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Effects of maternal separation, early handling, and gonadal sex on regional metabolic capacity of the preweanling rat brain

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Abstract

This is the first study to assess the effects of mother-infant separation on regional metabolic capacity in the preweanling rat brain. Mother-infant separation is generally known to be stressful for rat pups. Holtzman adolescent rats show a depressive-like behavioral phenotype after maternal separation during the preweanling period. However, information is lacking on the effects of maternal separation on the brains of rat pups. We addressed this issue by mapping the brains of preweanling Holtzman rat pups using cytochrome oxidase histochemistry, which reflects long-term changes in brain metabolic capacity, following two weeks of repeated, prolonged maternal separation, and compared this to both early handled and non-handled pups. Quantitative image analysis revealed that maternal separation reduced cytochrome oxidase activity in the medial prefrontal cortex and nucleus accumbens shell. Maternal separation reduced prefrontal cytochrome oxidase to a greater degree in female pups than in males. Early handling reduced cytochrome oxidase activity in the posterior parietal cortex, ventral tegmental area, and subiculum, but increased cytochrome oxidase activity in the lateral frontal cortex. The sex-dependent effects of early handling on cytochrome oxidase activity were limited to the medial prefrontal cortex. Regardless of separation group, females had greater cytochrome oxidase activity in the habenula and ventral tegmental area compared to males. These findings suggest that early life mother-infant separation results in dysfunction of prefrontal and mesolimbic regions in the preweanling rat brain that may contribute to behavioral changes later in life.

Keywords

cytochrome oxidase; preweanling rat brain; sex differences; maternal separation; prefrontal cortex

1. Introduction

Repeated mother-infant separation (also called maternal separation) is a known stressor in rats (Plotsky & Meaney, 1993), in which the mother is removed from the pups for prolonged periods (usually a 3- to 6-hour time period). The detrimental effects of mother-infant separation on the brain and behavior extend into adolescence and adulthood. Behavioral examples of the long-term effects of maternal separation include anxiety-like behavior (Daniels et al., 2004), depressive/hypoactive behavior (Spivey et al., 2008b), and behavioral

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hyperactivity and impulsivity (Daniels et al., 2009; Colorado et al., 2006; Kaneko et al., 1994; Jimenez-Vasquez et al., 2001). In particular, Spivey et al. (2008b) found that adolescent rats belonging to the Holtzman strain appear to be particularly susceptible to this depressive behavioral profile following maternal separation, as measured by reduced levels of general activity and risk-taking in the open field.

Long-term effects of maternal separation on the brain include, but are not limited to, changes to the production and binding of stress hormone receptors (Ladd et al., 2005), and alterations of dendritic morphology (Monroy et al., 2010) and neurotransmitter levels (Matthews et al., 2001) in the striatum, prefrontal cortex, and hippocampus. However, fewer studies have addressed the more short-term effects of maternal separation on the brain or behavior. Some have assessed the effects on hyperactive behavior (Daniels et al., 2009; Colorado et al., 2006) and response to cocaine (Marin & Planeta, 2004) in adolescence. More recent studies have shown decreases in glucocorticoid receptor mRNA and corticotrophin releasing factor-1 mRNA in the hippocampus of 14-day old rats separated for 3 hours (Litvin et al., 2010). Immediately following single 24-hour deprivations, increases in apoptosis in the hippocampus and various other brain regions are apparent (Zhang et al., 2002). Maternal separation stress also causes reductions in growth hormone and exaggerated hypothalamic-pituitary-adrenal (HPA) axis response in preweanling rat pups (Kuhn & Schanberg, 1998). However, it appears that there is a lack of investigation into the immediate effects of repeated prolonged maternal separation in the preweanling rat brain.

Therefore, this study aimed to assess the brain effects of 2 weeks of repeated maternal separation in the infant rat brain. Quantitative cytochrome oxidase (CO) histochemistry can be used as a marker for brain regional metabolic capacity (Gonzalez-Lima & Cada, 1998). CO builds up in the mitochondria of neurons as a reflection of increased demand for energy utilization (Wong-Riley et al., 1998). Specifically, CO is the rate-limiting enzyme in the mitochondrial electron transport chain in which ATP is formed from the breakdown of glucose, and therefore CO activity is tightly coupled to functional activity of a brain region (Wong-Riley et al., 1998). Energy demands over long periods of time are reflected in CO activity (Shumake & Gonzalez-Lima, 2003), and CO histochemistry is therefore an appropriate marker for studying the cumulative effects of behavioral and sex effects (Sakata et al., 2005).

We have previously demonstrated that adolescent Sprague-Dawley males and females show sex differences in brain CO activity (Spivey et al., 2008a), particularly in the prefrontal and posterior parietal cortical regions. Some neurochemical changes in the brain resulting from maternal separation have been reported to be sex-dependent. The prefrontal cortex (Zimmerberg & Brown, 1998; Matthews et al., 2001; Jimenez-Vasquez et al., 2001), hippocampus (Matthews et al., 2001), paraventricular nucleus of the hypothalamus (Genest et al., 2004), and striatum (Sircar et al., 2001) are among the regions reported to have maternal separation-related sex differences. However, it should be noted that Holtzman rats have shown diminished sex differences in antinociceptive responses (Terner et al., 2003) and in the effects of maternal separation on open-field behavior (Spivey et al., 2008b).

Based on the reviewed studies of brain changes produced by maternal separation (MS) and early handling (EH) from the existing literature, in combination with our previous characterization of depressive behavior in adolescent Holtzman rats (Spivey et al., 2008b), we hypothesized that MS and EH would alter regional metabolic capacity in a variety of brain regions, including the prefrontal cortex, posterior parietal cortex, nucleus accumbens shell and mesolimbic pathway. Additionally, we hypothesized that the effects of MS and EH on regional metabolic capacity could differ between males and females during the preweanling period.

2. Results

Table 1 shows the mean CO values for each group, by region. Sixty pups were divided into three separation groups at postnatal day 2 (P2): MS, maternal separation; EH, early handling; and SFR, standard facility rearing. Each of these three separation groups had males and females, making a total of six groups. For 10 days, the MS subjects were separated for 6 h daily, and the EH subjects were separated for 15 min daily. The SFR subjects were not separated. Their brains were collected at P13 and processed for CO quantitative histochemistry. The number of pups was 20 per separation condition but due to tissue damage in some subjects, the resulting n for each brain region in a group is provided in parentheses in Table 1. There were sixteen brain regions analyzed per subject. Each individual brain region was analyzed using a $2(\text{sex}) \times 3$ (separation group) univariate analysis of variance (ANOVA). This was followed by individual group contrasts. With these three separation conditions, there could be significant differences between MS and SFR groups, between EH and SFR groups, and between MS and EH groups. For the sake of clarity, below we will report sequentially 1) an overview of the results, 2) the main effects of separation, 3) the interaction between separation and sex, 4) the main effects of sex, and 5) the litter effects.

2.1. Overview

An overview of the results in Table 1 showed that EH resulted in the most widespread effects on CO activity, with increases in the lateral and medial prefrontal cortex, and decreases in the posterior parietal cortex, subiculum, and ventral tegmental area. In contrast, effects of MS were limited to decreases in CO activity in the nucleus accumbens and medial prefrontal cortex. Interactions between gonadal sex and separation group were consistent in the prefrontal cortical regions of MS animals, and to a lesser extent in EH animals. Main effects of sex were limited to the habenula and ventral tegmental area.

2.2. Main effects of separation

2.2.1. MS vs. SFR—MS resulted in lower CO activity compared to SFR in the deep layer of the medial prefrontal cortex (MS < SFR; $F(2,42) = 15.879$; $p < 0.001$).

2.2.2. EH vs. SFR—EH increased CO activity relative to SFR in the superficial layer of the lateral frontal cortex (EH > SFR; $F(2,43) = 10.513$; $p < 0.001$). EH decreased CO activity relative to SFR in the deep layers of the posterior parietal cortex (EH < SFR; $F(2,42) = 3.981$; $p = 0.026$), in the subiculum (EH < SFR; $F(2,47) = 5.370$; $p = 0.008$) and in the ventral tegmental area (EH < SFR; $F(2,47) = 7.542$; $p = 0.001$).

2.2.3. MS vs. EH—EH showed greater CO activity than MS in the lateral frontal cortex, in both the superficial (EH > MS; $F(2,43) = 10.513$; $p < 0.001$) and deep (EH > MS; $F(2,43) = 9.335$; $p = 0.001$) layers. EH also had greater CO activity than MS in the medial prefrontal cortex, in both the superficial (EH > MS; $F(2,42) = 7.246$; $p = 0.002$) and deep layers (EH > MS; $F(2,42) = 15.879$; $p < 0.001$). EH showed greater CO activity than MS in the shell of the nucleus accumbens (EH > MS; $F(2,46) = 4.642$; $p = 0.015$) and the caudate putamen (EH > MS; $F(2,36) = 3.380$; $p = 0.045$). Metabolic activity was reduced in EH animals relative to MS animals in both the subiculum (EH < MS; $F(2,47) = 5.371$; $p = 0.008$) and ventral tegmental area (EH < MS; $F(2,47) = 7.542$; $p = 0.001$).

2.3. Interaction between separation effects and sex

MS subjects showed the most sex-dependent separation effects in the frontal cortex. MS decreased metabolic activity in female pups more than male pups in the medial prefrontal and lateral frontal cortex, as reflected by significant sex $\times$ separation interactions for these

regions ($F(2,42) > 7.1$, $ps < 0.01$). Subsequent simple effects tests revealed that in all frontal cortex regions measured, MS females had lower CO activity than MS males (MS-F < MS-M; $ps < 0.003$). Conversely, in the deep layer of the medial prefrontal cortex, EH females had greater CO activity than EH males (EH-F > EH-M; $p < 0.05$). In addition, simple-effects tests showed that MS females had significantly lower CO values in all frontal cortical regions as compared to females from SFR and EH groups (MS-F < SFR-F; MS-F < EH-F; $ps < 0.01$), while there were no significant group differences in CO activity among males.

2.4. Main effects of sex

Two regions showed sex differences independent of separation condition, with females showing greater metabolic activity than males in the habenula ($F > M$; $F(1,42) = 4.636$; $p < 0.05$), and ventral tegmental area ($F > M$; $F(1,47) = 4.193$; $p < 0.05$).

2.5. Litter effects

Since there were only few litters used, we evaluated litter effects on CO activity using three-level hierarchical ANOVAs (this is a type of ANOVA in which comparisons are performed sequentially between separation groups, between litters/within groups, and between sexes/within litters, to identify separate influences contributing to the effects). Most regions did not show significant between-litter differences within separation groups (see Table 1). However, significant litter effects were noted for the lateral frontal cortex deep layer ($F(3,42) = 3.12$; $p < 0.05$), habenula ($F(3,41) = 5.00$; $p < 0.01$), ventral tegmental area ($F(3,46) = 12.3$; $p < 0.001$), and subiculum ($F(3,46) = 9.95$; $p < 0.001$). The analysis of separation effects for these regions should be viewed with caution because high between-litter variance could have either manufactured false differences or masked true differences related to separation condition.

In summary, the medial prefrontal cortex and accumbens showed separation effects not significantly confounded by litter, but separation effects detected in the VTA and subiculum were confounded by litter. However, because sex was modeled within litter in the hierarchical analysis, the sex differences observed in the habenula and VTA were not confounded by litter.

3. Discussion

In preweanling rats, prolonged (6 h/day for 10 days) mother-infant separation (MS) showed main effects in terms of reducing brain metabolic capacity in the deep layers of the medial prefrontal cortex, caudate putamen, and the shell of the nucleus accumbens. Early handling (EH) with brief separation (15 min/day for 10 days) had contrasting effects on brain metabolic capacity, increasing CO activity in the lateral frontal and medial prefrontal cortex compared to MS, but decreasing CO activity in the ventral tegmental area, subiculum, and posterior parietal cortex. In terms of sex differences, MS reduced brain metabolic capacity in the lateral frontal and medial prefrontal cortical regions to a greater degree in female pups than in males. All female pups, regardless of separation group, showed greater CO activity compared to males in the habenula and ventral tegmental area.

Our findings are consistent with existing literature which has demonstrated that maternal separation does affect the prefrontal cortex. For instance, corticotrophin-releasing hormone and glucocorticoid receptor mRNA levels are reduced in the prefrontal cortex of maternally separated rats (Ladd et al., 2005). Reductions in prefrontal cortical thickness (Spivey et al., 2009) and in dendritic processes of the prefrontal pyramidal neurons have also been demonstrated in maternally separated rats (Monroy et al., 2010; Pascual & Zamora-Leon,

2007). In addition, irregular patterns of interneuron development in the medial prefrontal cortex of maternally separated animals have been found in another rodent species (Helmeke et al., 2008). All of these previously reported findings are consistent with reductions in metabolic demands in the medial prefrontal cortex following prolonged repeated maternal separation. We also showed that among SFR pups that were not separated, P13 males appeared with lower frontal metabolic capacity than females. But after prolonged maternal separation, a marked metabolic hypofrontality was induced in MS females but not in MS males.

There are several behavioral implications of reductions in CO activity in the prefrontal cortex of preweanling rats. Prefrontal hypometabolism is strongly related to behavioral hyperactivity in boys with attention deficit hyperactivity disorder (ADHD) as well as male rats selectively bred as models of hyperactive behavior (Gonzalez-Lima and Sadile, 2000; Gonzalez-Lima, 2005). The medial prefrontal cortex is also implicated in depression in the human and animal literature (Shumake et al., 2000; Shumake and Gonzalez-Lima, 2003). Metabolic brain mapping in infant rats in particular, has shown that prefrontal cortical regions are involved in the maturation of behavioral inhibition (Nair et al., 2001a, 2001b). Perhaps hypometabolism in medial prefrontal cortex is also related to depression or behavioral inhibition dysfunction in general, and the behavioral outcome is dependent on the organism's predispositions to a certain stress-related psychopathology such as depression (Padilla et al., 2009). For example, Holtzman rats (as used in the present study) show a hypoactive phenotype after our MS treatment (Spivey et al., 2008b). Maternal separation may lead to hypoactive behavioral effects in Holtzman rats because of their greater genetic predisposition to show learned helplessness and depressive-like responses to stress (Wieland et al., 1986; Padilla et al., 2009).

Similarly, the sex differences we found in the preweanling brain may be related to different biological predispositions among males and females. Brain CO activity is a well-established index of metabolic capacity correlated with behavioral predispositions, especially sex effects in rats and other species (Sakata et al., 2000, 2002a, 2002b, 2005; Spivey et al., 2008a; Crews et al., 2009). As preweanlings, Holtzman females generally have higher metabolic capacity than males in most brain regions, including the prefrontal cortex. This supports previous findings in which female adolescent Holtzman rats show less risk-taking and short movement behavior in a novel open field compared to males, suggesting an increased capacity for behavioral inhibition (Spivey et al., 2008b). The MS-related sex differences during the preweanling period which are not present in adolescent behavior could be reflective of functional pathways still in the process of maturing (Shumake et al., 2004).

As compared to females, juvenile Sprague-Dawley male rats have lower prefrontal metabolic capacity under baseline conditions (Spivey et al., 2008a). This baseline sex difference is consistent with the lower prefrontal CO values observed in SFR Holtzman male pups. In addition, when preweanling Holtzman rats were subjected to MS stress the females showed the strongest frontal cortex metabolic reductions relative to SFR and EH groups. There are also sex-dependent strain differences in the behavioral response to MS. For example, after identical MS treatments, females of the Holtzman strain show hypoactive behavior; whereas Sprague-Dawley males show hyperactive behavior. Therefore, the effects of identical MS protocols on prefrontal metabolism and behavior are dependent on the rat's genetic background and sex, with Holtzman females being more predisposed to show hypoactivity.

In MS animals, hypometabolism in the accumbens shell may be functionally related to prefrontal hypometabolism because rat accumbens shell neurons are strongly regulated by excitatory afferents arising from the prefrontal cortex (Pennartz et al., 1994). Our findings

are also consistent with existing literature which has demonstrated that maternal separation affects the nucleus accumbens, in particular the shell (Zimmerberg and Brown, 1998; Andersen et al., 1999; Brake et al., 2004). This MS effect was similar for males and females, and may be linked to the hypoactive phenotype exhibited by Holtzman rats after MS vs. EH treatments (Spivey et al., 2008b). More generally, Holtzman rats show increased vulnerability to respond to stressful treatments with depressive-like behavior, such as increased rates of learned helplessness (Padilla et al., 2009). We interpret the reduction in MS pups as compared to EH pups as linked to the role of the nucleus accumbens shell in depressive-like behavior and antidepressant drug action (Shirayama & Chaki, 2006). In particular, metabolic reduction of the accumbens shell has been found to produce anhedonia, which is a key feature of human depression as well as of rats predisposed to develop learned helplessness (Shumake et al., 2005). This interpretation would be consistent with the depressive-like phenotype exhibited by Holtzman rats after MS and other types of stressors (Wieland et al., 1986; Spivey et al., 2008b; Padilla et al., 2009).

The lateral frontal cortex (often regarded as part of motor cortex in rats) was more metabolically active in EH as compared to MS animals, and this finding is likely related to the hypoactive behavior of Holtzman MS animals (Spivey et al., 2008b). Briefly, we previously found in Holtzman six-week-old rats a hypoactive phenotype for MS animals, but increases in risk-taking behaviors for EH animals. Increases in lateral frontal cortex metabolic capacity in EH animals would likely correspond to increased propensity for ambulatory activities, such as entering the exposed zone of an open field (Spivey et al., 2008b).

In conclusion, our findings are novel in that they are the first to report experience-based changes in regional metabolic capacity in preweanling rats as a result of early handling and mother-infant separation. The hypothesis that repeated prolonged mother-infant separation would alter prefrontal cortex regional metabolic capacity was supported. Our findings are consistent with existing literature that finds MS-dependent changes in prefrontal cortex and nucleus accumbens. This study is also the first to evaluate sex-dependent changes in metabolic capacity in the brain following two weeks of mother-infant separation.

4. Experimental Procedures

4.1. Subjects

Subjects were 60 Holtzman albino rat pups born to 6 timed-pregnant mothers bred in the colony of the Animal Resources Center at the University of Texas at Austin. Pregnant mothers were singly housed and maintained on a 12h/12h light/dark photoperiod, with lights on at 06:00 h and lights off at 18:00 h, in a room with temperature maintained at 22°C and 40% humidity. Food and water were available *ad libitum*. The day of birth was marked as postnatal day (P) 0. On P2, litters were culled to as close to ten pups as possible, always consisting of an equal male-to-female ratio. Anogenital distance was used to determine males from females at this age. All procedures were conducted in accordance with the guidelines of the National Institutes of Health in an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care-International, and the protocol was approved by the Institutional Animal Care and Use Committee.

4.2. Maternal separation protocol

The six litters were divided into three groups, as previously described (Colorado et al., 2006; Spivey et al., 2008a, 2008b): Maternal Separation (MS), in which separation consisted of 6 hours; Early Handled (EH), in which separation consisted of 15 minutes; and Standard Facility Reared (SFR), who were not handled at all except for the culling on P2 and

biweekly cage changes by facility staff. Separation protocols for MS and EH groups consisted of daily separation from P2 through P6; no separation from P7 to P8, and daily separation from P9 through P13, as in our other studies (Colorado et al., 2006; Spivey et al., 2008a, 2008b). Maternal separation began at 0730 h and ended at 1330 h daily, while early handling occurred from 1300 h to 1315 h daily. The dams were removed from the home cage, and all pups were removed and placed as a litter into a bedding-lined holding cage, and dams were returned to the home cage for the duration of the separation. Holding cages were then placed in an incubator (30–34°C) to maintain thermoregulation for the duration of the separation period. Upon return to the home cage, dams were again removed from the home cage, pups were returned to the cage, and the dams were returned to the pups.

4.3. Tissue preparation

Immediately following the final separation period on P13, pups were returned as a group to a holding cage, then transferred individually to a separate room and decapitated. Brains were rapidly extracted and frozen in isopentane. The brains were stored at –40°C until sectioning. Tissue was sectioned at 40 micrometers in a –20°C cryostat. The sections were kept frozen on slides at –40°C until processing for cytochrome oxidase histochemistry. Every third section was used for staining. Staining and quantification of neural tissue for cytochrome oxidase activity was used to determine the metabolic capacity of brain regions based on densitometric histochemical analysis of the sections and of brain paste standards of known cytochrome oxidase activity measured spectrophotometrically (Gonzalez-Lima & Cada, 1998).

Brain tissue was stained using the cytochrome oxidase histochemistry procedure previously described (Gonzalez-Lima & Cada, 1998). Slides were first treated in 10% sucrose phosphate buffer (0.1M, pH 7.6) containing 0.5% glutaraldehyde (Grade II) for 5 min. Three changes at 5 min each of 10% sucrose phosphate buffer were followed by 10 min in Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride, 10% sucrose, and 0.5% dimethylsulfoxide. The slides were then rinsed for 5 min in phosphate buffer and incubated at 37°C for 60 min in 700 ml of an oxygen-saturated reaction solution containing 350 mg of diaminobenzidine tetrahydrochloride (DAB), 52.5 mg of cytochrome *c*, 35 g of sucrose, 14 mg of catalase, and 1.75 ml of dimethylsulfoxide in phosphate buffer. To stop the reaction and fix the tissue, a 30 min immersion in 10% sucrose phosphate buffer with 4% formalin (v/v) was used before dehydrating with ethanol, clearing with xylene, and coverslipping with Permount.

Anatomically matched sections from male and female subjects were stained in the same batch to minimize the possibility of inter-batch variability during the comparison between subject groups. In addition, sets of homogenized tissue standards (10, 20, 40, 60, 80 micron-thick sections) were included with each batch of slides (Gonzalez-Lima & Cada, 1998). These standards were used to convert tissue optical density measures to cytochrome oxidase activity units via a regression equation based on their optical density and spectrophotometrically determined enzymatic activity.

Using an image-processing system (JAVA, Jandel Scientific, Corte Madera, CA), optical density was sampled from regions of interest. The size of the square-shaped sampling window was adjusted for each region so that it was as large as possible while still allowing for four, non-overlapping readings to be taken bilaterally. For each cortical region, optical density was sampled in both superficial (I–III) and deep (IV–VI) layers across three adjacent sections and averaged. Cortical regions were analyzed separately in superficial and deep layers because we have previously found differences between them in the cortex of six-week-old rats (Spivey et al., 2008a). These optical density values were then converted to

cytochrome oxidase activity units (micromol/min/g tissue wet weight), which were determined by spectrophotometry of cytochrome oxidase standards as described before.

4.4. Regions of interest

The maternal separation literature describes numerous alterations in both behavior and brain activity. The regions were selected based on studies regarding maternal separation and changes in brain activity. Hyperactive behavior has been reported as one long-term effect of MS (Colorado et al., 2006; Kaneko et al., 1994) and the relationship between hyperactive behavior and the prefrontal cortex is well documented (Sullivan & Brake, 2003). Dysfunction to the prefrontal cortex is also seen subsequent to MS (Monroy et al., 2010; Jimenez-Vasquez et al., 2001; Ladd et al., 2005). Many studies have used a myriad of measures to show brain differences in MS animals, including neurotransmitter levels, stress hormone levels, drug manipulations, and receptor binding studies. Alongside findings of behavioral hyperactivity, studies have shown increases in catecholamine levels in the striatum and dorsal hippocampus (Matthews et al., 2001), and changes in the nucleus accumbens in both MS (Monroy et al., 2010; Zimmerberg & Brown, 1998) and EH animals (Brake et al., 2004). Changes to the reward sensitivity in MS animals by way of reduced responding to sucrose solutions (Matthews, Wilkinson & Robbins, 1996) or cocaine (Matthews et al., 1999) was a rationale for considering dopamine related regions, including the ventral tegmental area, nucleus accumbens, striatum, and prefrontal cortex in the current study.

In order to account for divergent phenotypes found by various MS protocols, we also considered other studies that have reported hypoactive behavior following MS (Spivey et al., 2008b). Such a depressive phenotype in rats is associated with changes to the habenula, ventral tegmental area, striatum, prefrontal and infralimbic cortex, and hippocampus (Shumake et al., 2005). Due to the stressful nature of MS in infancy, many reports have listed changes to anxiety-like behaviors and defensive withdrawal in adolescence (Colorado et al., 2006), as well as alterations in neural activity in stress-related regions such as the paraventricular nucleus of the hypothalamus and central amygdala (Liu et al., 2000; Genest et al., 2004; Ladd et al., 2005; Plotsky et al., 2005). The posterior parietal cortex was of interest due to its relationship to attention and motor behaviors in rodents (Kolb & Walkey, 1987; Mattelli & Luppino, 2001), and is also reported to have less CO activity in males than females (Spivey et al., 2008a).

Cortical regions were assessed separately in terms of superficial (I – III) and deep (IV – VI) layers, based also on previous findings in which cytochrome oxidase levels showed group differences in superficial cortical layers but not deep layers (Gonzalez-Lima & Sadile, 2000). All regions of interest and corresponding Bregma levels (Paxinos & Watson, 1997; Gonzalez-Lima & Cada, 1998) are depicted in Figure 1.

4.5. Statistical analysis

Data from this experiment were analyzed using SPSS software (version 11.5, SPSS, Chicago, IL). Differences were considered significant at the two-tailed $p < 0.05$ level for all tests. A 2 (sex) $\times$ 3 (separation group) univariate ANOVA was used to assess separation group and sex differences in each region of interest (Spivey et al., 2008a). Additional post-hoc Bonferroni-corrected simple effects tests were utilized where indicated. Litter effects on CO activity were evaluated using three-level hierarchical ANOVAs.

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Figure 1.

Photographs of CO-stained hemisections (left) and corresponding coronal brain drawings (right) illustrating the locations of each region of interest by Bregma level according to the Paxinos and Watson (1997) and Gonzalez-Lima and Cada (1998) atlases. Abbreviations are listed in Table 1.

Table 1

Brain cytochrome oxidase activity (mean±S.E.M) after repeated maternal separation (MS), early handling (EH) or standard facility rearing (SFR) in preweanling rats.

Region	Abbrev.	Bregma	SFR		EH		MS		Effects ($p =$)		
			Male (n)	Female (n)	Male (n)	Female (n)	Male (n)	Female (n)	Separation ^a	Sex ^b	Sep.×sex ^c
Lateral frontal cortex, superficial	LFR-S	3.7	134±6 (6)	142±7 (5)	153±5 (10)	153±5 (10)	146±5 (9)	117±5 (9)	<0.001	0.086	0.003
Lateral frontal cortex, deep	LFR-D	3.7	137±6 (6)	145±7 (5)	153±5 (10)	151±5 (10)	146±5 (9)	116±5 (9)	<0.001	0.079	0.003
Medial prefrontal cortex, superficial	MPFC-S	3.7	125±6 (6)	142±7 (5)	138±5 (10)	139±5 (10)	133±5 (9)	107±5 (8)	0.002	0.506	0.002
Medial prefrontal cortex, deep	MPFC-D	3.7	136±7 (6)	139±7 (5)	144±5 (10)	160±5 (10)	134±6 (9)	109±6 (8)	<0.001	0.679	0.002
Nucleus accumbens (shell)	ACB	2.7	124±7 (9)	119±8 (6)	132±6 (10)	137±6 (10)	114±7 (8)	116±7 (9)	0.014	0.927	0.745
Infralimbic cortex	IL	2.7	126±6 (9)	118±7 (6)	127±6 (10)	134±6 (10)	116±6 (8)	115±6 (9)	0.051	0.951	0.498
Caudate putamen	CP	-1.8	143±8 (6)	148±9 (5)	161±7 (9)	148±7 (8)	139±7 (8)	130±8 (6)	0.045	0.419	0.505
Globus pallidus	GP	-1.8	97±8 (6)	109±8 (5)	117±6 (9)	105±7 (8)	103±7 (8)	88±8 (6)	0.119	0.396	0.200
Paraventricular nucleus	PVN	-1.8	118±6 (7)	107±7 (5)	104±4 (11)	100±5 (8)	102±5 (10)	116±5 (8)	0.163	0.957	0.066
Central amygdala	CEA	-1.8	131±5 (7)	113±7 (5)	115±4 (11)	118±5 (8)	118±5 (10)	124±5 (8)	0.528	0.471	0.072
Habenula	HB	-3.8	168±8 (8)	183±10 (5)	148±7 (11)	168±8 (9)	162±9 (7)	170±8 (8)	0.120	0.037	0.729
Posterior parietal cortex, superficial	PPA-S	-3.8	147±7 (8)	155±9 (5)	138±6 (11)	142±7 (9)	145±7 (7)	158±7 (11)	0.148	0.151	0.790
Posterior parietal cortex, deep	PPA-D	-3.8	126±5 (8)	141±6 (5)	121±4 (11)	121±4 (9)	127±5 (7)	134±5 (8)	0.026	0.057	0.343
Periaqueductal grey	DMPAG	-6.3	111±8 (8)	132±10 (5)	119±7 (11)	120±8 (9)	120±8 (9)	105±7 (11)	0.480	0.747	0.113
Ventral tegmental area	VTA	-6.3	98±8 (8)	109±10 (5)	77±7 (11)	86±7 (9)	98±7 (9)	117±7 (11)	0.001	0.046	0.748
Subiculum	SUB	-6.3	121±7 (8)	137±9 (5)	105±6 (11)	114±7 (9)	128±7 (9)	129±6 (11)	0.008	0.138	0.587

^aBold value indicates significant separation difference independent of sex.

^bBold value indicates significant sex difference independent of separation.

^cBold value indicates significant interaction between separation and sex.

^dBold value indicates significant confound of litter cohort on separation effects.