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Juvenile male rats display lower cortical metabolic capacity than females

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ABSTRACT

The juvenile brain undergoes marked maturational changes accompanied by major sex hormone changes. In particular, sex differences in neural substrates could underlie male-specific dysfunction in behavioral responses related to the prefrontal cortex. Sex differences in regional metabolic capacity of the cerebral cortex were investigated in juvenile Sprague-Dawley rats. At 6 weeks of age the brains were processed for quantitative histochemistry of cytochrome oxidase, a rate-limiting enzyme in cellular respiration, which is an index of brain metabolic capacity. Quantitative image analysis revealed a main effect of sex with males displaying lower regional metabolic capacity than females in the dorsolateral and orbital prefrontal cortex and in the posterior parietal cortex. In addition, males separated for 6 h/day from their mothers as pups showed greater ambulatory behavior in the novel open field and higher metabolism in the posterior parietal cortex relative to males separated for 15 min/day. This is the first study to show sex differences in brain metabolic capacity in regions such as the prefrontal cortex that may be hypometabolic in juvenile males relative to females.

Investigating the juvenile brain is important because during adolescence the brain undergoes marked maturational changes in cortical synaptic remodeling, neurogenesis, neurotransmitter receptors and transporters, accompanied by major sex hormone changes and corresponding physiological and behavioral differences [26,32]. Of interest for this study is that sex differences may also be evident in brain regional metabolic capacity, which may provide insight into sex differences in behavioral responses [7,8,26,27,29,30]. For example, sex differences in neural substrates could underlie male-specific dysfunction in behaviors related to the prefrontal cortex. In humans, prevalence of ADHD is higher in boys than girls [36]. A number of studies support the hypothesis that early androgen exposure in males facilitates their development of hyperactivity, impulsivity and inattention in a manner consistent with their higher expression of ADHD-like behaviors [17,20]. Specifically, the human literature and animal models indicate that young males with ADHD show frontal cortex hypoactivity and reduced volume of the cerebellum [9,31].

Additionally, we sought to assess changes in the brain resulting from prolonged vs. brief mother–infant separation to gain insight into any potential sex differences in brain metabolic capacity. The enzyme cytochrome oxidase (CO) is an appropriate histochemical marker for brain metabolic capacity [11]. Cytochrome oxidase, the rate-limiting enzyme in the mitochondrial electron transport chain, limits the production of ATP. The amounts of cytochrome oxidase reflect the metabolic demand of brain cells and are therefore indicative of general metabolic capacity in any given region of the brain [28]. We hypothesized that maturational changes in dendrites, the most energy-demanding compartment of neurons, may be mirrored by regional metabolic alterations. Therefore, the metabolic capacity in brain regions implicated in sex differences in juvenile behaviors such as ADHD [10,12,13] was measured using quantitative cytochrome oxidase histochemistry. We tested the hypothesis that juvenile male rats would show lower prefrontal cortical metabolic capacity as compared to females given the male-biased dysfunction in behavioral inhibition related to prefrontal cortex hypoactivity.

The study was conducted in accordance with the guidelines of the U.S. Department of Agriculture and National Institutes of Health in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International and all procedures were approved by the Institutional Animal Care and Use Committee. Subjects were forty (20 male, 20 female) newborn Sprague-Dawley rats from four pregnant females acquired from a commercial supplier (Harlan, Houston, TX). Four pregnant females were singly housed and maintained on a 12-h light/dark cycle in a temperature-controlled room at 22°C and 40% humidity. Food and water were available *ad libitum*. When the dams gave birth

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(postnatal day 0), the newborns were cross-fostered and culled so that each litter contained 5 males and 5 females.

Beginning on postnatal day (P) 2, the litters were randomly divided into two groups, each group containing 10 male and 10 female pups. One group was separated from the dams for 15 min, which is similar to the procedure used for handling of litters for cage cleaning in our animal facility; while the other group was separated from the dams for 6 h. During the separation period, the pups were kept in an incubator at 33 °C in order to maintain appropriate body temperature. Maternal separation occurred daily from P2 through P6; the animals were left undisturbed from P7 to P8, and separation occurred again daily from P9 to P13. Following P13, the subjects remained undisturbed until the day of weaning at P21, when they were separated from the mother and housed with littermates. Food and water continued to be freely available and the 12-h light/dark cycle was maintained. A recent paper from our laboratory reported that this separation protocol of 6 h per day for 10 days during postnatal days 2–13 resulted in behavioral hyperactivity/impulsivity in adolescent male Sprague-Dawley rats [6], a phenotype which is consistent with other similar studies [2,5,16,35]. We used the same separation procedures in this study to verify that maternal separation increases ambulatory behavior in the novel open field test [6].

At 6 weeks of age, the subjects were decapitated; the brains were removed and quickly frozen in isopentane. The brains were sectioned at 40 μm in a −20 °C cryostat. The sections were kept frozen on slides until processing with quantitative 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 [9].

Slides were first treated in 10% sucrose phosphate buffer (0.1 M, 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 remove the possibility of inter-batch variability during the comparison between subject groups. In addition, sets of homogenized tissue standards (10, 20, 40, 60, 80 μm-thick sections) were included with each batch of slides [11]. 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. These optical density values were then converted to cytochrome oxidase activity units (μmol/min/g tissue wet weight), which were determined by spectrophotometry of cytochrome oxidase standards as described before [9].

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) × 2 (separation group) univariate ANOVA was used to assess group and sex differences in behavior and the regions of interest, with cortical layers (superficial and deep) assessed separately. Additional Bonferroni-corrected simple effects tests were utilized post hoc.

The human literature suggests that there are alterations in dorsolateral and orbital prefrontal cortex and cerebellum in humans with ADHD. Regional measurements from functional MRI studies have also been used to identify sex differences both in patients with psychiatric disorders [9,14,15,19] and in normal patients with respect to cognitive tasks [4]. Dysfunction of these regions associated with ADHD merits a comparison within this study. Therefore, the implicated regions as well as adjacent cortical and cerebellar regions were analyzed: dorsal frontal, lateral frontal, agranular insular, lateral orbital, medial orbital, medial frontal, perirhinal cortex, posterior parietal cortex, entorhinal cortex, cerebellar cortex, vermis, and flocculus. Fig. 1 lists these 12 regions of interest and their corresponding Bregma levels according to the stereotaxic atlas of the rat brain by Paxinos and Watson and our cytochrome oxidase atlas of the rat brain [11,23].

The CO activity of each of the regions of interest is listed in Table 1, grouped for males and females because effects of sex dominated the CO differences. Generally, males were found to have lower CO activity relative to females in dorsolateral and orbital frontal cortical regions, posterior parietal cortex, lateral cerebellum and vermis.

In the cerebral cortex, males had consistently lower CO activity relative to females regardless of separation condition. This was found in the following regions: dorsal frontal cortex – deep ($F(1,32) = 5.830$; $p = 0.022$); lateral frontal cortex – deep ($F(1,32) = 9.728$; $p = 0.004$); agranular insular cortex – deep ($F(1,32) = 7.663$; $p = 0.009$); lateral orbital cortex – superficial ($F(1,32) = 9.203$; $p = 0.005$) and deep ($F(1,32) = 4.923$; $p = 0.034$); medial orbital cortex – superficial ($F(1,32) = 5.741$; $p = 0.023$) and deep ($F(1,32) = 6.012$; $p = 0.020$); and posterior parietal cortex – superficial ($F(1,32) = 5.787$; $p = 0.022$) and deep ($F(1,28) = 18.443$; $p < 0.001$). No significant sex differences were found in various superficial cortical regions as well as medial frontal, perirhinal and entorhinal cortical regions. In the cerebellum, males also showed less CO activity relative to females in the lateral cerebellar cortex ($F(1,29) = 10.51$, $p = 0.003$) and the vermis ($F(1,28) = 7.72$, $p = 0.01$). But there were no significant sex differences in other cerebellar regions such as the flocculus.

Maternal separation condition affected CO activity in only one region, the posterior parietal cortex. In the posterior parietal cortex there were main effects of both sex ($F(1,28) = 16.613$; $p < 0.001$) and separation condition ($F(1,28) = 4.861$; $p = 0.036$). While males had significantly lower CO activity relative to females within both separation conditions, Bonferroni-corrected simple effects post hoc tests showed that only males separated 6 h/day from their mothers as pups showed significantly greater CO activity in the posterior parietal cortex relative to males separated for 15 min/day (326 ± 13 vs. 283 ± 12; $p = 0.023$). No statistically significant effects relative to separation condition were found in any of the other regions. Fig. 2 behavioral data verified that males are more susceptible than females to show hyperactivity following maternal separation. In the novel open field test, separated males showed a 159% increase in ambulation as compared to 57% in females.

Cortical regions for CO analysis were selected based on animal models of hyperactivity and the human literature on ADHD. A genetic animal model of hyperactivity, the Naples high- and low-

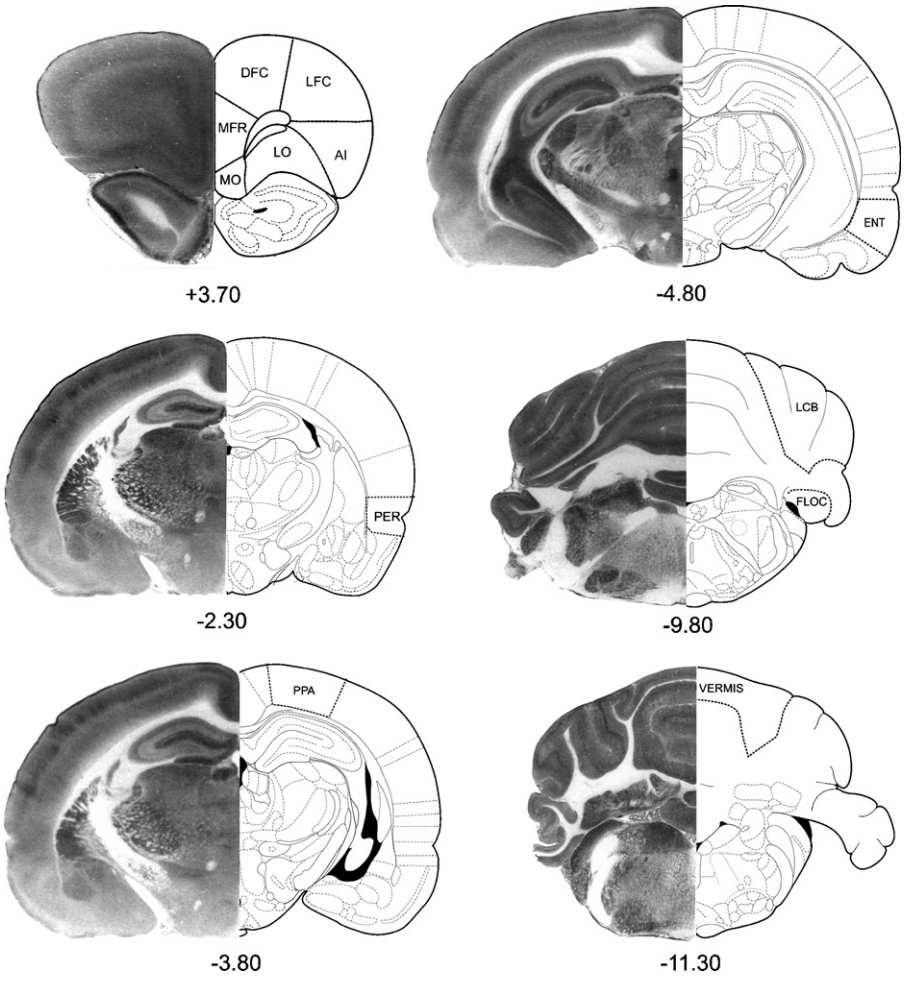

Fig. 1. Photographs of CO stained sections (left) and coronal brain diagrams (right) illustrating the locations of each region of interest by Bregma level according to the Paxinos and Watson [23] and Gonzalez-Lima and Cada [11] atlases. Abbreviations are listed in Table 1.

Table 1
Sex differences in brain cytochrome oxidase activity (mean ± S.E.M.) among juvenile rats

Region	Abbreviation	Bregma	Male	*N*	Female	*N*	Significance
Dorsal frontal cortex, superficial	DFC	3.7	196 ± 4	19	199 ± 4	17	
Dorsal frontal cortex, deep		**3.7**	**190 ± 3**	**19**	**203 ± 4**	**17**	*
Lateral frontal cortex, superficial	LFC	3.7	194 ± 4	19	206 ± 4	17	
Lateral frontal cortex, deep		**3.7**	**187 ± 3**	**19**	**203 ± 4**	**17**	*
Agranular insular cortex, superficial	AI	3.7	181 ± 3	19	187 ± 5	17	
Agranular insular cortex, deep		**3.7**	**187 ± 3**	**19**	**202 ± 4**	**17**	*
Lateral orbital cortex, superficial	LO	**3.7**	**189 ± 3**	**19**	**206 ± 4**	**17**	*
Lateral orbital cortex, deep		**3.7**	**190 ± 3**	**19**	**202 ± 4**	**17**	*
Medial orbital cortex, superficial	MO	**3.7**	**185 ± 3**	**19**	**199 ± 4**	**17**	*
Medial orbital cortex, deep		**3.7**	**183 ± 3**	**19**	**197 ± 4**	**17**	*
Medial frontal cortex, superficial	MFR	3.7	178 ± 3	19	187 ± 4	17	
Medial frontal cortex, deep		3.7	188 ± 3	19	197 ± 4	17	
Perirhinal cortex, superficial	PER	−2.3	266 ± 6	19	281 ± 10	16	
Perirhinal cortex, deep		−2.3	245 ± 6	19	253 ± 10	16	
Posterior parietal cortex, superficial	PPA	**−3.8**	**331 ± 11**	**18**	**359 ± 16**	**14**	*
Posterior parietal cortex, deep		**−3.8**	**302 ± 10**	**18**	**357 ± 9**	**14**	*
Entorhinal cortex, superficial	ENT	−4.8	273 ± 8	20	279 ± 11	20	
Entorhinal cortex, deep		−4.8	260 ± 5	20	265 ± 9	20	
Lateral cerebellum	LCB	**−9.8**	**297 ± 6**	**17**	**336 ± 12**	**12**	*
Vermis	VERMIS	**−11.3**	**306 ± 7**	**16**	**366 ± 23**	**12**	*
Flocculus	FLOC	−9.8	337 ± 6	17	350 ± 8	12	

Boldfaced with asterisk (*) indicates a significant sex difference ($p < 0.05$).

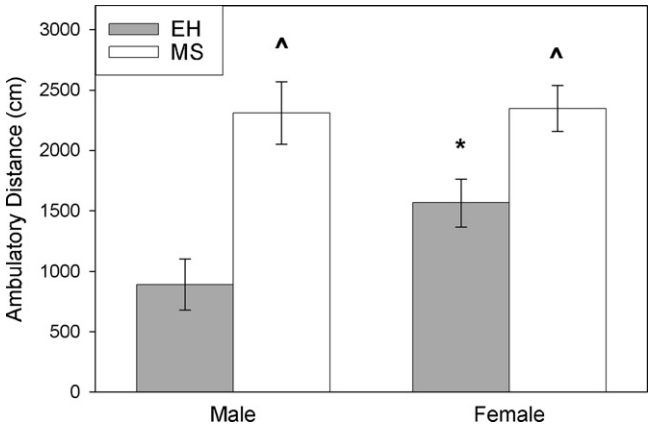

Fig. 2. Ambulatory distance traveled during 10 min in the novel open field test for early handled (EH) and maternally separated (MS) males and females. EH females showed 75% greater baseline activity than EH males. MS resulted in increased ambulation in both males and females. But MS males shower a greater increase (159% greater than EH) in ambulation relative to MS females (57% greater than EH). An asterisk (*) represents a significant ($p < 0.05$) sex difference within the separation group. A carat (^) represents a significant ($p < 0.01$) separation group difference within the sex.

excitability strains of rats, has been analyzed previously by us using this cytochrome oxidase mapping approach [10]. The Naples strains are Sprague-Dawley rats selectively bred for behavioral reactivity to spatial novelty [34] and are a suitable model with which to compare our subjects. Gonzalez-Lima and Sadile [12] conducted a mapping study of adult males of the Naples strains using CO histochemistry which showed group differences in the following cortical regions: prefrontal cortex, posterior parietal cortex, perirhinal cortex and entorhinal cortex. Additionally, this previous CO study found significant differences in the results when cortical areas were divided into superficial (I–III) and deep (IV–VI) cortical layers, so this methodology was applied to the sampling procedures in the current study. These areas are therefore of interest in order to compare cortical sex differences with those due to a genetic model of hyperactivity previously investigated with the same metabolic mapping approach. Our previous study with the Naples rat model of hyperactivity and the present study showed differences between superficial (I–III) and deep (IV–VI) layers in some frontal cortical regions. Superficial layers are primarily input layers while deep layers are for output. Hence hypometabolism in deep layers responsible for output pathways from the frontal cortex to other regions appear to be more relevant for the observed male preponderance to exhibit a hyperactive/impulsive phenotype.

Sex differences, regardless of separation condition, were evident in brain regional metabolic capacity in cortical regions of interest for this study. As hypothesized, a lower metabolic capacity was found in the prefrontal cortex of males compared to females. Hypometabolism in regions such as the dorsal frontal, lateral frontal, lateral orbital and medial orbital cortex is similar to findings in the human literature in which young males with ADHD show reduced metabolic activity, and potentially, dysfunction of, the prefrontal cortex, as well as reduced volume of the cerebellum [9,28]. Since increased metabolism of these prefrontal regions in rats and mice underlies the inhibition of behavior in both instrumental learning and classical conditioning [3,22], we interpret the sex difference in frontal metabolism as perhaps contributing to the greater frequency of juvenile males to display impaired behavioral inhibition characteristic of the hyperactive/impulsive phenotype.

It is possible that a sex difference in neural substrates could mean an underlying male-specific dysfunction in behaviors related to the prefrontal cortex. In humans, prevalence of ADHD is higher in boys than girls [36]. A number of studies support the hypothesis that early androgen exposure in males facilitates their predisposition to hyperactivity, impulsivity and inattention in a manner consistent with their higher expression of some ADHD-like behaviors [17,20]. Therefore, a relatively hypometabolic prefrontal cortex in males due to androgenization may render adolescent males more susceptible than females to show less behavioral inhibition and more impulsive behavior. The interpretation of a relatively hypometabolic prefrontal cortex as facilitating a hyperactive/impulsive phenotype is also consistent with our previous CO mapping study of the Naples strains of Sprague-Dawley rats [12]. The Naples high- and low-excitability rats were selectively bred to display either hyper-reactivity or hypo-reactivity to spatial novelty, respectively. The high-excitability rats are presumed to model the ADHD hyperactive/impulsive type, whereas the low-excitability rats model the ADHD predominantly inattentive type. The low-excitability rats show greater CO activity in the prefrontal cortex relative to the high-excitability rats [12]. Subjects with higher prefrontal cortex metabolic activity are more successful at inhibiting their behavior. For example, increased metabolic activity of the prefrontal cortex in normal rats is correlated with inhibition of behavior during extinction, in both instrumental goal-seeking behavior [22] and in classical conditioning [3,24]. Therefore, a relatively hypometabolic prefrontal cortex may make juvenile males less capable of inhibiting their behavior as compared to females.

Previous studies from our lab and others have demonstrated that sex differences in brain CO activity can be seen in the gecko [7,8,27,28] as well as in the human [33]. Because adolescence is a time of hormonal changes in the rat, we also sought to investigate whether there are sex-specific brain differences in juvenile rats subjected to prolonged vs. brief mother–infant separation. Some studies have identified certain separation protocols and rat strains in which sex-specific effects of mother–infant separation were found. For example, sex differences in adult rats following maternal separation include reductions in ambulation in females compared to males [25]. But no maternal separation studies have investigated sex differences in the brains of juvenile rats.

In our study, juvenile males with prolonged mother–infant separation had significantly greater CO activity in the posterior parietal cortex as compared to briefly separated animals. The posterior parietal cortex has been associated with higher order polymodal sensory integration, position sense for self and others, and attention mechanisms in both rats [18] and humans [1]. The integration of somatosensory information with visual stimuli is necessary to correlate the visual environment with the position of the body parts and external objects. Humans with posterior parietal cortex lesions have difficulty in attending to relevant stimuli. For example, patients with right posterior parietal cortex injury show a form of sensory neglect in which they ignore the left half of the body and objects in the visual field [1]. Although posterior parietal cortex activity is mainly regarded as related to the integration of different types of sensory inputs, its activity is also important for motor expression because the output of the posterior parietal cortex is synaptically linked to premotor and motor regions of the frontal cortex in both rats and humans [13,21]. Lower CO activity of frontal cortical regions may affect the output from these regions and render the males more impaired in behavioral inhibition. A relative CO increase in posterior parietal cortex may reflect the role of this region in attentional shift since following maternal separation subjects show evidence for greater attentional shifts [6].

In conclusion, the hypothesis that juvenile male rats would show lower prefrontal cortical metabolic capacity as compared to females was supported by the data. The lower cortical metabolic capacity measured by CO activity in males differentiates juvenile males from females regardless of maternal separation condition

or open field behavioral scores. The CO difference is a cortical sex difference present regardless of open field ambulatory scores. This sexually dimorphic lower metabolic capacity in males is proposed to impair cortically mediated behavioral inhibition more in males than females following maternal separation stress.

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