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Serotonergic lesions of the periaqueductal gray, a primary source of serotonin to the nucleus paragigantocellularis, facilitate sexual behavior in male rats

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Running Head: Serotonergic vlPAG lesions and sexual behavior

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Abstract

While selective serotonin reuptake inhibitors (SSRIs) are widely used to treat anxiety and depression, they also produce profound disruptions of sexual function including delayed orgasm/ejaculation. The nucleus paragigantocellularis (nPGi), a primary source of inhibition of ejaculation in male rats, contains receptors for serotonin (5-HT). The ventrolateral periaqueductal gray (vlPAG) provides serotonin to this region, thus providing an anatomical and neurochemical basis for serotonergic regulation of the nPGi. We hypothesize that 5-HT acting at the nPGi could underlie the SSRI-induced inhibition of ejaculation in rodents. To this end, we produced 5-HT lesions of the source of 5-HT to the nPGi (the vlPAG) and examined sexual behavior. Removing the source of 5-HT to the nPGi facilitated genital reflexes, but not other aspects of sexual behavior, consistent with our hypothesis. Namely, 5-HT lesions produced a significant increase in the mean number of ejaculations and a significant decrease in ejaculation latency as compared to sham lesioned animals, while latency to mating and the post-ejaculatory interval did not differ. These data suggest that the serotonergic vlPAG-nPGi pathway is an important regulatory mechanism for the inhibition of ejaculation in rats, and supports the hypothesis that this circuit contributes to SSRI-induced inhibition of ejaculation.

Keywords: genital reflexes, ejaculation, copulation, periaqueductal gray, SSRI

1 Introduction

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed class of drugs for individuals with the clinical diagnosis of depression (Arroll et al., 2009; Koenig and Thase, 2009). SSRIs increase synaptic availability of serotonin (5-HT) by blocking reuptake at axon terminals (Hiemke and Hartter, 2000; Richelson, 1994). A well-documented side effect of SSRI treatment is sexual dysfunction (Kennedy and Rizvi, 2009; Schweitzer et al., 2009), with symptoms of decreased libido, premature ejaculation, delayed ejaculation, and anorgasmia most commonly reported.

The interaction between 5-HT and the neural circuitry underlying mammalian sexual behavior is not completely understood, although experiments in the male rat have suggested that serotonin can either inhibit or facilitate sexual behavior, depending on the brain site in question and/or the type of receptor targeted (Bitran and Hull, 1987; de Jong et al., 2006). For example, systemic administration of 5-HT or 5-hydroxytryptophan (a precursor to 5-HT synthesis) to male rats increases latency to ejaculation (Ahlenius and Larsson, 1991; Gonzales et al., 1982), whereas the 5-HT synthesis inhibitor p-chlorophenylalanine decreases ejaculation latency (McIntosh and Barfield, 1984). In addition, general increases in 5-HT levels through chronic systemic administration of an SSRI produce delayed ejaculation in male rats (Ahlenius et al., 1980; de Jong et al., 2005; Mos et al., 1999; Vega Matuszcyk et al., 1998) much like in humans (Hsu and Shen, 1995). In these studies, 5-HT was administered or manipulated systematically; therefore the specific brain region(s) mediating the effects of 5-HT on sexual behavior are not known.

The nucleus paragigantocellularis (nPGi) of the rostroventrolateral medulla in rats, and the homologous structure in humans, the nucleus paragigantocellularis lateralis (Zec and Kinney, 2001), is the hypothesized site of descending inhibition of genital reflexes in both rats (Marson and McKenna, 1990) and humans (Johnson, 2006). The nPGi receives projections from upstream sites related to sexual behavior, including the paraventricular nucleus of the hypothalamus, medial preoptic area, and periaqueductal gray (Murphy and Hoffman, 2001; Murphy et al., 1999; Normandin and Murphy, 2008), and in turn, projects to the spinal motoneurons (pudendal motoneurons) innervating the bulbospongiosus and ischiocavernosus muscles (Hermann et al., 2003; Marson and Carson 3rd, 1999; Marson and McKenna, 1996; Tang et al., 1999), which are critical for ejaculation in male rats (Miura et al., 2001; Pescatori et al., 1993) and humans (Hsu et al., 2004; Shafik et al., 2009).

The rat nPGi and the human homologue contain 5-HT immunoreactive cells (Azmitia and Gannon, 1986; Marson and McKenna, 1992) and application of 5-HT to the spinal targets of the nPGi in male rats blocks the urethrogenital reflex (Marson and McKenna, 1992). Similarly, Clément, et al. (2007) found that in male rats, systemic administration of the SSRI dapoxetine reduced fictive ejaculatory reflexes, which was reversed by lesions of the nPGi. In addition, lesions of the nPGi in male rats block the inhibitory effects of systemic fluoxetine (an SSRI) on sexual behavior (Yells et al., 1994). These studies make it clear that 5-HT acts at the level of the spinal cord to inhibit genital reflexes, and that 5-HT from the nPGi is a necessary antecedent for normal inhibition of genital reflexes. It is unclear, however, whether 5-HT may also be acting at the level of the nPGi to produce the observed effects.

The nPGi, contains receptors for 5-HT including the 5-HT_{1A} (Thor et al., 1992, 1990), 5-HT_{1C} (Hoffman and Mezey, 1989), 5-HT_{2A} (Fay and Kubin, 2000; Fonseca et al., 2001), 5-HT_{2C} (Fonseca et al., 2001), and 5-HT_3 (Fonseca et al., 2001) subtypes, although only the 5-HT_{1A} and 5-HT_{2C} subtypes are found in abundance (Fonseca et al., 2001; Thor et al., 1990). The primary source of 5-HT to the nPGi is the ventrolateral periaqueductal gray (vlPAG). vlPAG neurons project directly to the nPGi (Murphy and Hoffman, 2001; Normandin and Murphy, 2008), and these vlPAG-nPGi cells co-localize with 5-HT (Bago et al., 2002) and sexual behavior-induced Fos (Normandin and Murphy, 2008), suggesting a role for the serotonergic vlPAG-nPGi pathway in male sexual behavior.

The present studies were designed to test the hypothesis that the effects of SSRIs on male sexual behavior are mediated, at least in part, via the nPGi. Our working hypothesis is that removal of the source of 5-HT to the nPGi would alter the expression of genital reflexes but not other aspects of sexual behavior.

2 Methods

2.1 Subjects

Thirty male Sprague-Dawley Rats (Rattus Norvegicus; Charles River; 275-375g) were same-sex double-housed in a temperature-controlled vivarium in reverse light (lights on 7:00pm, off 7:00am) with ad libitum access to food and water. All experiments were approved by the Georgia State Institutional Animal Care and Use Committee, with pain and suffering minimized in accordance with the Committee's policies.

2.2 Ovariectomy and gonadal-steroid replacement in stimulus females

To induce sexual receptivity, ovariectomized female rats were injected with β-estradiol-3-benzoate (10ug/0.1ml sesame oil s.c.; Sigma Aldrich) 48 hours before testing and progesterone (500ug/0.1ml sesame oil s.c.; Fluka) 4 hours before testing as previously described (Barfield and Lisk, 1970; McEwen et al., 1987; Normandin and Murphy, 2010; Quadagno et al., 1972).

2.3 vlPAG 5-HT lesions

All surgeries were performed under aseptic conditions. Male rats (n=30) were anesthetized by inhalation of isoflurane (2-5%; Henry Schein) and placed in a stereotaxic frame. The skull was leveled such that bregma and lambda were at the same dorsoventral plane, and a craniotomy was performed dorsal to the PAG. vlPAG serotonergic-specific lesions were produced by bilateral injection of 5,7-dihydroxytryptamine creatine sulfate (5,7-DHT; 3mg/ml in 0.9% saline 0.1% ascorbic acid; Fluka; n=22 males) using a 1µl Hamilton syringe. Sham animals received equivolume injections of vehicle (0.9% saline 0.1% ascorbic acid; n=8 males). Thirty minutes prior to 5,7-DHT administration, all animals received an injection of desipramine hydrochloride (25mg/kg in 0.9% Saline i.p.; Sigma) to block uptake of the 5-HT neurotoxin by noradrenergic transporters on noradrenergic cells (Bjorklund et al., 1975). The coordinates for the vlPAG were (in mm): AP-8.5 bregma, ML +/-0.75, DV-5.75. 5,7-DHT or vehicle (300nl/side) was slowly injected over 2 minutes and the syringe was left in place for 10 min. before being removed. A small amount of bone wax was placed in the burr-hole and the animals' skin was wound-clipped. Animals received buprenorphine (0.1mg/kg s.c.; Henry Schein) for pain relief and baytril (5mg/kg i.m.;

Henry Schein) as a prophylactic antibiotic. Animals recovered in clean heated cages before being returned to the housing facility.

2.4 Sexual behavior tests

Sexual behavior tests were conducted in acrylic aquariums (61cm x 30.5cm x 30.5cm). Animals were acclimated to the arena prior to the initiation of the experiment for 10 min. on two consecutive days. Three and six days later, males engaged in 1-hour mating bouts with stimulus females to gain sexual experience. A third mating bout on day nine served as the baseline (pre-lesion) measure. Experimental animals then underwent lesion (or sham lesion) surgery as described above. Following a seven-day recovery, a final 1-hour mating bout served as the post-lesion measure. All sexual behavior bouts were recorded and the number of mounts (attempted intromissions without the stereotyped pelvic thrust associated with an intromission), intromissions, and ejaculations, as well as the latency to begin mating (from placement of female into mating chamber to first mount or intromission), ejaculation latency, and post-ejaculatory interval was scored. Data were tabulated for each ejaculatory series and then compared across series.

2.5 Perfusion / fixation / tissue preparation

Following a euthanizing dose of SleepAway (0.5ml i.p.; Henry Schein), all animals were transcardially perfused with 250 ml of 0.9% sodium chloride/2% sodium nitrite, followed by 300 ml of 4% paraformaldehyde 2.5% acrolein (Polysciences) in 0.1 M phosphate buffer then 150ml of the sodium chloride/sodium nitrite solution. Following perfusion/fixation, brains were removed and stored at 4°C in 30% sucrose solution until sectioned. Brains were cut into 25µm coronal sections in a 1:4 series through the PAG

with a Leica 2000R freezing microtome and stored free-floating in cryoprotectant-antifreeze solution (Watson et al., 1986) at -20°C.

2.6 Immunohistochemistry

Midbrain tissue sectioned at 25µm in a 1:4 series was processed for NeuN or 5-HTP for lesion verification as previously described (Loyd and Murphy, 2006; Murphy and Hoffman, 2001). Briefly, sections were removed from the cryoprotectant solution, rinsed extensively in potassium phosphate buffered saline (KPBS; pH 7.4), and then reacted for 20 minutes in 1% sodium borohydride to remove excess aldehydes. Sections were then incubated in primary antibody solution directed against either NeuN (Millipore, MAB377; monoclonal, raised in mouse; 1:70,000) or 5-HTP (Immunostar, 24446; polyclonal, raised in rabbit; 1:1,000) in KPBS containing 0.1% Triton-X for 1 hour at room temperature followed by 48 hours at 4°C. After primary antibody incubation, tissue was rinsed in KPBS, incubated for 1 hour in biotinylated goat-anti mouse (mouse anti-NeuN primary antibody) or goat anti-rabbit (rabbit anti-5-HTP primary antibody) IgG (Jackson Immunoresearch) at a concentration of 1:600, rinsed in KPBS, followed by a 1-hour incubation in avidin-biotin peroxidase complex (Vector Labs, ABC Elite Kit PK-6100) at a concentration of 1:10. After rinsing in KPBS and sodium acetate (0.175 M; pH 6.5), NeuN and 5-HTP were visualized as a black reaction product using nickel sulfate intensified 3,3'-diaminobenzidine solution containing 0.08% hydrogen peroxide in sodium acetate buffer. The reaction product was terminated after approximately 15 minutes by rinsing in sodium acetate buffer. Sections were mounted out of saline onto gelatin-subbed slides, air dried overnight, dehydrated in a series of graded alcohols, cleared in Histoclear, and cover-slipped using Permount.

2.7 Lesion analysis

5,7-DHT lesion and vehicle sham lesion sites were verified by light-microscopic analysis of vlPAG-containing brain sections immunohistochemically stained for NeuN or 5-HTP. The position of the bottom of the injection track was noted in the NeuN stained sections, and the absence or presence of 5-HTP was noted in 5-HTP stained sections. Only those injections centered in the vlPAG and with an absence of 5-HTP immunoreactivity in the vlPAG were considered for analysis.

2.8 Statistical analysis

Planned comparisons of mean sexual behavior within the lesion or sham groups were analyzed with two-tailed paired t-tests. Between group (lesion vs. sham) comparisons of means of sexual behavior were conducted using independent sample t-tests (two-tailed). All statistical comparisons were made with the alpha value set at 0.05.

3 Results

3.1 Lesion Verification

An example of 5-HTP immunoreactivity in a lesioned animal and sham-lesioned animal is provided in Figure 1. Serotonergic lesions were restricted to the vlPAG in the lesion (n=9) group, with slight spread to the dorsal raphe nucleus, and encompassing an approximate 1mm^3 volume. Syringe paths in our sham-lesion group (n=7) ended just dorsal to, or within the vlPAG. The two animals with misplaced lesions (dorsal to the vlPAG) did not exhibit any obvious sexual behavior changes. In addition, no obvious changes in non-sexual behaviors were noted in any group.

3.2 Sexual Behavior

Serotonergic lesions of the vlPAG did not affect behaviors not directly related to genital reflex function. For example, there was no change from baseline in the mean latency to initiate copulation, or in the mean post-ejaculatory interval within either group (Figure 2). By contrast, serotonergic lesions of the vlPAG facilitated male sexual behavior in many measures related to genital reflex functions. While there was no change from baseline in the mean total of mounts or intromissions within either group (Figure 3A & B), there was an increase from baseline in the mean number of ejaculations within the lesion group ($p=0.005$) but not the sham group ($p=1.000$). In addition, the mean number of ejaculations in the sexual behavior test was significantly greater in the lesion group than in the sham group ($p=0.039$, Figure 3C). There was a decrease from baseline in the mean number of intromissions required for ejaculation within both groups (lesion: $p=0.004$; sham: $p=0.039$; Figure 3D). There was a trend approaching statistical significance for a decrease from baseline in the mean ejaculation latency within the lesion group ($p=0.054$; Figure 3E), and this trend is supported by the significantly shorter mean ejaculation latency in the sexual behavior test in the lesion group versus the sham group ($p=0.026$; Figure 3E).

4 Discussion

Overall, serotonergic lesions of the vlPAG in male rats produced a facilitation of sexual behavior. In particular, 5-HT lesions of the vlPAG significantly increased the number of ejaculations during the mating bout, and there was a trend for a decrease in the mean latency to ejaculation. 5-HT vlPAG lesioned animals also had significantly

shorter ejaculation latencies as compared to sham. There were no differences in the mean latency to mate, or in the post-ejaculatory interval for either group indicating that the effect of serotonergic vlPAG lesions is limited to those behaviors directly associated with genital reflexes.

The facilitation of sexual behavior we observed was specific to those measures associated with genital reflex function. Given the anatomical connections between vlPAG 5-HT cells and the nPGi (Bago et al., 2002; Normandin and Murphy, 2008; Underwood et al., 1999), as well as the concordance of effects in both vlPAG 5-HT lesions and nPGi lesions (Liu and Sachs, 1999; Yells et al., 1992), it is likely that the effects observed here are dependent on vlPAG 5-HT neurotransmission to the nPGi. In the context of a normally behaving animal, one would expect that 5-HT from the vlPAG serves to enhance nPGi activity, thereby increasing descending inhibition of genital reflexes. This serotonergic signal from the vlPAG would be particularly active in behavioral contexts where sexual behavior would not be appropriate (e.g. a "fight-or-flight" response) and likely "de-activated" when mating conditions were optimal (e.g. availability of a sexually receptive conspecific in the absence of a predator). This serotonergic vlPAG-nPGi pathway may effectively act as a gating mechanism for descending inhibition of genital reflexes.

Regulation of nPGi function (and thereby genital reflexes) through vlPAG is an interesting prospect, as midbrain periaqueductal gray (PAG) cells are known to be involved in the coordination of cardiovascular responses to anxiety and stress (Johnson et al., 2004; Moraes et al., 2008; Murphy et al., 1995), nociception (Haghparast and Ahmad-Molaei, 2009; Loyd et al., 2007), as well as social behaviors (Lonstein and

Stern, 1998; Pavesi et al., 2007), and could therefore signal the current behavioral context to the nPGi. With respect to sexual behavior, we have previously found that the medial preoptic area (MPOA) of the hypothalamus, a region critical in the expression of male sexual behavior (Arendash and Gorski, 1983; Malsbury, 1971), including genital reflexes (Giuliano et al., 1996; Marson and McKenna, 1994), provides input to the nPGi both directly (Normandin and Murphy, 2008) and through a PAG relay (Murphy and Hoffman, 2001). This MPOA-PAG pathway may provide necessary input to the nPGi regarding sexual "tone." Indeed electrolytic lesions of the PAG, including the vlPAG, blocks normal MPOA-elicited bulbospongiosus contractions (Marson, 2004), suggesting that the MPOA-vlPAG-nPGi circuit is required for the elicitation of genital reflexes.

The medially adjacent dorsal raphe nucleus (DR) also contains serotonergic neurons that may have been affected by our lesions. However, results from previous work suggest that our observed findings are primarily the result of vlPAG lesions, and not due to the spread of the toxin into the DR. For example, in male rats, lesions of the DR, or application of 5-HT or 5-HT agonists to the DR have no effect on male ejaculatory behavior (Albinsson et al., 1996; Fernandez-Guasti et al., 1992; Hillegaart, 1991), although one study has reported that 5-HT DR manipulations shortened both the latency to ejaculate and the post-ejaculatory interval (McIntosh and Barfield, 1984). Interestingly, lesions of the DR also have been found to facilitate lordosis in male rats treated with estradiol (Kakeyama and Yamanouchi, 1992), leading to speculation that the DR is not involved in male typical sexual behavior per se, but rather inhibits female-typical behavior in male rats.

5 Conclusions

Serotonergic lesions of the vlPAG in male rats facilitated genital reflex function in a manner similar to nPGi lesions. This result lends evidence to the functional serotonergic connectivity between the vlPAG and nPGi. This work has implications for the occurrence of SSRI-induced delayed ejaculation in human males, and in particular, suggests that these drugs may be producing an effect by increasing serotonergic neurotransmission within the vlPAG-nPGi pathway. Limiting serotonergic drugs for mood disorders in humans to receptors and loci that influence mood regulation systems, but not sexual behavior systems is an emerging area of research (Baldwin et al., 2006; Breuer et al., 2008; Kennedy and Rizvi, 2010). While it has been known that SSRIs can inhibit genital reflexes in male rats at the level of the spinal cord (Marson and McKenna, 1992), our work implies that this could also occur through the nPGi via vlPAG 5-HT input. Thus, in developing treatments for depression and anxiety, clinicians must consider both the spinal and supraspinal targets of serotonergic drugs in this genital reflex circuitry. In addition, SSRIs are often prescribed to men experiencing premature ejaculation (Hatzimouratidis et al., 2010; Hellstrom, 2009). A global increase in 5-HT is neither warranted nor desired in these patients, as other side effects may be incurred (Haddad and Dursun, 2008; Hellstrom, 2009) as a result of treatment. Targeting the serotonergic vlPAG pathway to the nPGi with appropriately specific drugs may be one way to circumvent global treatment of these patients with SSRIs. However, it remains to be elucidated which 5-HT receptor subtypes on nPGi cells are responsible for modulating nPGi activity.

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Figure 1 - Photomicrograph of sham lesion and lesion sites

A photomicrograph of 5-hydroxytryptophan (5-HTP) immunolabeled cells of the ventrolateral periaqueductal gray (vlPAG) and dorsal raphe (DR) is shown on the left, with a diagram (modified from Paxinos and Watson, 2005) at the approximate level of the tissue section shown on the right. Intra-vlPAG injection of vehicle (photomicrograph left) preserved 5-HTP immunoreactivity in the vlPAG. Intra-vlPAG injection of 5,7-dihydroxytryptamine (photomicrograph right) markedly reduced immunoreactivity for 5-HTP in the vlPAG. Lesion sites are indicating by the gray circles in the diagram. Aq=cerebral aqueduct.

Figure 2 - Measures of sexual behavior unrelated to genital reflex function

Serotonergic lesions of the vlPAG did not alter the latency to mating (A) or post-ejaculatory interval (B) from baseline to sexual behavior test within either the sham or lesion groups. Error bars = standard error of the mean (SEM).

Figure 3 - Measures of sexual behaviors related to genital reflex function

Serotonergic lesions of the vlPAG did not alter the mean number of mounts (A) or intromissions (B) from baseline to sexual behavior test within either group. However, such lesions significantly increased the mean number of ejaculations (C) within the lesion group, which was significantly greater than in sham group. The mean number of intromission per ejaculation (D) significantly decreased within both the sham and lesion group. The mean ejaculation latency (min.; E) decreased within the lesion group,

approaching significance, which was significantly less than the sham group. * = $p<0.05$, error bars = SEM.

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Figure 1

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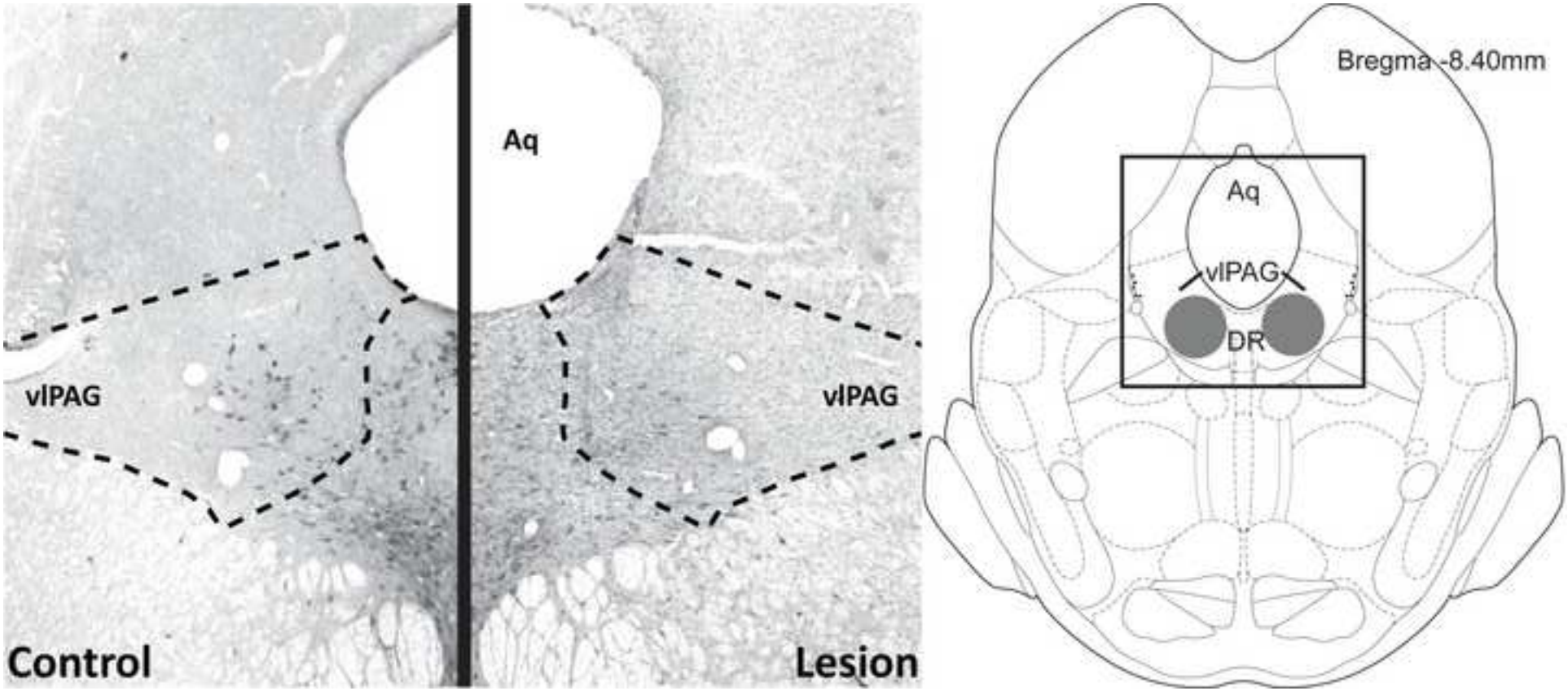

Figure 2

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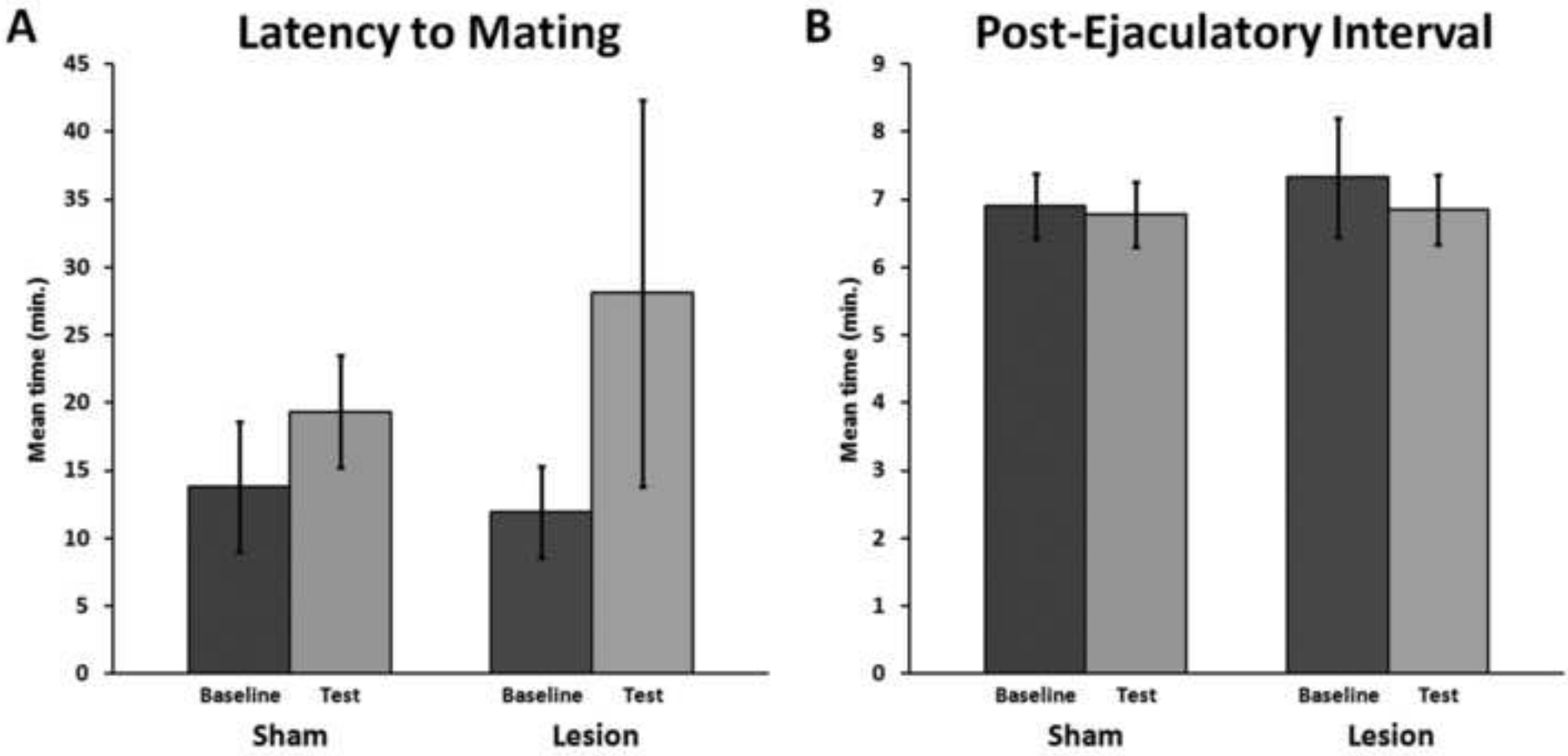

Figure 3

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