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Astrocytes in the Medial Preoptic Area Modulate Ejaculation Latency in an Experience-Dependent Fashion

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Abstract

While sexually experienced males copulate at a higher frequency than sexually inexperienced males, there is still a great deal of variability in their behavior. Within the medial preoptic area (mPOA) of the hypothalamus, glutamate modulates some of this variability. Glutamate levels, for example, increase during sexual activity, peaking with ejaculation and falling precipitously during the post-ejaculation interval. Whereas lower glutamate levels after ejaculation translates to longer post-ejaculatory intervals, administration of glutamate uptake inhibitors into the mPOA increases the number of ejaculations a male rat achieves over a mating bout, and reduces the latency to ejaculate once mating begins. Because astrocytes modulate the availability of neuronal glutamate, we hypothesized that differences in the number of GFAP-positive astrocytes in the mPOA may account for variability in sexual behavior. To this end, we examined whether the number of astrocytes in the mPOA related to ejaculation latency as well as to the duration of the post-ejaculatory interval (PEI) in sexually experienced and sexually inexperienced males. Results indicate that the number of astrocytes negatively correlated with latency to reach ejaculations in sexually inexperienced but not sexually experienced rats while the number of astrocytes and PEI were not related. Astrocyte numbers did not vary between inexperienced and experienced subjects indicating that astrocyte processes may differentially project to sex-relevant glutamatergic synapses or that glutamatergic innervation of the mPOA changes as a function of sexual experience.

Keywords

astrocytes; male sexual behavior; medial preoptic area

The medial preoptic area (mPOA) is a primary neural loci involved in the regulation of male sexual behavior. Several brain regions that receive sex-relevant sensory information innervate the mPOA (Simerly & Swanson, 1986), while its efferents project to various premotor regions involved in sexual response (Simerly & Swanson, 1988). As evidence of this role, lesions of the mPOA impair sexual behavior, while electrical stimulation decreases latency to ejaculate and the length of the post-ejaculatory interval (PEI) (Hull, Wood, & McKenna, 2006).

In conjunction with several other neurotransmitters, glutamate acts in the mPOA to regulate male sexual behavior (Dominguez, 2009). Supporting this role for glutamate, administration of L-Glutamate and D, L-homocysteic acid, a glutamate agonist, increase intracavernous pressure (Giuliano et al., 1996) and urethrogenital reflex (Marson & McKenna, 1994), respectively. Furthermore, glutamate levels rise in the mPOA during copulation, peak with ejaculation, and fall precipitously during the PEI, while reverse dialysis of glutamate uptake inhibitors decreased ejaculation latency and the PEI (Dominguez, Gil, & Hull, 2006). Finally, the magnitude of the glutamate decrease that follows ejaculation correlates with the duration of the PEI (Dominguez et al., 2006). Hence changes in glutamatergic response in the mPOA may very well underlie some of the individual differences seen in male sexual behavior.

Glutamate neurotransmission in the central nervous system is regulated by astrocytes (Anderson & Swanson, 2000). Glucose is converted to glutamate via the tricarboxylic acid cycle (Hertz, Dringen, Schousboe, & Robinson, 1999). Since neurons, but not astrocytes, lack pyruvate carboxylase, a crucial enzyme required in the tricarboxylic acid cycle, neurons cannot independently produce glutamate. Astrocytes synthesize both glutamate and its metabolic precursor glutamine; the latter is transported to neurons where it can be converted to glutamate or GABA (Hertz et al., 1999). Furthermore, astrocytes play a vital role in the maintenance of extracellular glutamate, as the glutamate transporter 1 (GLT-1), expressed primarily on astrocytes, is necessary for the clearance of extracellular glutamate (Rothstein et al., 1996). Finally, EAAC1 the main neuronal glutamate transporter is not found synaptically, and knockout studies suggest it is not necessary for glutamate uptake (Holmseth et al., 2012).

When considering that glutamate in the mPOA is important for male sexual behavior, particularly ejaculation, and since astrocytes regulate production and uptake of glutamate, we hypothesized that astrocytes will impact sexual response in male rats. To this end, we examined whether the number of astrocytes in the mPOA varied as a function of latency to ejaculate and the length of the post-ejaculatory interval in sexually experienced and sexually inexperienced male rats.

Method

Subjects

Thirty male Long Evans hooded rats (Harlan, Indianapolis), weighing 300–400 g, were single housed in large plastic cages on a 14:10-hr light–dark cycle, lights off at 10 a.m., with access to food and water ad libitum. Seventeen ovariectomized female conspecifics,

weighting 200–250 g, were double housed under identical conditions. All procedures were in accordance with the National Institutes of Health Guidelines for the Use of Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Surgery

Female subjects were ovariectomized under ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (8 mg/kg) anesthesia. A single incision was made through the abdominal wall, the uterine horns were then ligated with absorbable suture and the ovaries manually ablated. The abdominal wall was tied with absorbable suture and the skin incision was closed with sterile surgical staples. Aseptic techniques were used throughout surgery.

Sexual Behavior

Male subjects were randomly divided into sexually experienced ($n = 15$) and sexually inexperienced ($n = 15$) groups. Subjects assigned to the sexually experienced group received six 90-min sexual experiences with a hormonally primed novel female in their home cage. Sexual experience occurred between 12 p.m. and 4 p.m. in a behavior room separate from the animal colony. A maximum of eight males were present in each sexual experience session, with two sessions occurring on a given day. All sexual experience sessions occurred in the dark with one red light present to enable video recording.

Females were brought into behavioral estrus by subcutaneous injections of 10 μ g of estradiol 48-hr before and 500 μ g of progesterone 4-hr before being exposed to a novel male conspecific. Receptivity was determined by observing lordosis behavior upon presentation of a nonsubject male. Females who failed to display lordosis in response to a series of attempted mounts were removed from behavioral testing for that day.

During the sixth experience session male subjects were scored for sexual proficiency; operationalized as achieving two ejaculations and initiating a third mating bout in 90-min. Four males failed to meet criterion and were given a seventh sexual experience session, during which three of the four males reached the criteria for sexual proficiency.

Four to 7 days after the last sexual experience session all males underwent a sexual behavior test and were then sacrificed for immunohistochemistry. Subjects were randomly tested at a rate of eight to 12 subjects per day. During the test session the following behaviors were scored: mount, intromissions, latency to ejaculate from first mount, and the length of the PEI. Test sessions ended after the PEI, and subjects were then killed 1-hr after they ejaculated. Subjects who did not ejaculate within 1-hr were excluded from the study.

Histology

All subjects received a lethal dose of Euthasol (130 mg/kg) and transcardially perfused with 100 mL of PBS and 500 mL of 4% paraformaldehyde. Brains were removed and stored in 4% paraformaldehyde for 1 hr before being transferred to 30% sucrose and stored at 4 °C overnight; after 24-hr brains were transferred to another 30% sucrose solution and stored at 4 °C until sectioning. Brains were sliced at 35 μ m and stored in cryoprotectant (30%

ethylene glycol, 30% sucrose, and 0.0002% sodium azide in 0.1 M phosphate buffer [PB]) at -20°C .

Immunohistochemistry was then performed to visualize GFAP-positive astrocytes in the mPOA. Brain slices containing the mPOA were washed in 0.1M PB and incubated in a monoclonal mouse anti-GFAP antibody (1:4000, Santa Cruz Biotechnology, sc-71142) overnight. Tissues were further incubated in an anti-mouse biotinylated antibody (1:500, Vector) for 1-hr and enhanced with avidin/biotin (1:1000, ABC kit, Vector). GFAP immunoreactivity was visualized by incubating tissues in a 3,3'-Diaminobenzidine (DAB) with nickel sulfate intensification for 10 min. Between all incubations tissue was washed in 0.1 M PB 4×5 min. Slices were then mounted, dehydrated, and cover slipped in preparation for bright field microscopy.

Analysis

Bilateral bright field photomicrographs ($340 \times 430 \mu\text{m}$) were taken at 1,000x magnification of the rostral, central, and caudal mPOA. Photomicrographs of the central and caudal mPOA were taken in the medial preoptic nucleus (MPN), a central region of the mPOA (Swanson, 2004). Levels of the mPOA in each slice were identified in accordance with the Swanson rat brain atlas (Swanson, 2004). Sections containing the mPOA ranged from brain level 18–23 (Swanson, 2004). Levels 18–19 were considered the rostral mPOA, levels 20–21 the central mPOA, and levels 22–23 the caudal mPOA (see Figure 1). Two rostral, central, and caudal slices were photographed for each subject. Due to a slicing error, one subject had viable tissue only from the rostral mPOA; this subject was excluded from analysis.

The ventrolateral preoptic area (VLPO) served as a control region due to its proximity to the mPOA. One bilateral photomicrograph, between levels 19 and 20 (Swanson, 2004), was taken at 1,000x magnification.

A group-blind counter using the ImageJ (NIH) cell counter plugin, quantified astrocytes. The number of astrocytes was averaged across the entire mPOA as well as within the rostral, medial, and caudal subdivisions of the mPOA. Astrocytes were operationalized as three or more GFAP positive processes converging on GFAP positive cell body. It is important to point out that not all astrocytes express GFAP (Eng, Ghirnikar, & Lee, 2000); therefore, while we are confident that this approach helps us analyze the number of astrocytes in our subject, this method may have, nevertheless, underestimated the total number of astrocytes present in tissue.

Student's *t* tests were used to assess group behavioral differences as well as to compare astrocyte number between groups in the VLPO. A two-way analysis of variance (ANOVA) examined astrocyte number in the mPOA as a function anatomical subregion and sexual experience. Finally, Pearson's correlations were performed comparing the number of astrocytes with the latency to ejaculate as well as the duration of the PEI. A two-tailed significance test was used to assess significance of the number of astrocytes versus PEI; whereas a one-tailed significance test was used to assess the significance of the number of astrocytes versus ejaculation latency since pilot data suggested a negative correlation existed

between these two variables. All statistics were conducted using GraphPad Prism software (version 5.0a for Mac OS X).

Results

One of the animals in the experienced group did not meet the criteria for sexual experience on test day, while tissue from another animal was damaged during slicing, yielding a total of 13 of 15 for the experienced group. Four of the animals in the inexperienced group did not mate on testing day, and they were excluded, yielding 11 of 15 for the inexperienced group. Compared to sexually experienced males ($n = 13$), inexperienced subjects ($n = 11$) had larger ejaculation latencies, $t(22) = 4.292$, $p < .05$. Additionally, inexperienced subjects took significantly longer than experienced subjects to mount, $t(22) = 2.807$, $p < .05$ and intromit, $t(22) = 3.638$, $p < .05$. However, there were no group differences in the number of mounts, $t(22) = 0.457$, $p = .6523$ or intromissions, $t(22) = 0.357$, $p = .7246$. The duration of the PEI did not differ between groups, $t(22) = 0.436$, $p = .6673$.

A two-way ANOVA revealed that the number of astrocytes in the mPOA did not vary as a function of sexual experience, $F(1, 20) = 1.605$, $p = .2854$ or anatomical subregion, $F(1, 20) = 0.0981$, $p = .9067$ of the mPOA. There was no experience-by-region interaction, $F(1, 20) = .4250$, $p = .4250$. Additionally, sexual experience did not affect astrocyte number in the VLPO, $t(22) = 0.116$, $p = .9088$. Table 1 includes means and standard errors of the mean for copulatory behaviors of inexperienced and experienced subjects, in addition to cells counts.

Analysis with a Pearson's correlation revealed a significant negative correlation between the number of astrocytes in the mPOA and ejaculation latency in sexually inexperienced subjects, $r = -0.539$, $p < .05$ (Figure 2 and Figure 3A). This effect was not present in sexually experienced subjects, $r = -0.139$, $p = .3245$. Additionally, the number of astrocytes in the mPOA did not vary with the length of the PEI in experienced, $r = .089$, $p = .7716$ or inexperienced, $r = -0.399$, $p = .2237$ subjects. There was no correlation between astrocyte density and ejaculation latency or PEI in the VLPO regardless of sexual experience.

The number of astrocytes in the rostral, central, and caudal mPOA was compared with the ejaculatory latency to determine whether specific subregions of the mPOA were differentially involved in the relationship between astrocytes and latency. Pearson's correlations revealed there was a significant correlation between numbers of astrocytes in the central, $r = -0.532$, $p < .05$ (Figure 3C) and caudal, $r = -0.7378$, $p < .05$ (Figure 3D), but not the rostral, $r = -0.391$, $p = .2349$ (Figure 3B) mPOA.

Discussion

Here we demonstrated that the number of GFAP-positive astrocytes in the mPOA correlates with latency to ejaculate in sexually inexperienced males. Within the mPOA, this effect was observed in the central and caudal regions. This is in agreement with previous work, which demonstrates that the mPOA has functionally distinct subdivisions; the caudal region being important for primarily the regulation of consummatory sexual behavior in males (Balthazart & Ball, 2007). However, in sexually experienced subjects there was no relationship between the number of astrocytes and the latency to ejaculate. Additionally, there was no relationship

between astrocyte number and the length of the PEI regardless of sexual experience. The difference between sexually experienced and inexperienced animals is not completely unexpected, as the mPOA's role in the regulation of male sexual behavior has been shown to vary as a function of sexual experience. Namely, expression of the immediate early gene protein Fos is higher in sexually experienced males compared to sexually naïve males (Lumley & Hull, 1999). Additionally, lesions of the mPOA inhibit sexual behavior in sexually inexperienced rats, (De Jonge et al., 1989) whereas mPOA-specific lesions do not eliminate sexual behavior in sexually experienced rats (Arendash & Gorski, 1983).

Since the number of astrocytes did not differ between sexually inexperienced and experienced subjects, it is possible that astrocyte–neuron interactions in the mPOA changes as a function of experience. In fact, ensheathment of neurons by astrocytes varies as a function of multiple stimuli in other hypothalamic regions. Specifically, astrocytes differentially ensheath neurons in the suprachiasmatic nucleus as a function of light–dark cycles while astrocytes process in the rat arcuate nucleus and monkey preoptic area are modulated by the preestrus estrogen surge (Theodosios, Poulain, & Oliet, 2008). Additionally, astrocyte ensheathment of oxytocin neurons in the supraoptic nucleus of the hypothalamus has also been shown to differ in lactating and virgin female rats (Theodosios et al., 2008). Because the glutamate transporter GLT-1, found predominantly on astrocytes, is mostly expressed next to glutamatergic synapses (Moussawi, Riegel, Nair, & Kalivas, 2011) it is possible that, with sexual experience, astrocyte processes may project to glutamatergic synapses and more efficiently clear glutamate in response to sex related stimuli. It is also possible that GLT-1 is more readily expressed on processes apposed to glutamatergic synapses with increased sexual activity, since neural activity increases the expression of GLT-1 as well as the proximity between GLT-1 containing processes and synapses (Benediktsson et al., 2012). If so, animals with more astrocytes would better respond to sex-related stimuli, as more astrocytes would facilitate glutamate uptake by chance alone and thus prevent excess glutamate from acting on presynaptic metabotropic glutamate receptors (Kalivas, 2009).

It is also possible that subjects with more astrocytes have a greater number of glutamate transporters initially, but that astrocyte transporter levels equilibrate as a function of sexual experience. Glutamate exposure increases the expression of the glutamate aspartate transporter (GLAST) as well as increasing the rate of glutamate reuptake (Duan, Anderson, Stein, & Swanson, 1999). Enhancing neural activity increases the expression of GLT-1 near synapses while blocking neural activity decreases the expression of GLT-1 on astrocytes (Benediktsson et al., 2012). Additionally, astrocytes cultured alone only develop GLASTs whereas astrocytes cultured with neurons develop both GLAST and GLT-1 subtype transporters, suggesting neural activity modulates astrocyte glutamate response (Swanson et al., 1997).

Another possible explanation for the change observed in inexperienced but not experienced subjects is that secretion of astrocyte derived gliotransmitters is modulated by experience itself. Astrocytes release glutamate, ATP, D-Serine, and various cytokines, such as tumor necrosis factor alpha, which affect presynaptic and post-synaptic receptors as well as other astrocytes (Volterra & Meldolesi, 2005). In this event, astrocyte-derived glutamate may

initially play a large role in the sex related glutamatergic levels in the mPOA. However, with subsequent sexual experience glutamatergic afferents from other sex-related brain nuclei such as the medial amygdala (Hull & Dominguez, 2006) come to play a larger role in sex-related release of glutamate in the mPOA.

In conclusion, we observed a negative correlation between ejaculation latency and number of preoptic astrocytes in sexually inexperienced but not experienced animals. Since the number of astrocytes did not differ between the groups, it is possible that sexual experience influences astrocyte processes projecting to glutamatergic synapses in the mPOA. Additionally, astrocyte-derived glutamate might initially play a role in the modulation of sexual behavior, but with continued experience glutamatergic projections from other brain areas like the BnST and medial amygdala become dominant modulators.

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Figure 1.

(Top) Coronal plate (Swanson, 2004) delineating the rostral section of the mPOA (boxed) where cells were counted (middle). Coronal plate delineating a central section of the mPOA showing the area where cells were counted with a solid-black box for the mPOA or a dashed box for the VLPO (bottom). Coronal plate delineating a caudal section mPOA (boxed) where cells were counted. From *Brain maps: Structure of the Rat Brain* (3rd ed., pp. 51, 55, and 59), by L. W. Swanson, 2004 San Diego, CA. Copyright Academic Press. Adapted with permission.

A. SHORTER LATENCY TO EJACULATE **B. LONGER LATENCY TO EJACULATE** **C. VENTROLATERAL PREOPTIC AREA** **Figure 2.**

(A) A micrograph from a subject with higher number of GFAP-positive astrocytes and shorter ejaculation latency. (B) A micro-graph from a subject with lower number of GFAP-positive astrocytes and longer ejaculation latency. (C) A micrograph of GFAP-positive astrocytes in the VLPO. Photomicrographs were obtained from the central region of the mPOA; scale bar is 50 μ m.

Figure 3.

Graphs depicting the relationship between the number of astrocytes and ejaculation latency in male rats. (A) There is a negative correlation between the number of astrocytes in the mPOA and latency to ejaculate in sexually inexperienced males. This relationship is not present in (B) the rostral mPOA, but it is in the (C) central and (D) caudal regions.

Table 1**Copulatory Behavior**

Behavior	Age	Mean $\pm$ SEM	t-value	p-value
Mount latency	<i>Inexp.</i>	675.5 $\pm$ 258.4	2.807	< .05
	<i>Experienced</i>	10.89 $\pm$ 4.80		
Intromission latency	<i>Inexp.</i>	1003 $\pm$ 294.4	3.638	< .01
	<i>Experienced</i>	21.74 $\pm$ 5.39		
Ejaculation latency	<i>Inexp.</i>	1092 $\pm$ 191.3	4.292	< .01
	<i>Experienced</i>	306.1 $\pm$ 48.49		
PHI	<i>Inexp.</i>	517.5 $\pm$ 55.62	0.436	ns
	<i>Experienced</i>	489.9 $\pm$ 37.80		
Total mounts	<i>Inexp.</i>	6.273 $\pm$ 1.13	0.457	ns
	<i>Experienced</i>	5.462 $\pm$ 1.32		
Total intromissions	<i>Inexp.</i>	7.545 $\pm$ 1.19	0.357	ns
	<i>Experienced</i>	8.00 $\pm$ 0.60		
GFAP + Cells (mPOA)	<i>Inexp.</i>	37.58 $\pm$ 3.87	0.573	ns
	<i>Experienced</i>	40.00 $\pm$ 2.10		
GFAP + Cells (VLPO)	<i>Inexp.</i>	30.23 $\pm$ 2.01	0.116	ns
	<i>Experienced</i>	30.59 $\pm$ 2.40		

Note. Table summarizes the means and standard errors of the mean associated with copulatory behaviors and GFAP-positive astrocytes for experienced and inexperienced (*inexp.*) animals. Means and SEMs for latencies are reported in seconds; ns = not significant.