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Research report

Repeated interactions with females elevate metabolic capacity in the limbic system of male rats

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Abstract

The effect of heterosexual social experience on brain metabolic capacity was investigated by measuring the activity of cytochrome oxidase, a rate-limiting enzyme in oxidative metabolism. Male Sprague–Dawley rats were kept naïve or allowed to copulate with receptive females three (3F males) or 16 times (16F males). Throughout the vomeronasal system and other limbic areas, 16F males had elevated metabolic capacity relative to naïve and 3F males, whereas no significant differences in brain metabolism were found between 3F and naïve males. Behavioral differences were also found between 3F and 16F males. In a second experiment, we assessed differences in brain metabolism between sexually active and inactive males given only one opportunity to copulate and found no significant difference in neural metabolism between these males. This suggests that the differences found in the first experiment were primarily driven by differences in repeated experience rather than by sexual performance between 16F and 3F males. We speculate that these changes in brain metabolic capacity could be related to immediate early gene expression during copulation and could underlie the long-term behavioral changes accompanying heterosexual social experience.

Theme: Neural basis of behaviour

Topic: Neural plasticity

Keywords: Copulation; Male rat; Heterosexual experience; Metabolic capacity; Cytochrome oxidase

1. Introduction

Patterns of neural activity associated with the display of social, particularly sexual, behaviors in rodents have been extensively studied (reviewed in Refs. [47,53]), and most studies have utilized the expression of the immediate early gene (IEG), *c-fos*, as a marker for neural activity. However, other metabolic markers such as cytochrome oxidase (CO) activity have not been readily applied to the study of social behavior. Cytochrome oxidase is a rate-limiting enzyme in oxidative phosphorylation, the major energy-producing pathway in the brain [18], and an important marker of metabolic capacity [63]. Cytochrome oxidase activity is also a marker of the metabolic history of an area [22]; manipulations that decrease synaptic activity also decrease CO activity in efferent brain areas (e.g., Refs. [5,31,64]). The activity of CO is also sensitive to hormone manipulations in rodents and reptiles [10,12,35] and to sociosexual experience in lizards [13]. Unlike changes in *c-fos* expression, which represent evoked activity, changes in CO activity as a result of social interactions reflect the long-term effects of these interactions on neural metabolism.

Heterosexual social experience engenders not only changes in immediate neural activity but also long-term behavioral changes. For example, relative to sexually naïve males, experienced male rats display copulation longer after castration and begin copulating sooner after testosterone administration [40,56]. Consequently, assessing changes in CO activity with heterosexual social experience might provide information on the neurobiological correlates underlying these long-term changes in behavior.

In these experiments we examined the degree to which varying amounts of heterosexual interaction affect CO activity in limbic brain areas in male rats. We predicted that repeated interactions with females would repeatedly

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activate the neural circuit underlying copulatory behavior, and that this repeated neural activation would lead to increases in CO activity in brain nuclei within the circuit. Further, we predicted that the degree of metabolic change would be related to the amount of IEG induction during copulation and hormonal stimulation. Finally, to bolster the notion that differences in neural metabolism are driven by repeated experience and not by differences in sexual performance, we also investigated whether neural metabolic differences existed between copulating and noncopulating male rats given only one test with a female.

2. Materials and methods

2.1. Animals

Sprague–Dawley male rats (300–400 g) from a colony maintained at the University of Texas at Austin were used in the study. Males and females were separated at weaning, and males were isosexually housed in groups of 3–5. Males were purchased when they reached ~200 g and housed individually. Food and water were provided ad lib. Individuals were kept on a 12:12 light–dark photocycle with lights off at 13.00 h. Ambient temperature was kept constant at 25 °C.

2.2. Procedure

Two separate experiments were conducted. For both experiments, all males were tested with sexually receptive females during the first 4 h of the dark period. Ovariectomized female rats were made receptive with an injection of 17β-estradiol benzoate (10 μg) followed by an injection of progesterone (500 μg) 48 h later, or by implanting ovariectomized females subcutaneously with 17β-estradiol benzoate (Sigma, St. Louis, MO, USA) (5 mm; approximately 4–5 mg). All females were screened for sexual receptivity with a stud male before testing.

In experiment 1, gonadally intact males were divided into three groups: socially naïve males (NAÏVE; $n=9$), males exposed to receptive females three times (3F males; $n=9$), and males exposed to receptive females 16 times (16F males; $n=10$). The experimental design is summarized in Fig. 1. NAÏVE males were placed in an empty plexiglass testing arena (40×40×30 cm) covered with fresh wood chips for 35 min on 3 days; each 'test' was separated by 5 days. The 3F males were also placed in the test arena using the same schedule. However, in this group, after a 10 min habituation period, a sexually receptive female was introduced, and these males were allowed to copulate for 25 min. The 16F males were treated identically to the 3F group, except that on the days between tests in the test chamber, these males were allowed to copulate with receptive females in their home cage for 1 h. 16F males were also allowed to copulate with females in their home cage (1 h) for 3 consecutive days after the third test in the test chamber. This design was selected to control for the amount of handling across groups, as differences in handling can lead to different neural activity patterns (see Ref. [38]).

The copulatory behavior of 3F and 16F males was recorded in the test chambers. Mount latency, latency to ejaculate, and frequency of ejaculations were recorded. The latency to ejaculate was defined as the time since the entry of the female to the time of ejaculation; this is different from 'ejaculation latency', which is the interval between first intromission and first ejaculation. If a male did not mount or ejaculate during the test, the maximum latency score (1500 s) was given. Behavioral observations were also taken on the last 2 days of copulation in the 16F group (i.e., days 15 and 16), and on these 2 days only latency to ejaculate and ejaculation frequencies were recorded. These observations were designed to assess whether all males in this group were copulating as some 16F males showed minimal copulatory behavior in the test chamber. Because males were observed for 1 h, males that failed to ejaculate were given the maximum latency score of 3600 s.

Four days following the final test (i.e., day 17), all males were anesthetized with sodium pentobarbital (80 mg/kg) and decapitated. All animals were killed between 08.00

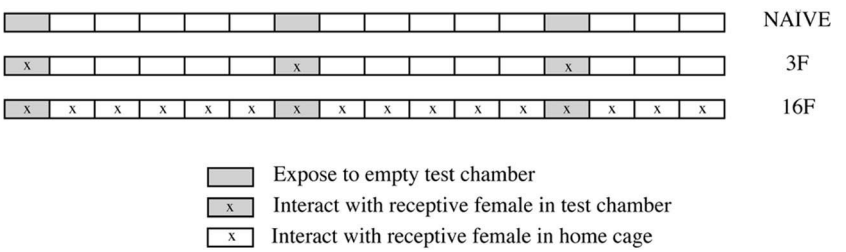

Fig. 1. Experimental design. Socially naïve males (NAÏVE) were placed in an empty test chamber on three occasions, each separated by 5 days. Males with limited exposure to females (3F males) were allowed to copulate with a sexually receptive female for 25 min in the test chamber on three different occasions. Males with extensive heterosexual experience (16F males) were handled identically to 3F males but on the days between tests in the test chamber, these males were allowed to copulated with a receptive female for 1 h in their home cage. 16F males also copulate with females in their home cage for 3 days after their last test in the test chamber. All males were killed on the fourth day after their last test.

and 12.00 h. Brains were rapidly frozen in isopentane, sectioned at 40 μm in a Reichert–Jung cryostat, and stored at −40 °C until processing for CO activity.

Behavioral differences were found between 3F and 16F males in experiment 1, and we did not know whether the neural differences observed were due to differences in the amount of heterosexual social experience or due to behavioral differences. If the neural differences between 16F and 3F males were due to differences in copulatory performance (see Results), then we predicted that among individuals with limited and equal exposure to females, poor copulators would have elevated metabolic capacity within the limbic system relative to effective copulators. On the other hand, if neural differences between 16F and 3F males are due primarily to differences in the amount of heterosexual experiences, we predicted that there would be no neural differences between poor and effective copulators given limited and identical exposures to females. In experiment 2, we assessed neural differences in brain metabolism between copulating and noncopulating males given only one exposure to a receptive female (i.e., same amount of exposure to females but different in sexual behavior). Gonadally intact males were tested in the test arena (40×40×30 cm) with a female previously screened for sexual receptivity. During this test, latency to ejaculate and number of ejaculations were noted. Only males that ejaculated at least two times with a female (COPULATORS; $n=9$) and males that did not ejaculate with the female (NONCOPULATORS; $n=8$) were included in this study. Thereafter, males were kept in isolation for 16 days, then were anesthetized with sodium pentobarbital (80 mg/kg) and decapitated. This design kept constant the time from first test to sacrifice across both experiments. Brains were collected and sectioned as described in experiment 1.

2.3. Cytochrome oxidase histochemistry and imaging

Detailed protocols for quantitative CO histochemistry have previously been published [21]. Briefly, slides were first treated in 10% sucrose phosphate buffer (0.1 M, pH 7.6) containing 0.5% glutaraldehyde for 5 min. This step facilitates the adherence of sections to slides and does not affect the enzymatic activity of CO [23]. Slides were then rinsed 4× in 10% sucrose phosphate buffer (5 min each), then incubated for 10 min in Tris buffer (0.05 M, pH 7.6) containing 275 mg/l cobalt chloride (Sigma), 10% sucrose, and 0.5% dimethylsulfoxide (DMSO) (Sigma). Slides were subsequently rinsed for 5 min in phosphate buffer then incubated at 37 °C for 60 min in 700 ml of an oxygen-saturated reaction solution containing 350 mg diaminobenzidine tetrahydrochloride (Sigma), 52.5 mg cytochrome *c* (Sigma), 35 g sucrose, 14 mg catalase (Sigma), and 1.75 ml DMSO (Sigma) in 700 ml phosphate buffer. To stop the reaction and fix the tissue, slides were then immersed in 10% sucrose phosphate buffer with 4% (v/v) formalin for 30 min. Thereafter, slides were dehydrated through a series of alcohols (30, 50, 70, 95% 2×, 100% 2×) then cleared with xylene (EM Science) and coverslipped with Permount (EM Science).

Optical density (OD) of brain sections and standards were measured using an image-processing system consisting of a high-gain camera (Javelin Electronics), a Targa-M8 image capture board, a 486 computer, Sony color monitor, DC-powered illuminator, and JAVA software (Jandel Scientific, San Rafael, CA, USA). The system was calibrated using an optical density step tablet (Kodak Calibration Tablet No. 2). Four OD measurements were taken per nucleus on each section, and 2–3 sections were imaged for a single nucleus. All measurements were taken unilaterally for each subject, and the side of the brain was randomly selected across individuals. The experimenter was blind to the treatment of the animals during data collection. Optical density values for each nucleus were then averaged and converted into activity units (μmol/min/g tissue wet weight) using a regression based on brain homogenate standards included in each batch [23]. Brain homogenates served as internal calibration standards to control for factors that affect staining intensity. Standards were made by homogenizing whole brains of 12 naïve male rats at 4 °C followed by rapid freezing in isopentane. The CO activity of the homogenate was then spectrophotometically assessed [6]. Within each reaction, at least two slides with brain homogenates are included, and on each slide are sections cut at varying thickness (10, 20, 40, 60, and 80 μm). The OD of these sections were then regressed on the known CO activity of the sections of varying thickness. This regression is used to convert OD into a standard unit of activity; this allows for the aggregation of data from different batches. Altogether, a total of four batches were run, and in each batch all groups were represented. The R^2 values for the regressions in each of the four batches were 0.94, 0.94, 0.96, and 0.95. The mean±S.E.M. OD of the standards across the batches is presented is Fig. 2, and on average OD readings had standard errors <7% of means.

Locations of specific nuclei were identified using a stereotaxis atlas [51] and a CO atlas of the rat brain (Fig. 3) [24]. The brain areas of interest were the medial preoptic area (MPOA), periventricular preoptic area (PvPOA), lateral preoptic area (LPOA), medial division of the bed nucleus of the stria terminalis (BNSTM), medial amygdala (MeA), ventromedial nucleus of the hypothalamus (VMN), habenula (HAB), anterior cortical amygdalar nucleus (ACo), central amygdala (CeA), anterior hypothalamic nucleus (AH), and paraventricular nucleus of the hypothalamus (PVN). Several subregions of the MPOA, BNSTM, MeA, VMN, PvPOA, and HAB were imaged. Within the MPOA the rostral and caudal areas were imaged (rMPOA and cMPOA, respectively), and within the BNSTM, the anterior and posteromedial subnuclei were imaged (BNSTMa and BNSTMpm, respective-

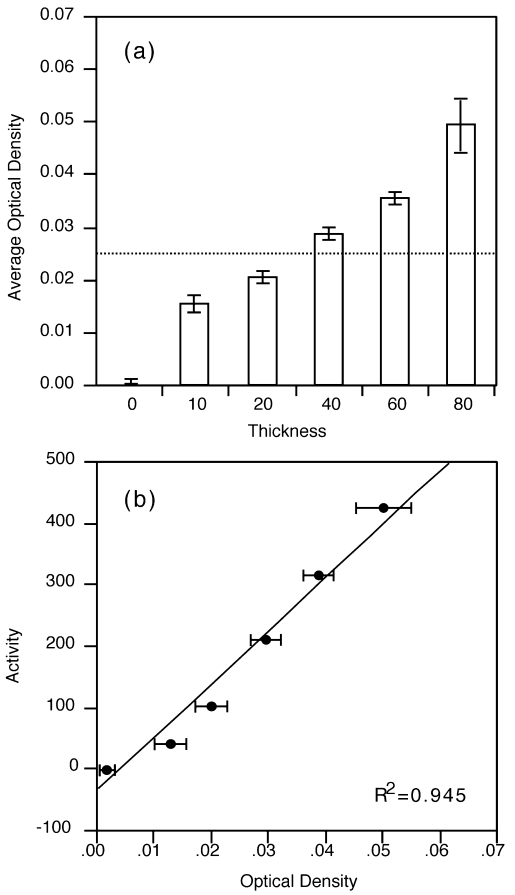

Fig. 2. (a) Mean+S.E.M. optical density of standards (10, 20, 40, 60, 80 μm) included in each batch. The value of the 0 μm section was obtained by imaging a blank area of the slide in which the standards were mounted. Optical density readings, on average, had standard errors <7% of the means. (b) Average regression line plotting the optical densities of the standards against their known activities as determined by spectrophotometry. The average R^2 value across the four batches is also given.

ly). Within the MeA, the anterodorsal, posterodorsal and posteroventral subnuclei were imaged (MeAD, MePD, and MePV, respectively). Within the VMN, the dorsomedial and ventrolateral divisions were imaged (VMNdm and VMNvl, respectively), and within the PvPOA, the anteroventral PvPOA (AVPV) and periventricular hypothalamic nucleus (Pe) were examined. Within the HAB, the medial and lateral nuclei were imaged (MHAB and LHAB, respectively).

2.4. Statistical analyses

For experiment 1, in areas in which multiple subregions were imaged (MPOA, BSNTM, MeA, VMN, PvPOA, and HAB) metabolic capacity was analyzed using a repeated-measures multivariate analysis of variance (MANOVA) with Experience (NAÏVE, 3F, or 16F) as the independent variable. In experiment 2, MANOVAs were also used but Group (COPULATOR vs. NONCOPULATOR) was the independent variable. Metabolic capacity of the subregions of the nuclei (e.g., rMPOA and cMPOA) were the dependent variables (Subregion). If the main effect of group was significant, multivariate contrasts were conducted. In the MeA, when the effect of Subregion was significant, we ran separate MANOVAs that included only two subregions: (MeAD–MePD); (MeAD–MePV); and (MePD–MePV). If the interaction between Group and the within-subject variable was significant, separate univariate analyses of variance (ANOVAs) were conducted for each subregion, and Tukey's HSD test was used for post hoc contrasts. We selected Pillai's trace as our multivariate test statistic because it is robust to deviations from multivariate normality and homogeneity in variance–covariance matrices [49]. For all the analyses, we set $\alpha=0.05$.

For the rest of the brain regions, one-way ANOVAs were calculated with Experience (experiment 1) or Group (experiment 2) as the independent variable ($\alpha=0.05$), and post hoc contrasts were calculated using Tukey's HSD.

In addition to statistical analyses on brain metabolism, behavioral differences between 3F and 16F males on the test days were assessed using a repeated-measures MANOVA. In this model, Group (3F and 16F) was the independent variable, and behavioral scores across the three tests were the dependent variables (Test).

Finally, in experiment 1, behavioral scores were correlated with metabolic capacity in all brain areas within both the 3F and 16F groups using Pearson's product-moment correlations. Correlations on the data set including both 3F and 16F groups were not performed because significant correlations found in this analysis would likely be due to group differences in both behavior and brain metabolism. In order to minimize the number correlations performed in each group, only one behavioral parameter—average latency to ejaculate (across the three tests in test chamber)—was correlated with brain metabolism. Because of the large number of correlations analyzed, we set $\alpha=0.01$ to reduce Type I error. Brain-behavior correlations were also computed for COPULATORS in experiment 2.

All statistics were done using Version 3.2 of JMP [59] for the Apple Macintosh.

3. Results

3.1. Experiment 1: Effects of repeated exposure to females on brain metabolism and behavior

3.1.1. Brain metabolism

Generally speaking, metabolic capacity in key limbic brain areas was not significantly different between 3F males and NAÏVE males, but 16F males had significantly elevated CO activity in many brain regions. The results are summarized in Table 1.

In the MPOA (rMPOA and cMPOA), there was a significant effect of Experience [$F(2,25)=13.6$, $P=0.001$]

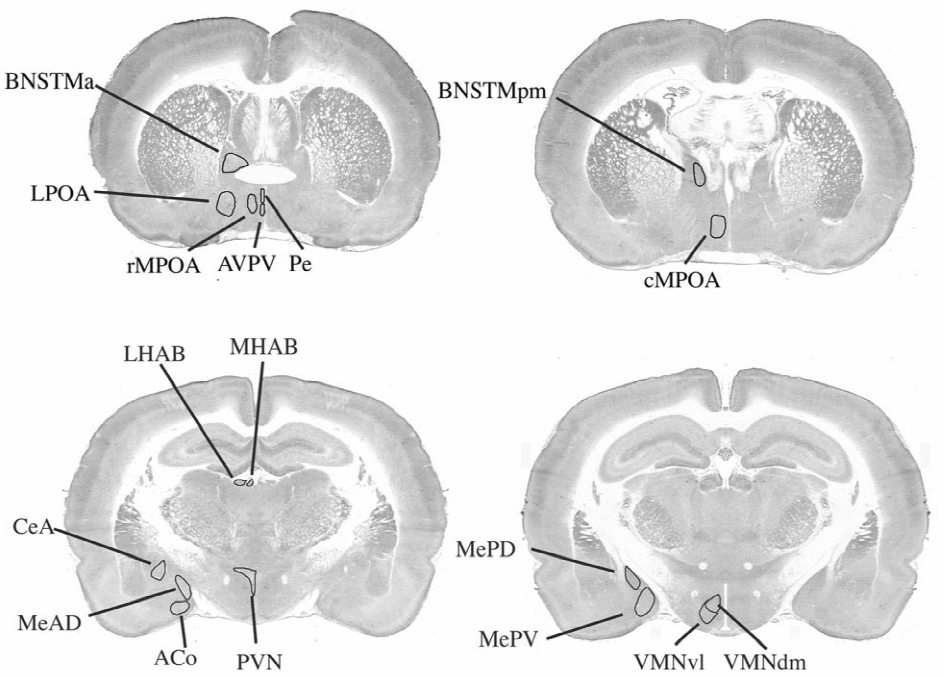

Fig. 3. Regions of interest on brains stained for cytochrome oxidase activity. Darker areas represent brain regions with elevated metabolic capacity.

and significant Experience×Subregion interaction [$F(2,25)=4.4$, $P=0.023$] (Table 1). Overall, 16F males had elevated CO activity in the MPOA relative to NAÏVE and 3F males ($P<0.001$ for both contrasts), whereas no difference existed between NAÏVE and 3F males. In the cMPOA, there was a significant effect of Experience [$F(2,25)=21.4$, $P<0.001$], and post hoc contrasts revealed that 16F males had elevated CO activity relative to both NAÏVE and 3F males. In the rMPOA there was only a trend for CO activity to increase with experience. There was no significant difference between 3F and NAÏVE males in either the rMPOA or cMPOA.

In the BNSTM (BNSTMa and BNSTMpm), there was a significant effect of Experience [$F(2,25)=15.8$, $P<0.001$]

Table 1
Least squared means for cytochrome oxidase activity (μmol/min/g tissue wet weight) in limbic brain regions in socially naïve males (NAÏVE, $n=9$), males with three opportunities to copulate (3F, $n=9$), and males with 16 opportunities to copulate (16F, $n=10$)

Brain region	NAÏVE	3F	16F	Contrast
Medial preoptic area—rostral (rMPOA)	218±9	230±9	244±8	ns
Medial preoptic area—caudal (cMPOA)	214±7	218±7	270±7	16F>3F, NAÏVE
Medial division of the bed nucleus of the stria terminalis—anterior (BNSTMa)	204±8	215±8	239±8	16F>NAÏVE
Medial division of the bed nucleus of the stria terminalis—posteromedial (BNSTMpm)	219±9	218±9	282±8	16F>3F, NAÏVE
Medial amygdala—anterodorsal (MeAD)	206±14	177±14	243±13	16F>3F, NAÏVE
Medial amygdala—posterodorsal (MePD)	221±10	196±10	267±9	16F>3F, NAÏVE
Medial amygdala—posteroventral (MePV)	213±9	192±9	257±9	16F>3F, NAÏVE
Ventromedial nucleus of the hypothalamus—dorsomedial (VMNdm)	247±14	209±14	276±13	16F>3F
Ventromedial nucleus of the hypothalamus—ventrolateral (VMNvl)	219±13	198±13	244±13	16F>3F
Medial habenula (MHAB)	243±14	222±14	280±14	16F>3F, NAÏVE
Lateral habenula (LHAB)	258±12	242±12	305±12	16F>3F, NAÏVE
Periventricular hypothalamic nucleus (Pe)	202±11	212±11	251±12	16F>NAÏVE
Anteroventral periventricular preoptic area (AVPV)	211±9	229±9	240±10	ns
Central nucleus of the amygdala (CeA)	234±13	188±13	262±13	16F>3F
Paraventricular nucleus of the hypothalamus (PVN)	180±9	156±9	190±9	16F>3F
Anterior cortical nucleus of the amygdala (ACo)	199±13	204±13	202±13	ns
Lateral preoptic area (LPOA)	211±7	227±7	233±6	ns
Anterior hypothalamic area (AH)	252±12	244±12	276±11	ns

Note: values are means±S.E.M.s.

(Table 1) and of Subregion [$F(1,25)=12.9$, $P=0.001$]. Overall, 16F males had elevated CO activity relative to NAÏVE and 3F males ($P<0.001$), whereas no significant difference existed between NAÏVE and 3F males. Activity in the BNSTMa was significantly lower than in the BNSTMpm. There was also a significant interaction between Experience and Subregion [$F(2,25)=4.9$, $P=0.017$], and this was due, in part, to the fact that heterosexual social experience had a greater effect in the BNSTMpm than in the BNSTMa. In both the BNSTMa [$F(2,25)=4.8$, $P=0.017$] and the BNSTMpm [$F(2,25)=18.3$, $P=0.001$] there was a significant effect of Experience. In the BNSTMpm 16F males had significantly elevated CO activity relative to both 3F and NAÏVE males, and in the BNSTMa 16F males had elevated metabolic capacity relative only to NAÏVE males.

In the MeA (MeAD, MePD, and MePV), there was a significant effect of Experience [$F(2,25)=12.0$, $P<0.001$] and Subregion [$F(2,24)=7.4$, $P=0.003$] (Table 1). Overall, 16F males had elevated CO activity relative to 3F and NAÏVE males ($P<0.006$ for both contrasts), whereas no significant difference existed between NAÏVE and 3F males. Activity in the MePD was greater than in the MeAD [$F(1,25)=11.1$, $P=0.003$] and MePV [$F(1,25)=9.5$, $P=0.005$].

In the VMN (VMNdm and VMNvl), there was a significant effect of Experience [$F(2,25)=4.5$, $P=0.021$] (Table 1). Overall, 16F males had greater CO activity in the VMN relative only to 3F ($P<0.001$).

In the PvPOA (AVPV and Pe), there was a significant Experience×Subregion interaction [$F(2,22)=4.7$, $P=0.020$] (Table 1). There was a significant effect of Experience in the Pe [$F(2,23)=5.2$, $P=0.014$] but not in the AVPV, and post hoc contrasts revealed that in the Pe 16F males had increased CO activity relative to NAÏVE males.

In the HAB (MHAB and LHAB), there was a significant effect of Experience, [$F(2,24)=6.4$, $P=0.006$] and Subregion [$F(1,24)=22.4$, $P<0.001$] (Table 1). Overall, 16F males had elevated CO activity relative to NAÏVE ($P=0.002$) and 3F males ($P=0.023$). Metabolic capacity in the LHAB was significantly greater than in the MHAB.

Experience also affected CO activity in the PVN [$F(2,24)=3.5$, $P=0.047$] and CeA [$F(2,24)=8.1$, $P=0.002$] (Table 1), and post hoc contrasts revealed that, in both the PVN and CeA, 16F males had elevated metabolic capacity relative to 3F males. Experience did not affect metabolic capacity in the LPOA, ACo, and AH.

3.1.2. Copulatory behavior

Copulatory behavior in the test arena also differed between 3F and 16F males (Table 2). Though mount latencies did not differ significantly across groups, latencies to ejaculate did differ [$F(1,17)=7.6$, $P=0.013$]. 16F males had elevated latencies to ejaculate relative to 3F males. The interval from first mount to ejaculation also differed across groups [$F(1,17)=6.6$, $P=0.020$], and again 16F males had elevated scores. Finally, ejaculatory frequencies differed across groups [$F(1,17)=15.2$, $P<0.001$], and 16F males had lower ejaculatory frequencies than 3F males. For none of the parameters were the effects of Test number or Group×Test interaction significant.

The differences between the groups were not due to the fact that 16F males were sexually inactive. In the observations on days 15 and 16, it was noted that all 16F males ejaculated at least once, and the number of ejaculations ranged from 1 to 5.

3.1.3. Correlations

Within both the 3F and 16F groups, average latency to ejaculate was correlated against CO activity in all brain areas examined ($\alpha=0.01$ because of large number of correlations). Average latency to ejaculate was not significantly correlated with neural metabolic capacity in 3F males. However, among 16F males, metabolic capacity in the MePD ($r=0.84$), MePV ($r=0.82$), and CeA ($r=0.90$) was significantly correlated with average latency to ejaculate (Table 3).

3.2. Experiment 2: The effect of baseline differences in copulatory behavior on brain metabolism

Generally speaking, metabolic capacity in key limbic brain areas was not significantly different between COPULATORS and NONCOPULATORS (Table 4).

In the MPOA (rMPOA and cMPOA), there was no significant effect of Group or Subregion.

In the BNSTM (BNSTMa and BNSTMpm), there was a significant effect of Subregion [$F(1,14)=7.9$, $P=0.014$] but not of Group; activity in the BNSTMpm was significantly greater than in the BNSTMa.

In the MeA (MeAD, MePD, and MePV), there was a significant effect of Subregion [$F(2,14)=13.5$, $P<0.001$] but not of Group. Activity in the MePD [$F(1,15)=26.7$, $P<0.001$] and MePV [$F(1,15)=14.7$, $P=0.002$] was significantly greater than in the MeAD.

Table 2
Average mount latency and latency to ejaculate of 3F and 16F males on each of the observations in the test arena

Group	*n*	Average mount latency (s)			Average latency to ejaculate (s)		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
3F	9	135±41	83±36	46±6	906±77	698±126*	787±149
16F	10	82±21	286±126	137±84	1087±104	1210±123	1078±119

Note: values are means±S.E.M.s.
*$P<0.05$, unpaired Studentized *t*-test.

Table 3
Correlation between average latency to ejaculate and metabolic capacity in limbic brain areas of 3F (experiment 1), 16F (experiment 1), and COPULATORS (experiment 2)

Brain region	3F		16F		COPULATORS	
	r	*n*	*r*	*n*	*r*	*n*
Medial preoptic area—rostral (rMPOA)	−0.12	9	0.31	10	−0.28	8
Medial preoptic area—caudal (cMPOA)	0.07	9	−0.06	10	−0.05	9
Medial division of the bed nucleus of the stria terminalis—anterior (BNSTMa)	−0.18	9	0.28	10	−0.25	8
Medial division of the bed nucleus of the stria terminalis—posteromedial (BNSTMpm)	0.52	9	0.01	10	0.03	9
Medial amygdala—anterodorsal (MeAD)	0.00	9	0.74	10	−0.21	9
Medial amygdala—posterodorsal (MePD)	0.32	9	*0.84	10	0.05	9
Medial amygdala—posteroventral (MePV)	0.45	9	*0.82	10	−0.32	9
Ventromedial nucleus of the hypothalamus—dorsomedial (VMNdm)	0.06	9	0.71	10	0.07	9
Ventromedial nucleus of the hypothalamus—ventrolateral (VMNvl)	−0.07	9	0.71	10	0.39	9
Medial habenula (MHAB)	0.34	9	−0.01	9	0.30	9
Lateral habenula (LHAB)	0.33	9	0.05	9	0.32	9
Periventricular hypothalamic nucleus (Pe)	−0.67	9	0.73	8	−0.16	9
Anteroventral periventricular preoptic area (AVPV)	−0.71	9	0.56	7	−0.31	9
Central nucleus of the amygdala (CeA)	0.23	9	*0.90	10	0.09	9
Paraventricular nucleus of the hypothalamus (PVN)	0.71	9	0.74	9	−0.09	9
Anterior cortical nucleus of the amygdala (ACo)	−0.76	9	0.56	9	−0.04	9
Lateral preoptic area (LPOA)	−0.02	9	0.04	10	−0.40	8
Anterior hypothalamic area (AH)	0.01	9	0.42	10	0.49	9

*$P<0.01$.

In the VMN (VMNdm and VMNvl), there was a significant effect of Subregion [$F(1,15)=44.6$, $P<0.001$] but not of Group. Metabolic capacity in the VMNdm was significantly greater than that in the VMNvl.

In the PvPOA (AVPV and Pe), there was a significant effect of Subregion [$F(1,15)=5.7$, $P=0.031$] but not of Group. Metabolic capacity in the AVPV was greater than that in the Pe.

Table 4
Least squared means for cytochrome oxidase activity (μmol/min/g tissue wet weight) in limbic brain regions of NONCOPULATORS ($n=8$) and COPULATORS ($n=9$)

Brain region	NONCOPULATORS	COPULATORS
Medial preoptic area—rostral (rMPOA)	236±9	243±9
Medial preoptic area—caudal (cMPOA)	243±14	235±12
Medial division of the bed nucleus of the stria terminalis—anterior (BNSTMa)	234±12	248±12
Medial division of the bed nucleus of the stria terminalis—posteromedial (BNSTMpm)	260±14	268±14
Medial amygdala—anterodorsal (MeAD)	188±7	175±7
Medial amygdala—posterodorsal (MePD)	229±12	219±11
Medial amygdala—posteroventral (MePV)	221±13	208±13
Ventromedial nucleus of the hypothalamus—dorsomedial (VMNdm)	224±7	210±7
Ventromedial nucleus of the hypothalamus—ventrolateral (VMNvl)	203±7	189±7
Medial habenula (MHAB)	202±10	208±9
Lateral habenula (LHAB)	217±10	222±9
Periventricular hypothalamic nucleus (Pe)	207±11	220±10
Anteroventral periventricular preoptic area (AVPV)	214±12	230±11
Central nucleus of the amygdala (CeA)	221±15	199±14
Paraventricular nucleus of the hypothalamus (PVN)	173±11	159±10
Anterior cortical nucleus of amygdala (ACo)	190±7	180±7
Lateral preoptic area (LPOA)	225±8	235±8
Anterior hypothalamic area (AH)	209±7	205±8

Note: values are mean±S.E.M.s.

In the HAB (MHAB and LHAB), there was a significant effect of Subregion [$F(1,15)=22.7$, $P<0.001$] but not of Group. Activity in the LHAB was significantly higher than in the MHAB.

There were also no differences between COPULATORS and NONCOPULATORS in metabolic capacity in the LPOA, AH, ACo, CeA, and PVN.

Altogether, this suggests there are no differences in neural metabolic phenotype between males the copulate and males that fail to copulate on their first test and, moreover, that the neural differences found in experiment 1 are not due to differences in copulatory behavior across groups.

3.2.1. Correlations

None of the correlations between average latency to ejaculate and brain metabolism was significant within COPULATORS (Table 3).

4. Discussion

While many studies have investigated changes in neural activity during copulatory behavior (reviewed in Refs. [47,53]), none have examined the long-term changes in metabolism (cytochrome oxidase activity) in the mammalian brain following heterosexual social experience. This investigation is important because heterosexual experience can induce long-term changes in behavior [47,61], and changes in neural metabolism could underlie these behavioral modifications. Because CO is a rate-limiting enzyme in ATP production [22,63,65], it serves as an important marker of changes in metabolic capacity. Here we show that extensive, but not limited, opportunity to copulate with females leads to elevations in CO activity in many limbic nuclei (experiment 1) and that this difference is not likely due to differences in sexual vigor across groups (experiment 2). We propose that these neural metabolic changes are related to repeated immediate early gene expression during sexual interactions and to hormonal stimulation and could underlie experience-dependent changes in behavior.

4.1. Experience-dependent changes in neural metabolism

We found that males with sixteen daily opportunities to copulate with females (16F males) but not males with three opportunities to copulate (3F males) had increased neural metabolism in limbic brain nuclei such as the MPOA, Pe, BNSTM, MeA, VMN, HAB, CeA, and PVN (Table 1). In the MPOA, Pe, BNSTM, MeA, and HAB, 16F males had elevated CO activity relative to naïve males and 3F males. In the VMN, CeA, and PVN, 16F males had elevated metabolic capacity relative only to 3F males. No significant changes in neural metabolism were found in areas such as the LPOA, ACo, AVPV and AH. With the exception of the BNSTM, metabolic capacity in all brain areas that showed experience-dependent changes was similar among males with no heterosexual experience (NAÏVE), one heterosexual experience (COPULATORS and NONCOPULATORS), and three heterosexual experiences (3F) and was elevated only in males with 16 heterosexual experiences (16F). We hypothesize that these neural differences are driven by increased sexual interactions with females and not by differences in copulatory behavior. That latter is suggested by the fact that males with equal and limited copulatory experience that differ in copulatory performance have identical neural metabolic phenotypes.

The MPOA is considered the central integrative brain area underlying the expression of male-typical copulatory behavior [47], and the MPOA showed one of the largest increases in metabolic capacity. Within the MPOA, the degree of change in CO activity was much larger in the caudal MPOA than in the rostral MPOA. Copulation induces greater increases in Fos expression in the caudal MPOA than rostral MPOA in quail [46], and in male hamsters, caudal MPOA lesions lead to greater decrements in sexual behavior than rostral MPOA lesions [55]. Altogether, this suggests that areas that are more critical in the expression of copulatory behavior are more metabolically plastic.

The MeA also showed experience-dependent increases in metabolic capacity. Interestingly, in both the MeA and MPOA, experience-dependent changes in Fos induction have also been noted. In rats, sexually experienced, but not naïve, males show elevations in Fos expression in the MPOA after exposure to female odors [2,7,8], and sexually experienced males show greater Fos expression in the MPOA after ejaculation relative to males ejaculating for the first time [42]. In hamsters, sexually experienced males express significantly more Fos in the MePD and MPOA after exposure to a clean, test chamber in which they have previously copulated relative to naïve males exposed to the same test arena [38]. In gerbils, Fos expression in the sexually dimorphic area of the MPOA (SDN–MPOA) is significantly increased when a male is placed in an area associated with copulation but not when placed in a familiar cage not associated with copulation [29]. Furthermore, lesions in the MeA or SDN–MPOA have differential effects in experienced vs. naïve male rats [1,15,28,39]. Taken together, these results suggest that amygdalar and preoptic areas might underlie experience-dependent changes in behavior and that changes in CO activity may be manifestations of these changes.

Though the VMN is traditionally thought of as an area regulating female sexual behavior [52], we noted that with increased interactions with females CO activity increased in the VMN. This is not surprising because copulation induces *c-fos* expression in the VMN of male rats [25], because the VMN is replete with androgen receptors [57], and because antagonism of androgen receptors in the VMN significantly inhibits copulatory behavior [45]. Likewise, lesions of the basal hypothalamus that include the VMN

inhibit the expression of courtship behaviors in male green anole lizards [19], red-sided garter snakes [20], and ring doves [4]. Interestingly, extensive heterosexual social experience also elevates CO activity in the VMN of male leopard geckos [13], suggesting that this could be an evolutionarily-conserved brain area of social plasticity.

We found that three opportunities to copulate with a female was not sufficient to induce metabolic changes in brain nuclei. On the other hand, substantial behavioral changes have been found to occur even after one copulation [33,41]. The lack of neural metabolic differences between NAÏVE and 3F males might be due to the fact that opportunities to copulate were separated by 5 days for 3F males; three daily opportunities might have exerted greater effects on CO activity. Alternatively, elevations in CO activity might have been found in 3F males if they, like 16F males, were killed the day following their last interaction with a female. It is also possible that rapid behavioral changes are mediated not by long-term metabolic mechanisms but by rapid changes in neurochemical systems such as dopamine [34] or serotonin (e.g., Ref. [58]).

4.2. Mechanisms of metabolic change

Cytochrome oxidase is a holoenzyme composed of 13 proteins; 10 are encoded in the nuclear genome and three, including the major catalytic subunits, are encoded in the mitochondrial genome [32,65]. Cytochrome oxidase activity is regulated, primarily, by the abundance of the holoenzyme in the mitochondria [30,31]. Therefore, changes in CO activity are likely to be due to changes in the expression of mitochondrial genes. Immediate early genes such as *c-fos* and sex steroid hormone receptors acting as transcription factors could affect the expression of the catalytic subunits [14,48,62]. Copulatory behavior consistently induces Fos expression in the MPOA, BNSTM, and MeA (reviewed in Ref. [53]), as well as short-term surges in sex steroid hormones such as testosterone [36,37]. Androgens affect Na^+/K^+-ATPase activity [27], the expression of the mitochondrial gene encoding for CO catalytic subunit I [11], and CO activity [12]. Therefore, the direct activation of neural circuits and/or androgenic stimulation accompanying sociosexual behavior might produce these metabolic changes.

Interestingly, there appears to be a relationship between the amount of change in brain metabolism, the pattern of Fos induction during copulation, and androgen receptor (AR) mRNA abundance. Areas that show Fos induction during copulation and high AR mRNA expression (e.g., MPOA, BNSTM, MeA, VMN, CeA) [25,53,60] tended to show increases in CO activity with extensive heterosexual experience. In contrast, areas that show neither Fos induction during copulation nor high AR mRNA expression (e.g., LPOA and AVPV) tended not to show increases in CO activity with experience. Experience-dependent changes were also found in the Pe, an area that expresses moderate AR mRNA but does not show FOS increases during copulation; this suggests that increased androgen stimulation during heterosexual interactions may be sufficient to increase metabolic capacity. The lack of metabolic changes in the AH argues against the sufficiency of Fos to induce metabolic changes. The fact that AR is expressed in a substantial proportion of neurons showing Fos increases during copulation [26] also suggests that the two might work together to induce CO changes.

4.3. Behavior and the relationship between neural metabolism and behavior

In the first experiment, we found several unexpected behavioral differences. Because previous studies documented that the latency to copulate decreased with increased heterosexual experience (e.g., Refs. [16,42]), we predicted that behavior would change over time in both 3F and 16F males and that 16F males, because of their increased experience, would show quicker copulatory behavior relative to 3F males. Neither of these predictions was supported. Rather, relative to 3F males, 16F males had longer latencies to ejaculate and lower ejaculation frequencies.

These findings could be due to strain and/or genotype differences in behavioral plasticity following heterosexual experience, as has been found in mice [43,44,54]. In this study we used male Sprague–Dawley rats, whereas other studies that have documented significant behavioral changes used Wistar or Long–Evans rats (e.g., Refs. [16,40–42]). Male Sprague–Dawley rats have been found to be less sexually active than Wistar rats [17], and this could explain the lack of significant behavioral changes across time. The significant behavioral difference between 3F and 16F males might also be due to exhaustion from repeated sexual activity, and one way to test this would be to test both groups of males after a period of rest. In the current study, 16F males were given daily opportunities to copulate with females, and this design was selected to maximize the probability of altering neural metabolism. In other studies in which experience-dependent facilitation of copulatory behavior was documented, tests with females are spaced at least 3 days apart to allow for recovery from the effects of sexual exercise (e.g., Refs. [3,16,40,42]). Therefore, daily experience with females could have altered the ejaculatory behavior of 16F males.

The relationship between copulatory behavior and neural metabolism differed among the groups of males in experiments 1 and 2 (i.e., 3F, 16F and COPULATORS). Whereas brain-behavior correlations were not statistically significant in 3F males or COPULATORS, there was a significant positive correlation between average latency to ejaculate and metabolic capacity in the MePD, MePV and CeA in 16F males (Table 3). In other words, males that took longer to ejaculate had elevated metabolic capacity in the amygdala. The posterodorsal medial amygdala (MePD) is selectively activated during ejaculation [9], and lesions

of this nucleus delay sexual satiety in male hamsters [50]. It is possible that males with longer latencies to ejaculate have higher ejaculatory thresholds and might require more intromissions before ejaculation. Increased intromissions might lead to greater brain activity, and this, in turn, could translate into elevated metabolic capacity, at least in the amygdala. That the correlation is significant only in 16F males suggests that the relationship is driven by repeated heterosexual experiences.

Interactions with females involve a number of different types of experiences. Therefore, it is possible that different aspects of interactions with females differentially contribute to long-term changes in brain metabolism and behavior. Though we propose that sexual interactions are paramount in producing these metabolic changes, we cannot separate the contributions of different social interactions; we know only that the total experience alters brain metabolism. It is possible that the exercise associated with repeated copulation and/or the repeated exposure to female odors were paramount in producing these neural changes. As this was a first attempt to manipulate neural metabolism, our main objective was to maximize the potential to induce brain changes. It would be interesting to assess the degree to which penile stimulation, ejaculation, and sensory stimulation from the female (olfactory, visual, and/or tactile) contribute to these neural metabolic changes.

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