓*	↓*
medial geniculate (MG)	↓(R)	—	↓*	↓* _c
midline nuclei: centromedial, intermediodorsal, paraventricular, reunions, rhomboid (CM, IMD, PV, Re, Rh)	—	↑	↓* _{Re}	—

	MS	NMS	CF	CON
	CF vs. CON	CF vs. CON	MS vs. NMS	MS vs. NMS
posterior (Po)	—	↓*	↓*	↓* ^c
ventral posteromedial, ventral posterolateral n. (VPM, VPL)	—	↓*	—	↓*
White matter (anterior commissures, corpus callosum, internal and external capsule, cerebral peduncle, deep cerebral white matter, forceps major and minor; ac, cc, ic, ec, cp, dcw, fmj, fmi)	↑* ^c	—	↑* ^c	↑* ^c

Significant increases (↑) or decreases (↓) are bilateral across the cerebral hemispheres, except as indicated (L=left only, R=right only). Significance is shown at the voxel level for clusters of greater than 100 contiguous voxels (* $P < 0.005$, and $P < 0.05$), as well as significance at the cluster level after correction for multiple comparisons (^c $P < 0.005$). Landmarks indicated are per the Paxinos and Watson Rat Brain Atlas (Paxinos and Watson, 2007). A full table of significant differences can be found in the supplementary table online.