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Activation of NMDA receptors in lumbar spinothalamic cells is required for ejaculation

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Abstract

Introduction—The sexual reflex ejaculation is controlled by a spinal ejaculation generator located in the lumbosacral spinal cord. A population of spinothalamic (LSt) neurons forms a key component of this generator, as manipulations of LSt cells either block or trigger ejaculation. However, it is currently unknown which afferent signals contribute to the activation of LSt cells and ejaculation.

Aim—The current study tested the hypothesis that glutamate, via activation of N-Methyl-D-aspartic acid (NMDA) receptors in LSt cells, is a key regulator of ejaculation.

Methods—Expression of phosphorylated NMDA receptor subunit 1 (NR1) was investigated following mating, or following ejaculation induced by electrical stimulation of the dorsal penile nerve (DPN) in anesthetized, spinalized male rats. Next, the effects of intraspinal delivery of NMDA receptor antagonist AP-5 on DPN stimulation-induced ejaculation were examined. Moreover, the ability of intraspinal delivery of NMDA to trigger ejaculation was examined. Finally, the site of action of NMDA was determined by studying effects of NMDA in male rats with LSt cell-specific lesions.

Main Outcome Measures—Expression of NR1 and phosphorylated NR1 in LSt cells was analyzed. Electromyographic recordings of the bulbocavernosus muscle (BCM) were recorded in anesthetized, spinalized rats following stimulation of the DPN, and delivery of AP-5 or NMDA.

Results—Results indicate that the NR1 receptors are activated in LSt cells following ejaculation in mating animals or induced by DPN stimulation in anesthetized, spinalized animals. Moreover, NR1 activation in LSt cells is an essential trigger for rhythmic BCM bursting, as DPN stimulation-induced reflexes were absent following administration of NMDA receptor antagonist in the L3-L4 spinal area, and were triggered by NMDA. NMDA effects were dependent on intact LSt cells and were absent in LSt-lesioned males.

Conclusion—These results demonstrate that glutamate, via activation of NMDA receptors in LSt cells, is a key afferent signal for ejaculation.

Introduction

Ejaculation is a reflex mediated by a spinal ejaculation generator that consists of LSt cells and their projections to autonomic and motor areas within the lumbosacral spinal cord¹⁻⁵. Previous studies from our laboratory and others have indicated that LSt cells (see table 1 for list of all abbreviations) are essential for ejaculation as LSt cell lesions ablate ejaculatory behavior in intact mating animals⁴. In addition, electrical microstimulation in the area of LSt cells can trigger ejaculation⁶. Moreover, LSt cells express Fos and phosphorylated ERK (pERK) with ejaculatory behavior in male rats⁷⁻⁸. Finally, it was recently shown that stimulation of the dorsal penile nerve (DPN) in male rats with complete spinal transection,

which triggers bursting of the bulbocavernosus muscle (BCM) in patterns characteristic of ejaculation, induced pERK in LSt cells⁷. Moreover, rhythmic contractions of the BCM induced by stimulation of the DPN can be prevented by inhibition of activation of mitogen-activated protein (MAP) kinase signaling in LSt cells⁷. Hence, it is clear that LSt cells play an essential role in control of ejaculation and that activation of these cells via the MAP kinase pathway is required. However, it is currently unknown which neurotransmitters are involved in activation of LSt cells. Therefore, the goal of the current study is to test the hypothesis that glutamate is a critical neurotransmitter for activation of LSt cells and subsequent ejaculation.

Glutamate, the main excitatory neurotransmitter throughout the central nervous system⁹, appears a likely candidate for the activation of ejaculation. Two prominent lumbosacral spinal reflexes that involve pudendal nerve fibers, micturition and erection, have been reported to be mediated by glutamatergic neurotransmission. Regular urinary bladder function, including urine continence, is regulated by reflex activity of pudendal nerves innervating the urethral sphincter¹⁰. Both NMDA and AMPA receptors have been demonstrated to mediate bladder activity through lumbosacral spinal mechanisms¹¹. Sensory information from the genitals inducing erection is conveyed via the DPN to a lumbosacral network of interneurons¹². Moreover, the physiological process of erection is mediated by glutamatergic neurotransmission and is disrupted in male rats by intrathecal application of both NMDA and AMPA antagonists¹³. Thus, glutamate is implicated in the function of various spinal reflexes.

The current study tested the hypothesis that ejaculatory reflexes are triggered via activation of the NMDA receptors in LSt cells. This hypothesis was tested by observing the expression of phosphorylated NR1 (pNR1) in LSt cells in response to mating as well as stimulation of the DPN in anesthetized, spinalized male rats. The NR1 subunit was chosen as it must be present for functional glutamatergic neurotransmission and has nearly ubiquitous expression throughout the central nervous system^{14,15}. The binding of glutamate to NMDA receptors induces calcium influx and activates associated secondary messengers, which subsequently induce receptor phosphorylation. Thus, pNR1 can be used as an appropriate marker of receptor activation¹⁶. Furthermore, it was tested if the ejaculatory reflex following DPN stimulation was abolished through the pharmacological blockade of NMDA receptors in the area of LSt cells, and induced by the administration of NMDA. Together, these studies aim to enhance the knowledge of the spinal ejaculation generator and the nature of activation inputs to the LSt cells in particular.

Materials and Methods

Animals

Adult male Sprague Dawley Rats (200-250g) were obtained from Charles River (Sherbrooke, Quebec, Canada) and housed in pairs in Plexiglas cages. The colony room was maintained on a 12/12 light-dark cycle (lights off at 10 am). Food and water were available *ad libitum*. Female Sprague-Dawley rats were bilaterally ovariectomized and received a subcutaneous implant containing estradiol benzoate (5%). Sexual receptivity was induced by administration of 500 µg (in 0.1 mL sesame oil) 5 hours before testing. All procedures were approved by the Animal Care Committee at the University of Western Ontario and conform to guidelines outlined by the Canadian Council on Animal Care.

Ejaculation-induced pNR1

Mating-induced pNR1—Male rats were tested for mating behavior with females in test cages. Male rats were divided in 3 experimental groups: (1) males that displayed mounts

(average of 20 mounts) with vaginally masked females (n=4); (2) males that displayed mounts and intromissions (average of 10 intromissions), but no ejaculations (n=6); (3) males that displayed one ejaculation (n=5; average of 13 intromissions). Animals were sacrificed 30 minutes following behavior. This time interval was based on previous studies examining mating-induced pNR1 in the brain¹⁶. Control rats were also placed in test cages, but were not exposed to a female, and were sacrificed 40 minutes after placement in test cage, which is an equivalent amount of time compared to the experimental groups (n=4). Animals were anesthetized with sodium pentobarbital and perfused transcardially with 5 mL 0.9% saline solution followed by 500 mL of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). Spinal cords were removed and post-fixed for 1 hour in the same fixative, after which they were placed in cryoprotective solution (20% sucrose in PB with 0.01% sodium azide) until further processing and analysis of pNR1.

DPN-stimulation induced pNR1—Male rats (n=22) were anesthetized with urethane (1.5 g/kg, i.p.), a laminectomy between thoracic levels T6-T8 was performed, and the spinal cord was completely transected at the same level. Following transection, animals did not receive manipulation for 6 hours to allow for injury-induced pNR1 to dissipate (personal observations, data not shown). The pelvic cavities were then opened and both connective tissue and muscle was dissected away to expose the DPN and BCM. A bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI) was placed bilaterally on the DPN. Silver recording electrodes were inserted bilaterally into the BCM and a ground electrode was inserted into the muscle of the right thigh. Recording electrodes were connected to the PowerLab/4SP Data Acquisition System (ADInstruments, Inc., Colorado Springs, CO). Electrical stimulation consisted of square wave pulses of 1 ms duration, 4 V at 30 Hz for 10 seconds. One group of males (n=18) received electrical stimulation of the DPN, while another group (n=4) had electrodes positioned as described, but did not receive stimulation. Electrical stimulation of the DPN evoked a rhythmic bursting response of the BCM in 100% of animals. BCM activity was observed and recorded using PowerLab Chart 5 (v5.0.2; ADInstruments, Inc.). Animals were sacrificed 5, 15, 30 or 60 minutes following stimulation (n=4-5 each) or control sham (n=4 consisting of 1 per time point) procedures as described above and spinal cord were analyzed for pNR1.

Immunohistochemistry

The spinal tissue was sectioned using a freezing microtome (Thermo Fisher Scientific, Walldorf, Germany) into 12 parallel series of 35 μ m coronal sections in cryoprotectant solution (30% sucrose, 30% ethylene glycol in 0.1 M sodium phosphate buffer (PB) with 0.01% sodium azide) and stored at -20° C until further processing. For all immunohistochemistry procedures, free floating sections were rinsed thoroughly with 0.1 M saline buffered sodium phosphate (PBS) between incubations and all incubations were performed at room temperature with gentle agitation. Sections were blocked with 1% H₂O₂ in PBS for 10 minutes, then incubated in incubation solution (PBS containing 0.1% bovine serum albumin and 0.4% Triton X-100 (BP151-500, Thermo Fisher Scientific, Ottawa, Ontario)) for 1 hour. Primary antibody incubations were performed in the incubation solution overnight at room temperature.

Galanin/NR1, galanin/VGlut1, galanin/VGlut2, or galanin/pNR1 dual fluorescence—One series of lumbosacral sections for each animal was incubated overnight with goat anti-NMDAR1 (n=4 control animals; 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), guinea pig anti VGlut1 or VGlut2 (n=4 control animals; AB5905 or AB5907, 1:10,000; Chemicon, Temecula, CA), or rabbit anti-pNR1 (all mating and DPN-stimulation groups; 1:12,000; #06-041; Upstate Biotechnology, Inc., Lake Placid, NY)

followed by incubations with biotinylated rabbit anti-goat, biotinylated goat anti-guinea pig, or biotinylated goat anti-rabbit (1:500 in incubation solution, 1 hour; Vector Laboratories, Burlingame, CA), avidin horseradish peroxidase complex (ABC-elite, 1:1,000 in PBS, 1 hour; Vector Laboratories), biotinylated tyramine (1:250 in PBS containing 1 μ l 3% H₂O₂ per 1 ml PBS, 10 minutes; NEL700/700A; PerkinElmer Life Sciences, Boston, MA), and Alexa 488-conjugated streptavidin (1:100 in PBS, 30 minutes; Molecular Probes/Invitrogen, Burlington, Ontario). Next, sections were incubated overnight with a rabbit raised antibody recognizing galanin (LSt cell marker⁴; 1:3,000; T-4334; Bachem, Torrance, CA) and with Cy3 conjugated donkey anti-rabbit (1:200 in PBS, 30 minutes; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were mounted onto plus charged glass slides and coverslipped with gelvatol. Controls included omission of primary antibodies which resulted in lack of labeling in appropriate wavelengths.

Data Analysis

Galanin/VGlu1 or VGlu2 expression—Sections were analyzed with a Zeiss laser-scanning confocal microscope system (Zeiss LSM510; Carl Zeiss MicroImaging, Inc., Thornwood, NY). CY3-fluorescence was imaged with a 567 nm emission filter and a HeNe laser, and Alexa 488 with a 505 nm emission filter and Argon laser. Images were created by projecting several 1 μ m optical sections obtained at 1 μ m intervals through galanin-IR neurons in the z-axis (63x magnification).

Galanin/NR1 and galanin/pNR1 expression—Sections were analyzed on a fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Percentages of galanin-positive cells that express NR1 was counted and analyzed for 4 animals. For pNR1 analysis, percentages of galanin-positive cells expressing pNR1 were analyzed, and data are expressed as the mean percentages of LSt cells that express pNR1. One-way ANOVA followed by Fisher's LSD tests were used to measure statistically significant differences between experimental groups and the control group.

Pharmacological Manipulations

Intraspinal L3-4 AP-5 administration—Male rats (n=13) were anesthetized, spinalized, and the DPN was exposed as described above. The DPN was electrically stimulated to verify that animals received a complete spinalization, after which no further manipulation was performed for at least 6 hours. A second laminectomy was then performed at the L3-L4 spinal segment and drugs were delivered bilaterally via two micropipettes. One group of animals (n=9) received a bilateral intraspinal injection of NMDA receptor antagonist D-2-Amino-5-phosphonovaleric acid (AP-5; 1.5 μ m ventral; 4 $\times$ 1 μ L, 10 mM; A8054; Sigma-Aldrich Canada Ltd., Oakville, Ontario) 5 minutes prior to DPN stimulation. Control animals (n=4) received a bilateral intraspinal injection of vehicle (4 $\times$ 1 μ L, 0.9% saline). BCM activity was observed and recorded. Figure 1A illustrates the time line of this experiment.

Intraspinal NMDA administration—Male rats (n=22) were anesthetized and spinalized as previously described. Six hours following transection, a second laminectomy was performed at the L3-L4 spinal segment. At this level, the dura mater was punctured and drugs were delivered via two micropipettes unilaterally into the L3-L4 spinal segment. Animals received unilateral injections of different doses of NMDA (1.5 μ m ventral; 2 $\times$ 2 μ L, M3262; Sigma): 1 mM (n=5), 100 μ M (n=6), 50 μ M (n=4) or 10 μ M (n=4). Control animals (n=6) received a unilateral intraspinal injection of vehicle (2 $\times$ 2 μ L, 0.9% Saline). BCM activity was observed and recorded during and following injection. These animals were sacrificed and perfused 5 minutes following injections as previously described. Figure 1B illustrates the time line of this experiment.

Intraspinal NMDA administration in LSt cell lesioned males—Male rats (n=18) were anesthetized with a ketamine/xylazine mixture (87/13%, i.p.) and a laminectomy was performed to expose the L3-L4 spinal segment. The selective neurotoxin SSP-SAP (1.5 μ m ventral; 12 $\times$ 1 μ L, 4 ng/ μ L; Advanced Targeting Systems, San Diego, CA) was infused bilaterally into the spinal cord as previously described by Truitt and Coolen⁴. This lesion technique has previously been described to result in LSt cell-specific lesions without loss of other surrounding neurons in the spinal cord⁴. Animals were allowed to recover for two weeks after which they were deeply anesthetized and spinalized as described above. The L3-L4 spinal segment was again exposed, the dura mater punctured and drugs were delivered as described above. NMDA (50 μ M) was infused and BCM activity was observed and recorded. These animals were perfused as described above for lesion verification (analysis of galanin immunoreactivity).

LSt lesion verification—One series of lumbosacral sections were immunoprocessed for galanin. Sections were incubated overnight with rabbit anti-galanin (1:60,000; T-4334; Bachem), followed by incubations with biotinylated goat anti-rabbit (1:500 in incubation solution, 1 hour; Vector Laboratories), avidin horseradish peroxidase complex (ABC-elite, 1:1,000 in PBS, 1 hour; Vector Laboratories). Next, sections were incubated for 10 minutes in 0.02% diaminobenzidine (DAB) in 0.1 M PB containing 0.012% hydrogen peroxide, resulting in a reddish-brown reaction product. Sections were mounted onto plus charged glass slides and coverslipped with Di-N-Butyl Phthalate in Xylene (DPX; Electron Microcopy Sciences, Hatfield, PA). Numbers of galanin cells were counted. Animals that expressed less than 1/3 of the numbers of galanin cells in intact animals were considered lesioned (n=8; average 0.18 ± 0.06 cell per section). All others were classified as incomplete or misplaced lesion (“mis-lesioned”; see Truitt and Coolen⁴) males (n=7; average 1.18 ± 0.11 cell per section).

Analysis of BCM activity—EMG activity of the BCM is expressed as mean number of events (termed bursts) above baseline (fluctuations less than 2.5% above baseline are ignored), analyzed using PowerLab Chart 5 (v5.0.2; ADInstruments, Inc.) (see Figure 2 for representative EMG and analysis). For the NMDA- and APV-injected groups, BCM contractions were analyzed for 70 seconds after injection (corresponds to the typical length of the DPN-induced reflex (personal observations, also see Figure 2). One-way ANOVA and Fisher LSD tests were used to compare numbers of bursts between control and drug-groups as well as between dosages. For the AP-5-injected animals, a two-way ANOVA and Fisher LSD tests were used to compare numbers of bursts between control and drug-groups as well as before and after treatment (within same animals). For the LSt lesion study, t-tests were used to analyze differences in numbers of bursts between lesioned and mis-lesioned animals.

Results

LSt cells receive glutamatergic input and express NMDA receptors

Qualitative confocal analysis of lumbosacral spinal cord tissue revealed that LSt cells receive direct inputs from glutamatergic axon terminals containing vesicular glutamate transporters 1 and 2 (VGlut1 and VGlut2; Figure 3). Moreover, quantitative analysis of lumbosacral spinal cord tissue revealed that 100% of LSt cells express NR1 (Figure 4).

Mating activates NMDA receptors in LSt cells

Mating activated NMDA receptors in LSt cells, evidenced by increased pNR1 expression in LSt cells ($F(3,12)=36.466$; $p<0.001$) (Figure 5). Furthermore, the percentages of LSt cells expressing pNR1 increased with increased sexual activity. Display of mounts resulted in

significantly increased percentages of LSt cells expressing pNR1 relative to control groups ($p=0.004$). Mounts and intromissions further increased percentages of LSt cells expressing pNR1 relative to mounts ($p=0.017$) and control groups ($p<0.001$). The highest percentage of pNR1-expressing LSt cells was observed following ejaculation, which resulted in significantly increased percentages of LSt cells expressing pNR1 relative to intromissions ($p=0.003$), mounts and control groups ($p<0.001$; Figure 6A).

DPN stimulation activates NMDA receptors in LSt cells

Ejaculation induced by stimulation of the DPN resulted in activation of NR1 in LSt cells ($F(4,17)=93.008$; $p<0.001$) (Figure 5). Percentages of LSt cells expressing pNR1 was significantly increased 5, 15, 30 and 60 minutes following DPN stimulation relative to control animals ($p<0.001$; Figure 6B).

AP-5 administration abolishes the ejaculatory reflex

To investigate if NMDA receptors are essential for the ejaculatory reflex, AP-5 was delivered at the general location of the LSt cells. Overall, there was a significant effect of intraspinal AP-5 infusion when compared to vehicle treatment ($F(1,14)13.664$; $p=0.002$), or compared to BCM bursting activity prior to infusion ($F(1,14)16.536$; $p=0.001$). Post hoc analyses revealed that the number of BCM events was significantly reduced compared to responses elicited prior to drug infusion in the same animals ($p<0.001$) and vehicle-treated controls ($p<0.001$; Figure 7). Vehicle infusions did not alter the ejaculatory reflex or the mean number of bursts compared to prior responses elicited in the same animals.

NMDA administration induces BCM bursting activity

To investigate if activation of NMDA receptors is sufficient to induce ejaculation, intraspinal injections of NMDA were delivered at the general location of the LSt cells. Intraspinal administration of NMDA induced BCM activity in the absence of DPN stimulation ($F(4,20)=8.961$; $p<0.001$). The BCM response was significantly induced by NMDA infusions of 1 mM ($p<0.001$), 100 μ M ($p<0.001$) and 50 μ M ($p<0.018$), but not 10 μ M compared to vehicle injections (Figure 8).

NMDA-induced BCM activity requires LSt cells

NMDA infusions in the LSt area were sufficient to trigger BCM activity. However, at the higher doses, the BCM EMG patterns no longer displayed the characteristic rhythmic bursting pattern typically observed with DPN stimulation or ejaculation, suggesting that NMDA may also act on downstream target areas of LSt cells. Therefore, to demonstrate that NMDA is acting primarily on LSt cells, LSt cell lesioned males were examined for responses to NMDA (50 μ M) infusions. Indeed, NMDA did not induce BCM EMG activity in LSt-lesioned males, as number of bursts was significantly reduced compared to mis-lesioned males ($p<0.001$) (Figure 9).

Discussion

The current results indicate that NMDA receptors are activated in LSt cells following ejaculation in mating animals and induced by DPN stimulation in anesthetized, spinalized animals. Activation of NMDA receptors in LSt cells is an essential trigger for DPN-induced BCM bursting, as DPN stimulation-induced reflexes were absent following administration of AP-5 in the L3-L4 spinal area. Furthermore, activation of NMDA receptors in LSt cells is sufficient to drive BCM activity, as NMDA administration induced the ejaculatory reflex in the absence of DPN stimulation. Finally, NMDA acts primarily on LSt cells, as NMDA administration in LSt-lesioned animals did not elicit the BCM bursting reflex.

In support of the finding that NMDA receptor activation is required for the initiation of ejaculation, the phosphorylation of NMDA receptors in LSt cells appears associated with the onset of ejaculation as it was observed following mounts and intromissions, albeit to a lesser extent. The afferent inputs that trigger activation of NMDA receptors in LSt cells are currently unknown, although the most likely activation of LSt cells is via DPN nerve fibers. The densest distribution of DPN sensory afferent terminals is located in the superficial laminae of the L5 and L6 lumbar segments¹⁷ and glutamate is expressed in DPN sensory afferents in male rats¹². Stimulation of the pudendal nerve results in increased Fos-immunoreactive nuclei in the L5-S1 spinal segments, with the majority of these nuclei being located in the superficial dorsal horn and the medial gray in the DGC¹⁸. These patterns of Fos-immunoreactivity correspond with the distribution of pudendal nerve sensory afferent fibers, and overlap was found with vesicular glutamate transporters¹⁸. Furthermore, stimulation-induced Fos was observed bilaterally in the medial gray of the L3-L4 segments, corresponding to the location of LSt cells¹⁸. It is currently not clear if the source of glutamate activating the LSt cells indeed derives from DPN nerve fibers or from interneurons. DPN fibers have been shown to contain glutamate¹², but direct connections of DPN axons in close apposition to LSt cells have yet to be identified. Therefore, it appears likely that DPN nerve fibers activate LSt cells via a multisynaptic pathway including glutamatergic interneurons. The current data suggest that LSt cells receive close contacts from both vesicular glutamate transporters 1 and 2-expressing axon terminals, with the latter appearing most abundant. Although the exact location of the source of this glutamate has not been determined in the current study, it is possible that these contacts originate from interneurons in layers II-IV of the lumbar spinal cord, where VGlut1 and VGlut 2-mRNA expressing neurons are particularly abundant¹⁹. In addition, NR1 and pNR1 immunoreactivity was not confined to LSt cells, and instead was also observed in the vicinity of LSt cells and surrounding layers of the lumbosacral spinal cord, as previously reported²⁰⁻²¹ (also see Figure 5). LSt cell lesions completely ablate both ejaculatory behavior⁴ and NMDA-induced BCM bursting activity, indicating the essential role of LSt cells for ejaculation and the action of NMDA directly on LSt cells. However, it is possible that NR1 activation in cells that in turn project to LSt cells may contribute to ejaculation or BCM bursting activity. Moreover, even though it is possible that LSt cells receive supraspinal glutamate afferents, such inputs are not required for the activation of NMDA receptors as spinalization did not prevent DPN stimulation induced activation. Yet, on the other hand, NMDA receptors in LSt cells were activated by spinalization and pNR1 was observed in LSt cells for up to 4 hours following this injury (Staudt and Coolen, unpublished observations). Such activation of NMDA receptors may be consistent with increased glutamate release due to severing of supraspinal afferents^{22,23}.

In addition to a role for glutamate in the control of ejaculation, studies of erectile function¹³ or bladder function²⁴ have demonstrated that reflexes evoked by electrical stimulation of the dorsal penile or pudendal nerve are dependent on glutamatergic excitatory transmission. Pudendal nerve reflexes regulating bladder function and the external urethral sphincter are depressed following administration of NMDA and/or AMPA antagonists²⁴, and bladder activity and excitation via the external urethral sphincter triggered by a pelvic-pudendal nerve reflex are depressed following administration of NMDA or AMPA antagonists, and induced by intrathecal administration of glutamate or NMDA¹⁰. In the present study, low levels of pNR1 expression were observed throughout the lumbosacral spinal cord and in LSt cells in the absence of mating, suggesting a basal level of glutamate release and NMDA receptor activation. Thus, pNR1 expression in these areas may have additional roles such as control of bladder function.

Although these studies indicate that NMDA receptors play a role in the activation of LSt cells, other neurotransmitters and neuropeptides are likely involved in the afferent inputs to

LSt cells. AMPA receptors may have additional roles in modulating the ejaculatory reflex, as AMPA receptors are expressed in the general area of the spinal cord where LSt cells are located²⁵. Expression of AMPA receptor subunits in LSt cells remains to be determined. Furthermore, the presence of NK-1R on LSt cells also suggests that substance P may regulate ejaculation⁴. Indeed, blockade of these receptors at the L3 spinal level significantly decreases ejaculations induced by D3 dopamine receptor agonist 7-OH-DPAT in anesthetized male rats²⁶. However, the capacity of intrathecal or intraspinal injections of substance P to elicit ejaculation has yet to be determined.

In conclusion, the current data provide insight into the nature of the neurotransmitters involved in the activation of ejaculation through LSt cells and specifically show that activation of NMDA receptors in LSt cells is essential for triggering the ejaculatory reflex as indicated by DPN-stimulated BCM activity. Therefore, NMDA receptors may provide a target for development of future treatments for sexual dysfunction related to ejaculation. Although rodent studies have explored the role of glutamate in sexual function^{16, 27}, clinical studies are in their infancy and drugs targeting glutamate receptors are currently in development. A recent study has demonstrated that sexual dysfunction related to selective serotonin reuptake inhibitor (SSRI) treatment for depression is related to glutamatergic, but not serotonergic genes²⁸. Specifically, genetic polymorphisms in glutamatergic genes were associated with decreased libido, difficulty achieving orgasm, and erectile dysfunction. Although the mechanism by which SSRIs influence glutamatergic neurotransmission is unknown, SSRIs are used off-label to treat premature ejaculation²⁹. In another clinical study, the anti-inflammatory drug Tramadol, which also acts as an NMDA antagonist and SSRI, has successfully been demonstrated as an off-label treatment for premature ejaculation³⁰⁻³¹. Finally, NMDA and other glutamate receptor drugs are being developed for treatment of psychiatric disorders, including mood disorders³², and may have potential additional benefits for sexual dysfunction.

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Figure 1.

Timeline for pharmacological manipulations. For antagonist studies (**A**), animals were spinalized (*SCT*) and tested for the ejaculatory reflex 1 hour afterward (*Stim*). AP-5 was delivered to LSt cells via intraspinal injection 6 hours following initial stimulation (*AP-5*). Animals were then stimulated 5 minutes following drug delivery (*Stim*) and perfused 5 minutes following stimulation (*Perfuse*). For agonist studies (**B**), animals were spinalized (*SCT*) and left undisturbed for 6 hours, at which time NMDA was delivered to LSt cells via intraspinal injections (*NMDA*). Animals were perfused 5 minutes following drug delivery (*Perfuse*).

Figure 2.

Analysis of the characteristic EMG bursting pattern of the BCM induced by DPN stimulation. Dashed lines depict time of DPN stimulation followed by 70 seconds of EMG recording (**A**). Section of trace enclosed by box indicates 18 seconds of recording (**A**), which is then expanded to demonstrate a series of bursts (**B**). EMG recording is further expanded to shown an individual cluster of bursts, 2 seconds in duration (**C**). BCM EMG activity is analyzed as events above baseline (fluctuations less than 2.5% above baseline are ignored) using PowerLab Chart 5 analysis software. These events are termed bursts and are indicated by the filled circles.

Figure 3. LSt cells receive glutamatergic input. LSt cells are contacted by axon terminals containing VGlut1 (**A**) and VGlut2 (**B**). Confocal images, 1 μ m optical sections. Scale bar = 100 μ m.

Figure 4. LSt cells express NMDA receptors. 100% of LSt cells (A; galanin, green) express NMDA receptor subunit 1 (NR1; red; B; merged image in C). Scale bar = 100 μ m.

Figure 5.

Expression of pNR1 in LSt cells. LSt cells (galanin; red; **A, D, G, J**) expressed pNR1 (green; **B, E, H, K**; merged images in **C, F, I, L**) following ejaculation in mating animals (**D-F**) or DPN stimulation in anesthetized, spinalized males (**J-L**). Control animals that did not mate (**A-C**), and control spinalized animals without stimulation of the DPN (**G-I**) did not express any pNR1 in LSt cells. cc = central canal; Scale bar = 100 μ m.

Figure 6. Quantitative analysis of pNR1 expression in LSt cells following mating (**A**: *C*: control males; *M*: mounting only; *I*: mounts and intromissions; *E*: ejaculation), or DPN stimulation (**B**). * indicates significant difference from all other groups (**A**) or from control values (**B**).

Figure 7.

Characteristic bursting pattern of the BCM induced by DPN stimulation is abolished by pre-treatment with AP-5. Male rats were stimulated prior to intraspinal (**A**, **C**) drug treatments to verify bursting pattern. Subsequent intraspinal injection of vehicle (**B**) did not affect BCM bursting. In contrast, subsequent intraspinal (**D**) injection of AP-5 abolished BCM bursting upon DPN stimulation. The number of DPN-stimulation induced BCM bursts was significantly reduced in animals treated with intraspinal AP-5 compared to DPN-stimulation induced BCM bursting prior to infusion or vehicle infusions (**E**). Dashed lines depict time of stimulation followed by 70 seconds of recording. * indicates significant difference from vehicle treatment and responses prior to AP-5 infusion.

Figure 8.

Intraspinal NMDA injections induced bursting of the BCM in the absence of DPN stimulation. NMDA injections of different concentrations induced BCM bursting (**B**: 50 μ M; **C** 100 μ M; **D**: 1mM), whereas saline injections did not result in a bursting pattern (**A**). The number of BCM bursts was significantly induced by increasing doses of NMDA (50 μ M – 1 mM), compared to NMDA injections of 10 μ M and saline (**E**). Solid lines beneath traces represent the 70 second recording period in which BCM EMG activity was analyzed. * indicates significant difference from NMDA injections of 10 μ M and saline.

Figure 9.

LSt cells are required for the effects of NMDA on BCM bursting, as intraspinal NMDA injections did not induce BCM bursting in LSt-lesioned males (**C** and **D** show two representative animals), while BCM bursting was induced in mis-lesioned control animals (**A** and **B** show two representative animals). Solid lines beneath traces represent the 70 second recording period in which BCM EMG activity was analyzed and **E** shows the significant reduction in number of BCM bursts in lesioned compared to mis-lesioned males. * indicates significant difference.

Table 1

List of abbreviations used in the text.

List of Abbreviations	
AMPA:	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCM:	bulbocavernosus muscle
DPN:	dorsal penile nerve
ERK:	extracellular signal-regulated kinase
LSt:	Lumbar spinothalamic cells
NMDA:	N-Methyl-D-aspartic acid
NR1:	NMDA receptor subunit 1
pERK:	phosphorylated extracellular signal-regulated kinase
pNR1:	phosphorylated NMDA receptor subunit 1