

ORIGINAL RESEARCH—BASIC SCIENCE

Activation of Gastrin-releasing Peptide Receptors in the Lumbosacral Spinal Cord is Required for Ejaculation in Male Rats

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ABSTRACT

Introduction. Ejaculation is a complex reflex mediated by a spinal ejaculation generator located in the lumbosacral spinal cord and consisting of a population of lumbar spinothalamic (LSt) neurons. LSt neurons and their intraspinal axonal projections contain several neuropeptides, including gastrin-releasing peptide (GRP).

Aim. To test the hypothesis that GRP is critically involved in mediating ejaculation by acting in autonomic and motor areas of the lumbosacral spinal cord, utilizing a physiological paradigm to investigate ejaculatory reflexes in isolation of supraspinal inputs.

Methods. Dual immunohistochemistry for GRP and galanin was performed to investigate co-expression of GRP in LSt cells of control male rats. Next, anesthetized, spinalized male rats received intrathecal infusions of either GRP antagonist RC-3095 (0, 10, or 20 nmol/10 μ L) or GRP (0, 0.2, 0.5 nmol/10 μ L). Ejaculatory reflexes were induced by electrical stimulation of the dorsal penile nerve (DPN) which reliably triggers rhythmic increases in seminal vesicle pressure (SVP) and contractions of the bulbocavernosus muscle (BCM), indicative of the emission and expulsion phases of ejaculation, respectively.

Main Outcome Measures. GRP in LSt cells was expressed as percentages of co-expression. SVP and electromyographic recording (EMG) of BCM activity following drug treatment and DPN stimulation were recorded and analyzed for numbers of SVP increases, BCM events and bursts.

Results. GRP was exclusively expressed in LSt cells and axons. Intrathecal infusion of RC-3095, but not saline, blocked SVP increases and BCM bursting induced by DPN stimulation. Intrathecal infusions of GRP, but not saline, triggered SVP increases and BCM bursting in 43–66% of animals and facilitated SVP increases and BCM bursting induced by subthreshold DPN stimulation in all animals.

Conclusion. These data support a critical role for GRP for control of the emission and expulsion phases of ejaculation in male rats by acting in LSt target areas in the lumbosacral spinal cord. **Kozyrev N, Lehman MN, and Coolen LM. Activation of gastrin-releasing peptide receptors in the lumbosacral spinal cord is required for ejaculation in male rats. J Sex Med 2012;9:1303–1318.**

Key Words. Spinal Cord; Gastrin-releasing Peptide; Ejaculation; Reproduction; Autonomic; Motor Neuron

Introduction

Male sexual behavior is a complex and rewarding behavior that ultimately results in ejaculation [1]. Ejaculation is defined as the expulsion of seminal fluid from the urethral meatus [2,3] and comprises two phases: emission and expulsion. Emission refers to the secretion of seminal fluids

from the accessory sex glands, contraction of the ductus deferens, and closure of the bladder neck and external urethral sphincter. Expulsion refers to the forceful ejection of semen, which is produced by the rhythmic contractions of the striated perineal and smooth muscles of the urethra, and the bulbocavernosus muscle (BCM) in particular [4–7].

It is well known that ejaculation is a reflex and that the central components that regulate the ejaculatory reflex are located in the lumbosacral spinal cord [3,8]. Indeed, ejaculatory reflexes remain intact in animals and men with complete spinal cord transection [3,8,9]. Thus, ejaculation is mediated by a spinal control center, referred to as a spinal ejaculation generator [2,3,10], spinal pattern generator [11], or spinal pacemaker [12]. This spinal ejaculation generator is suggested to coordinate sympathetic, parasympathetic, and motor outflow to induce the emission and expulsion phases of ejaculation. In addition, the spinal ejaculation generator is hypothesized to integrate this autonomic and motor outflow with sensory inputs associated with the summation of sexual activity, thereby triggering ejaculation.

The spinal ejaculation generator is composed of a population of interneurons in the central gray of lumbar levels L3–4 that play a pivotal role in the control of ejaculation, and are hypothesized to convert sensory signals into motor or secretory outputs [13] (Figure 1). This population of interneurons is located in lamina 10 and the medial portion of lamina 7 of lumbar segments 3 and 4 (L3–4), contain the neuropeptides galanin, cholecystokinin, gastrin releasing peptide, and enkepha-

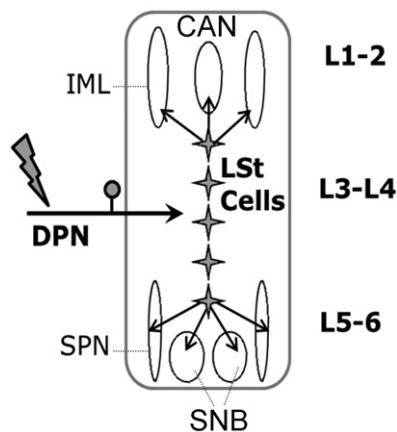


Figure 1 Schematic diagram of the spinal ejaculation generator. Lumbar spinothalamic (LSt) cells receive sensory inputs related to the summation of sexual activity via the dorsal nerve of the penis (DPN), triggering activation of the LSt cells. LSt cells send axonal projections to preganglionic sympathetic neurons in the central autonomic nucleus (CAN) and intermediolateral cell column (IML), to preganglionic parasympathetic neurons in the sacral parasympathetic nucleus (SPN), and to pudendal motorneurons in the sacral nucleus of the bulbocavernosus (SNB). It is hypothesized that the neuropeptides expressed in the axon terminals of the LSt cells trigger ejaculatory reflexes by acting in the LSt target areas.

lin [14–18], and have projections to a nucleus in the thalamus: the parvocellular subparafascicular thalamic nucleus [14,16,17]. Based on their anatomical location in the spinal cord and thalamic projections, these particular cells are referred to as lumbar spinothalamic (LSt) cells [19].

Moreover, LSt cells have additional characteristics that are consistent with their role as a critical component of the spinal ejaculation generator. Mating-related sensory inputs induce activation of LSt cells, thereby triggering ejaculation [8,12,20,21]. These sensory inputs are relayed via the sensory branch of the pudendal nerve, or dorsal penile nerve (DPN) [22,23] (Figure 1). Mating or stimulation of the DPN, which reliably triggers ejaculatory reflexes in anesthetized and spinalized male rats, induces expression of markers of neural activation cFos [17] and phosphorylated extracellular signal-regulated kinases 1 and 2 (pERK) [22] in LSt cells. Moreover, inhibition of mitogen-activated protein (MAP) extracellular signal-regulated kinase (MEK) completely abolished ejaculation induced by DPN stimulation [22]. Therefore, DPN fibers transmit sensory inputs to LSt cells and, in turn, trigger ejaculation via activation of the MAP kinase pathway.

LSt cells are ideally positioned to coordinate the outflow necessary for ejaculation (Figure 1). LSt cells have axonal projections to target regions of the spinal cord implicated in ejaculatory control [24–26]. Specifically, LSt cells project to preganglionic sympathetic neurons in the intermediolateral cell column (IML) and central autonomic nucleus (CAN) of the thoracolumbar spinal cord and to the preganglionic parasympathetic neurons in the sacral parasympathetic nucleus (SPN) of the lumbosacral spinal cord [19,26] and motor neurons in the spinal nucleus of the bulbocavernosus (SNB) [18,25]. LSt cells and their axonal projections contain several neuropeptides, including galanin, cholecystokinin, enkephalin, and gastrin-releasing peptide (GRP) [14–18]. Thus, we hypothesize that these neuropeptides act in the LSt target areas to mediate ejaculatory reflexes. In support of this hypothesis, GRP, a member of the family of bombesin-like peptides [27,28], has been implicated in male erectile and ejaculatory function in spinally-intact males utilizing the *ex copula* reflex paradigm [18]. However, in order to study the role of GRP in ejaculatory reflexes, it is necessary to eliminate supraspinal influences on the spinal ejaculation generator.

Therefore, the purpose of the present study was to test the role of GRP in the control of ejaculation using a physiologically relevant model of ejacula-

tion involving electrical stimulation of the DPN in anesthetized, spinalized male rats. Stimulation of the DPN reliably triggers ejaculation in all mammals, including rats [8], monkeys [29], and humans [30]. Moreover, in anesthetized and spinalized male rats, DPN stimulation elicits rhythmic contractions of the BCM similar to those in observed in a mating animal during ejaculation [20,23], and reflexive penile erections are evident [8,31]. The aim of the present study was twofold. First, it was confirmed that GRP is expressed in LSt cells and their axon terminals in autonomic and motor areas in the lumbosacral spinal cord. Next, it was tested whether GRP receptor activation is required to trigger the emission and expulsion phases of ejaculation and whether GRP is sufficient to trigger ejaculatory reflexes in the absence of DPN stimulation.

Materials and Methods

Animals

Adult male Sprague Dawley rats (4–5 months) were obtained from Charles River (Sherbrook, Quebec, Canada and Wilmington, MA, USA) and were housed in pairs in standard housing cages on a 12-hour light/dark cycle with lights off at 10 PM. Food and water were available *ad libitum*. All procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and conform to the guidelines outlined by the National Institutes of Health.

Experimental Designs

Intrathecal Infusion of GRP Receptor Antagonist RC-3095

Male rats were anesthetized with urethane (1.5 g/kg, i.p.), a laminectomy was performed between the sixth and the eighth thoracic spinal segments and the spinal cord was completely transected. Next, the dorsal penile nerve (DPN) and the muscle of the bulbocavernosus (BCM) were exposed and the surrounding connective tissue was removed. In preparation for electromyographic (EMG) recordings, silver recording electrodes, connected to the PowerLab/7SP Data Acquisition System (AD Instruments, Inc., Colorado Springs, CO, USA) were inserted bilaterally into the BCM and a ground electrode was placed into the muscle of the right thigh. A bipolar stimulating electrode connected to the SD9 Square Pulse Stimulator (Grass Technologies, West Warwick, RI, USA) was positioned directly on top of the DPN. Initial

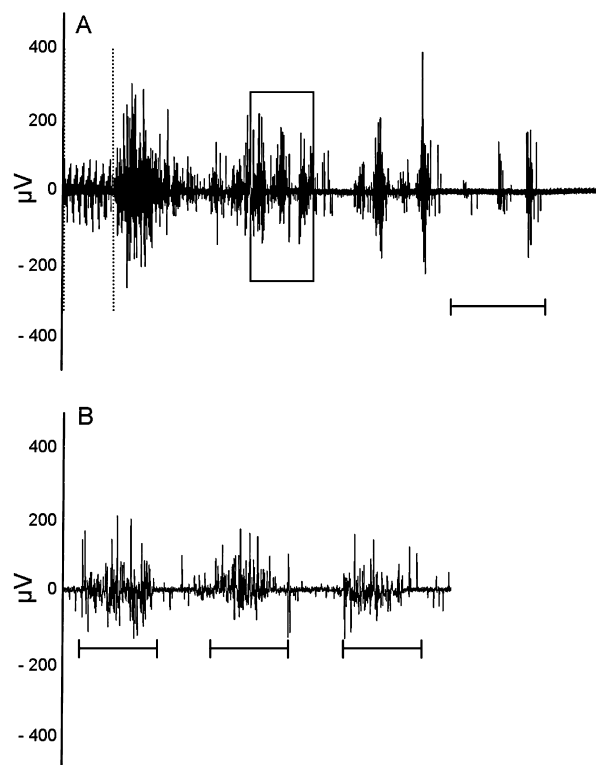


Figure 2 Example of a characteristic BCM EMG activity induced by DPN stimulation. Dashed lines in the EMG trace in (A) represent the end of DPN stimulation followed by 90 seconds of EMG recording showing BCM events (activity above baseline) and clusters of events, i.e., BCM bursts. Time is presented on horizontal scale (Scale bar in A represents 20 seconds) and activity amplitude (in μV) on vertical scale. The three bursts indicated by the box (16 seconds of recording) are shown on an enlarged time scale in (B) to clearly show the clusters of BCM events within bursts (each burst is indicated by a bracket) and the return to baseline activity between bursts.

stimulation of the DPN was performed in order to verify the completeness of the spinal cord transection, indicated by a rhythmic bursting pattern of the BCM (Figure 2). Electrical stimulation of the DPN comprised square wave pulses of 1 ms duration, 4 V at 60 Hz for 10 seconds. These stimulation parameters have previously been demonstrated to trigger rhythmic bursting of the BCM indicative of the expulsion phase of ejaculation in 100% of control animals [22,23]. Experiments started 2 hours following transection of the spinal cord to allow for potential acute effects of spinal cord transection to subside. A second laminectomy was performed at spinal level T10 in preparation for intrathecal administration of neuropeptides. A small incision was made in the dura mater at the site of the second laminectomy and a polyethylene

catheter (Caly-Adams PE-10, Parsippany, NJ, USA) was inserted into the subarachnoid space at lumbar levels, to deliver drugs presumably to the entire lumbosacral spinal cord. First, 10 μ L/minute of saline was infused and BCM EMG activity was recorded for 25 minutes. Afterwards, the DPN was stimulated twice with 5-minute intervals between stimulation, at 30 Hz and 60 Hz in a counterbalanced manner (testing trial 1; control trial). BCM activity was recorded for 90 seconds, the time period for a typical BCM bursting activity [22,23]. One hour later, the procedure was repeated in the same animals, which now received a 10 μ L infusion (over 1 minute) of either 0.9% saline (N = 10) or one of two doses of the GRP receptor antagonist RC-3095, 20 nmol (N = 9) and 10 nmol (N = 6) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). BCM activity was recorded for 25 minutes, after which the DPN was stimulated at 30 Hz and 60 Hz (as described earlier in a counterbalanced manner), and the BCM EMG activity was recorded (testing trial 2; drug trial).

Intrathecal Infusion of GRP²⁰⁻²⁹ Agonist

A separate group of male rats was anesthetized, spinalized, and prepared as described previously. Males received intrathecal 10 μ L infusions of 0.9% saline (N = 11) and BCM EMG was recorded for 10 minutes following infusion. Then, the DPN was stimulated at 5 HZ, 10 HZ, 30 HZ and 60 HZ in a randomized and counterbalanced manner, and BCM EMG was recorded for a period of 90 seconds following stimulation (testing trial 1; control trial). One hour after the last DPN stimulation, the procedure was repeated in the same group of animals, which now received 10 μ L intrathecal infusions of either 0.9% saline (N = 11) or one of two doses of GRP agonist (GRP²⁰⁻²⁹ 0.2 nmol, N = 7; 0.5 nmol, N = 7 [Phoenix Pharmaceuticals Inc. USA, Burlingame, CA, USA]) and BCM EMG was recorded for 10 minutes after infusion of vehicle or drug. Next, the DPN was stimulated at 5, 10, 30, and 60 HZ in a counterbalanced order as described earlier (testing trial 2; drug trial).

BCM EMG Analysis

BCM EMG recordings were analyzed for the 25 or 10 minutes following the infusion of the antagonist or agonist, respectively, and for 90 seconds following each DPN stimulation. The numbers of events and bursts, as well as latencies to first event, were analyzed. The analysis of

numbers of events was performed using LabChart 7 (AD Instruments Inc.), and all events that were above baseline activity were recorded [22]. In addition, numbers of bursts were analyzed by two independent observers blinded to experimental treatment groups. Bursts were defined as a group of 10 or more events, without return to baseline activity between events. These criteria were based on a detailed analysis of the BCM bursting activity in a subgroup of control animals (N = 15). The average number of events per burst, number of bursts, and time intervals between successive bursts during a period of 90 seconds following a 30 or 60 Hz DPN stimulation were calculated. There was an average of 48.56 (ranging from 10–253) or 62.57 (ranging from 10–315) BCM events per burst after 30 and 60 Hz DPN stimulation, respectively. Furthermore, there was a mean of 7.4 bursts (ranging between 3–12 bursts) or 8.07 bursts (ranging between 4–12 bursts) following 30 or 60 Hz resp. Analysis of time (second) between bursts revealed that there was an average of 2.54 seconds between successive bursts (ranging from 0.2–7 seconds).

Effects of antagonist treatments on numbers of events or bursts were compared both within animals (between the two testing trials [control and drug trials] and within the same drug treatment group) and between groups (within each of the two testing trials, and between drug treatment groups), separately for the 30 or 60 Hz stimulations, using two-way repeated ANOVA (factors: Drug treatment and Testing trial) and Holm-Sidak post hoc tests. Effects of agonist treatments on numbers of events or bursts were compared within animals (between the five stimulations [following infusion without stimulation, 5, 10, 30, 60 Hz]) and between treatment groups (within each stimulation setting), separately for each of the two testing trials, using a two-way repeated ANOVA (factors: Drug treatment and Stimulation) and Holm-Sidak post hoc tests. A 95% confidence level was used for all tests.

Immunohistochemistry Experiments

GRP Expression in LSt Cells

In order to verify that LSt cells and their axon terminals in autonomic and motor areas express GRP, eight male rats (four spinal intact and four spinalized 6–8 hours prior) were perfused transcardially with 10 mL 0.9% saline followed by 500 mL of 4% paraformaldehyde in phosphate buffer (0.1 PB). Spinal cords were removed and postfixed for 1 hour in the same fixative and subsequently placed in cryoprotective solution (20% sucrose in 0.1 PB

with 0.01% sodium azide) until further processing for immunohistochemical detection of GRP and galanin, a marker for LSt cells [13,17,20,22,23].

DPN Stimulation-induced pERK after RC-3095

To verify that GRP antagonist did not prevent activation of LSt cells, expression of phosphorylation of ERK (pERK) in LSt cells was examined following stimulation of the DPN [22]. Male rats (N = 6) were anesthetized and spinalized as described above. Two hours after spinalization, the DPN and BCM were exposed and vehicle (saline, N = 3) or drug (RC-3095; Sigma-Aldrich Canada Inc.; 20 nmol; N = 3) were infused in a volume of 10 μ L intrathecally as described previously. Twenty-five minutes later, the DPN was stimulated at 30 Hz and BCM EMG activity was recorded for 90 seconds. Five minutes following DPN stimulation, animals were perfused transcardially with 5 mL 0.9% saline solution and 500 mL of 4% paraformaldehyde in 0.1 M PB. Spinal cords were removed and postfixed for 1 hour in the same fixative and subsequently placed in cryoprotective solution (20% sucrose in 0.1 PB with 0.01% sodium azide) until further processing for immunohistochemical detection of pERK and galanin. The stimulation parameters and time of perfusion were previously shown to be optimal for visualization of pERK expression in LSt cells [22]. Moreover, it was previously shown that spinalization and exposure of DPN and BCM without electrical stimulation of the DPN did not induce pERK expression in LSt cells [22], hence negative control groups were not included in this experiment.

Immunohistochemistry

Spinal cords were 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 PB with 0.01% sodium azide) and stored at -20°C until further processing. Free floating sections containing lower thoracic, lumbar, and sacral spinal levels were thoroughly rinsed in 0.1 M saline buffered sodium phosphate (PBS) between incubations and blocked with 1% H_2O_2 for 10 minutes prior to incubation. All antibody incubations were performed in incubation solution containing 0.1% bovine serum albumin and 0.4% Triton X-100 (BP151-500, Thermo Fisher Scientific, Ottawa, Ontario, Canada) in PBS at room temperature with gentle agitation.

Galanin/GRP Dual Fluorescence

One series of sections for four animals (two spinal intact and two spinalized) were dual stained for galanin and GRP. Sections were incubated overnight with rabbit anti-galanin (1:60,000; T-4334; Bachem, Torrance, CA, USA) and with biotinylated goat anti-rabbit for one hour (1:500; Vector Laboratories, Burlingame, CA, USA), avidin horseradish peroxidase complex (ABC-elite, 1:1,000 in PBS; 1 hour; Vector Laboratories), biotinylated tyramine (BT; 1:250 in PBS containing 1 $\mu\text{L}/\text{mL}$ of 3% H_2O_2 for 10; NEL700/700A; PerkinElmer Life Sciences, Boston, MA, USA), and Alexa 488-conjugated streptavidin (1:100 in PBS, 30 minutes (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Next, sections were incubated with rabbit anti-GRP (1:2,000; Phoenix [H-027-13]) and Alexa 555-conjugated goat anti-rabbit for 30 minutes in PBS (1:100; Jackson ImmunoResearch Laboratories) and mounted on plus charged slides, cover-slipped with gelvatol and stored in the dark at 4°C . One series of sections of each of four animals (two spinal intact and two spinalized) was stained using this same procedure, but were first processed for GRP immunoreactivity (-ir; using rabbit-anti-GRP; 1:40,000) and subsequently for galanin (rabbit anti-galanin; 1:3,000).

Galanin/pERK Dual Fluorescence

One series of sections were processed using the same protocol as described earlier using rabbit anti-pERK (1:1,000; overnight; Cell Signaling # 26 L) and rabbit anti-galanin (1:3,000).

For all procedures, the omission of primary antibodies resulted in a complete loss of signal at specific wavelengths and all primary antibodies have been previously characterized [17,22,23].

Data Analysis

Analysis for Galanin/GRP Co-localization

Sections of the lumbosacral spinal cord containing LSt cells were analyzed for expression of GRP using a Leica DM5000B (Leica Microsystems, Wetzlar, Germany). In all eight animals, included in the analysis, all galanin-ir and CGRP-ir cells were analyzed for co-expression of GRP-ir or galanin-ir, respectively. The percentages of galanin-ir co-expressing GRP and vice versa were determined for each animal and a group mean was calculated. In addition, the co-localization of galanin and GRP was analyzed in LSt axons in the intermediolateral cell column (IML), central autonomic nucleus (CAN), sacral parasympathetic nucleus (SPN), and spinal nucleus of the bulbocav-

ernosus (SNB) using a Zeiss laser-scanning confocal microscope system (Zeiss LSM510; Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Alexa 555-fluorescence was imaged using a 567 nm emission filter and a HeNe laser and Alexa 488 was imaged using a 505 nm filter and Argon laser. Images were generated by projecting several 1 μm optical sections collected at 1 μm intervals through areas of interest in the z-axis using a 63x water-immersion objective.

pERK Expression in LSt Cells

pERK expression in LSt cells was analyzed on a DM5000B Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Specifically, all lumbar cells expressing galanin-ir were analyzed for expression of pERK. Data are expressed as the mean percentages of LSt cells that express pERK for each animal. The group means were calculated and compared between animals that received an infusion of RC-3095 (20 nmol) and saline-treated controls, using Student *t*-tests with 95% confidence levels.

Pressure Recording in Seminal Vesicles

Based on findings from the above described experiments, an additional experiment was conducted to analyze pressure in the seminal vesicles (SV), which is considered an indicator of the emission phase ([32]). Spinal cords were transected, and BCM and DPN were exposed as described above. In addition, the right SV was exposed by coeliotomy. SV pressure (SVP) was measured using a pressure catheter (AD Instruments Inc., model number: SPR-671(1.4 F, Single, Straight, 15 cm, Ny) attached to a catheter interface cable (AD Instruments Inc., model number: AEC-IOD) and connected to a Bridge AMP (AD Instruments Inc.). The pressure catheter was inserted into the lumen of the SV and secured prior to recording. SVP and BCM EMG (as described earlier) were simultaneously recorded and analyzed using LabChart 7 (AD Instruments Inc.). There were three groups of animals ($N = 3$ per group). The first group of control animals received DPN stimulation at 5, 10, 30, and 60 Hz 10 minutes after intrathecal saline infusion (control Trial 1) and identical treatment in trial 2 (control Trial 2). The second group of animals received intrathecal saline and DPN stimulation at 30 and 60 Hz 25 minutes later (control Trial 1), and subsequent intrathecal RC-3095 (20 nmol; Trial 2; drug trial) again followed by DPN stimulation at 30 and 60 Hz 25 minutes after each infusion. The third group of animals received intrathecal saline and DPN stimulation at 5, 10, 30, and 60 Hz 10 minutes later (Trial 1; control trial) and subsequent

GRP agonist (GRP²⁰⁻²⁹; Trial 2; drug trial) followed by DPN stimulation at 5, 10, 30, and 60 Hz 10 minutes after each infusion.

Analysis: BCM EMG was analyzed as described and numbers of bursts were compared within animals and between trials 1 and 2 using *t*-tests or the Mann-Whitney–Wilcoxon test. SVP was analyzed using LabChart7 (AD Instruments Inc.). SVP increased simultaneously with BCM EMG bursts and the numbers of times that increased SVP occurred were counted and compared within animals between trials 1 and 2 using *t*-tests with confidence levels of 95%.

Results

GRP Expression in LSt Cells and Axons

GRP was co-expressed by all LSt cells, based on analysis of 119 galanin-IR cells in eight animals (Figure 3). Moreover, 100% GRP-IR neurons in the lumbosacral spinal cord co-expressed galanin-IR, suggesting that GRP is exclusively expressed by LSt cells. There was no effect of spinal transection on co-expression of galanin and GRP-ir. In addition, qualitative analysis showed that virtually all galanin-IR axons in IML, CAN, SPN, and SNB co-expressed GRP (Figure 3). Hence, GRP may be released in autonomic and motor areas to mediate ejaculation. However, a few GRP-IR fibers that did not co-express galanin-IR were detected dorsal to the SPN (Figure 3J–L), raising the possibility that the SPN also receives a minor GRP input from cells other than LSt neurons and outside the lumbosacral spinal cord.

GRP Receptor Antagonist Blocked Ejaculatory Reflexes

GRP receptor antagonist RC-3095 significantly reduced DPN stimulation-induced ejaculatory reflexes at both 30 and 60 Hz stimulation frequencies as evidenced by the reduced numbers of BCM events and bursts (Figure 4C–F). There were main effects of testing trial on the numbers of BCM events for both 30 ($F(1, 43) 13.760$; $P = 0.002$; Figure 4C) and 60 Hz stimulation frequencies ($F(1, 41) 12.727$; $P = 0.003$; Figure 4D). Post hoc analyses revealed that animals treated with either dose of RC-3095 during the second, drug trial (trial 2; Figure 4B) had significantly decreased BCM events in response to 30 and 60 Hz DPN stimulation compared to their DPN stimulation-induced BCM events following saline treatment in their first control trial (trial 1; 60 Hz: $P = 0.008$ (10 nmol); $P = 0.040$ (20 nmol); 30 Hz: $P = 0.022$

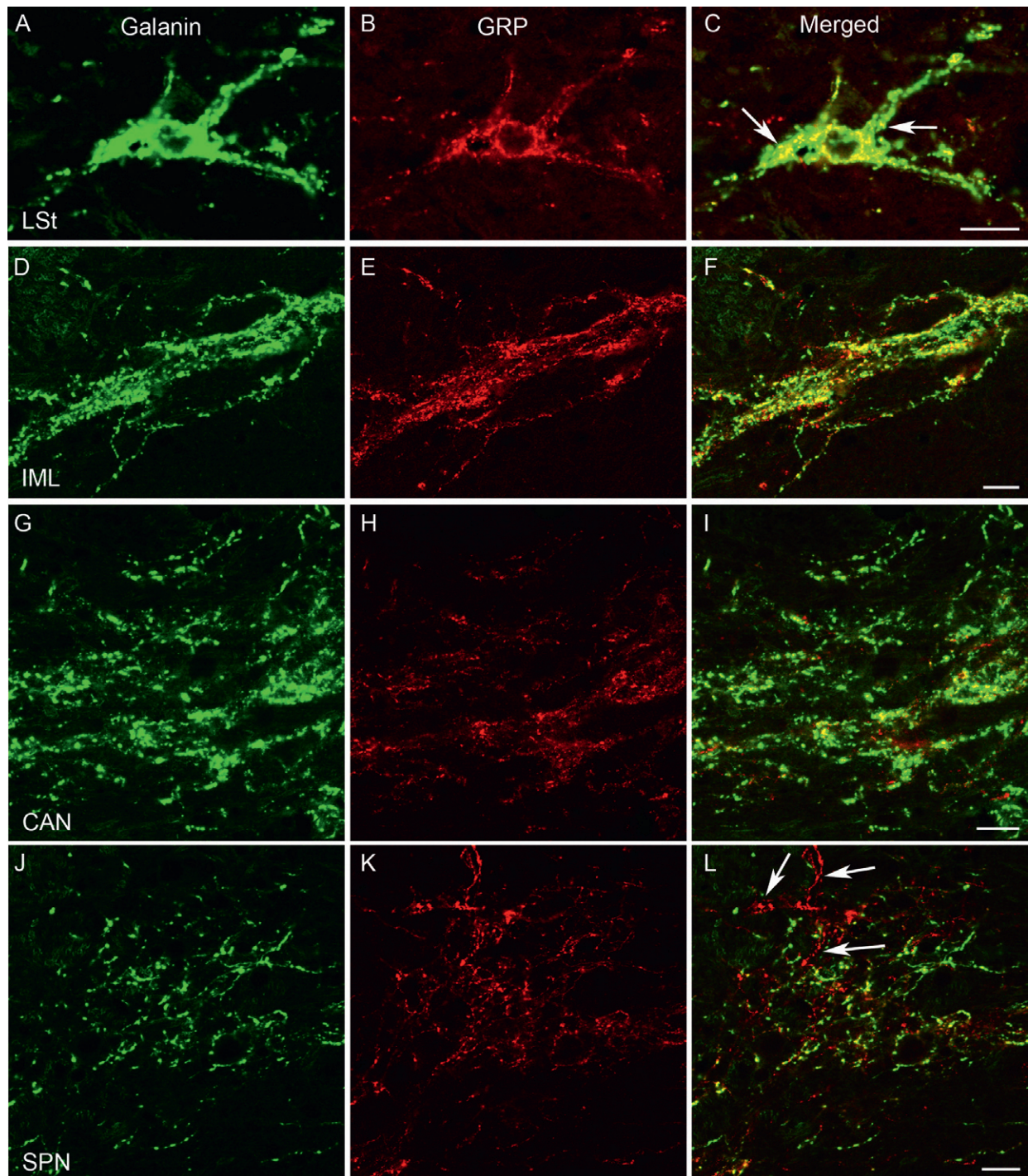


Figure 3 Confocal images (1 μm optical sections) illustrating the co-expression of galanin-ir (A) and GRP-ir (B) in LSt cells (A–C), IML (D–F), CAN (G–I), and SPN (J–L) in a spinal intact male rat. Galanin-ir was immunoprocessed using TSA amplification, while GRP was immunoprocessed using a conjugated secondary antibody. Arrows in (C) indicate putative close contacts of galanin/GRP-ir axon terminals with LSt cells. Arrows in L indicate GRP-ir fibers dorsal to the SPN that do not co-express galanin-ir. Scale bars indicate 20 μm .

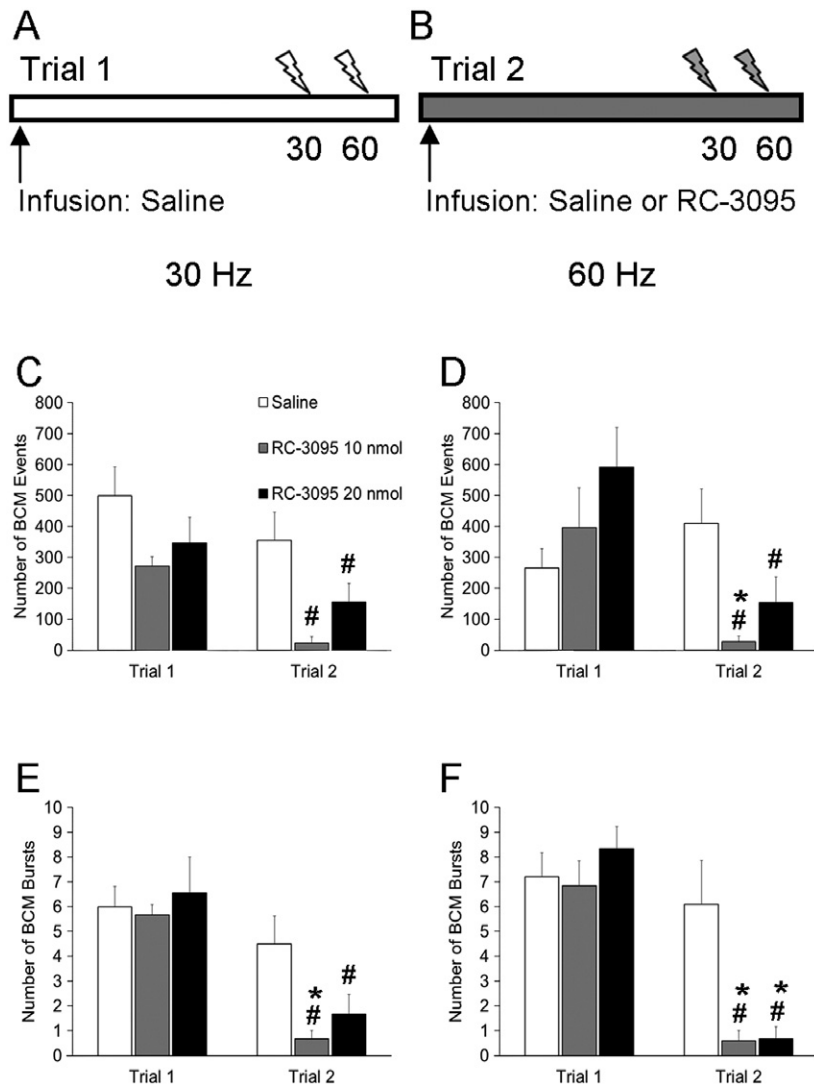


Figure 4 Quantitative analysis of BCM events and bursts following infusions with GRP receptor antagonist RC-3095 During Trial 1 (A; control trial), all groups received saline infusions (arrow) and 30 and 60 Hz DPN stimulation (bolt arrows; in counterbalanced manner). During Trial 2 (B; drug trial), the same groups received saline or RC-3095 (10 or 20 nmol) infusions (arrow) followed by 30 and 60 Hz DPN stimulation (bolt arrows; in counterbalanced manner). Numbers of BCM events are shown in (C) (30 Hz) and (D) (60 Hz); while numbers of BCM bursts are shown in (E) (30 Hz) and (F) (60 Hz). # denotes significant differences from Trial 1 within the same treatment group. * indicates significant differences from controls within the same testing trial.

(10 nmol); $P = 0.029$ (20 nmol); Figures 4C, D and 5C, D). There were no significant differences between the two groups treated with the lower and higher doses of RC-3095. Saline-treated animals did not differ in their BCM events during trial 2 compared to trial 1 (30 Hz: $P = 0.130$; 60 Hz: $P = 0.280$, Figures 4C, D and 5A, B), demonstrating that the reduction in BCM events was not an effect of repeated testing, but rather of RC-3095 treatment. Similarly, there were main effects of testing trial on the numbers of BCM bursts for both 30 Hz ($F(1,48) = 56.209$; $P < 0.001$; Figure 4E) and 60 Hz ($F(1,48) = 41.878$; $P < 0.001$; Figure 4F) stimulation frequencies. Males treated with either dose of RC-3095 during trial 2 (drug trial) had significantly lower numbers of BCM bursts compared to trial 1 (control trial) with saline treatment (30 Hz: $P < 0.001$ [10 nmol],

60 Hz: $P < 0.001$ [20 nmol]). Once again, there was no effect of the dosage of the antagonist and of repeated testing, as saline-treated animals did not differ in the numbers of BCM bursts during the second compared to the first testing trial (Figure 4E, F).

A significant interaction between drug treatment and testing trial was detected for numbers of BCM events at the 60 Hz stimulation ($F(2,48) = 6.941$; $P = 0.005$). Post hoc analyses demonstrated that during trial 2 (drug trial), animals treated with 10 nmol RC-3095 had significantly fewer numbers of BCM events induced by 60 Hz stimulation compared to saline controls ($P = 0.004$; Figure 4D). A trend towards significance was observed in animals treated with the higher dose of RC-3095 (20 nmol) and received 60 Hz DPN stimulation ($P = 0.06$; Figure 4D).

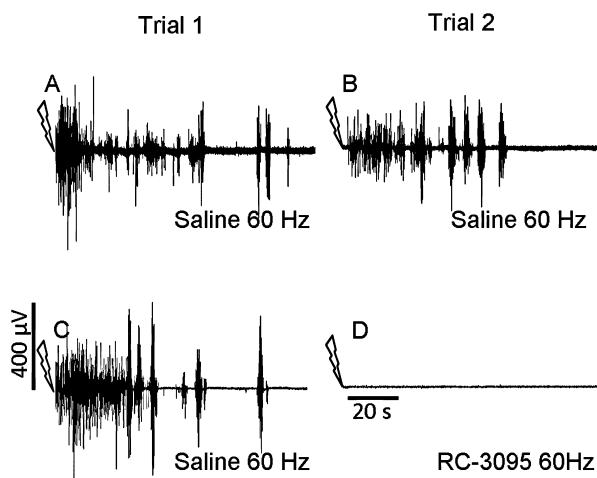


Figure 5 Representative EMG recording traces depicting BCM activity induced by 60 Hz DPN stimulation (indicated by arrow) following intrathecal infusions of saline or GRP antagonist RC-3095. DPN stimulation induced characteristic BCM bursting following infusion of saline in trial 1 (A, C) and again following saline in trial 2 (B; same animals as A). In contrast, infusion of the GRP antagonist RC-3095 (20 nmol) in trial 2 completely prevented BCM activity (D; same animal as C). Each trace represents 90 seconds of BCM activity.

Interactions between drug treatment and testing trial were also detected for numbers of BCM bursts at 30 Hz ($F(2,48) = 5.250$; $P = 0.014$) and 60 Hz ($F(2,48) = 6.941$; $P = 0.005$) stimulations. Specifically, during trial 2 (drug trial), animals treated with the lower dose of RC-3095 had significantly lower numbers of BCM bursts compared to saline controls for both 30 Hz ($P = 0.01$; Figure 4E) and 60 Hz ($P < 0.001$; Figure 4F) stimulations and animals treated with the higher dose had fewer BCM bursts compared to saline controls following 60 Hz stimulation ($P = 0.003$; Figure 4F). There were no significant differences between the two groups treated with the lower and higher doses of RC-3095. Finally, there were no differences in numbers of BCM events nor bursts between groups during the first control trial, when

all groups were treated with saline (Figure 4C–F), demonstrating that group differences during the second testing trial were caused by drug treatments, rather than differences in BCM bursting responses.

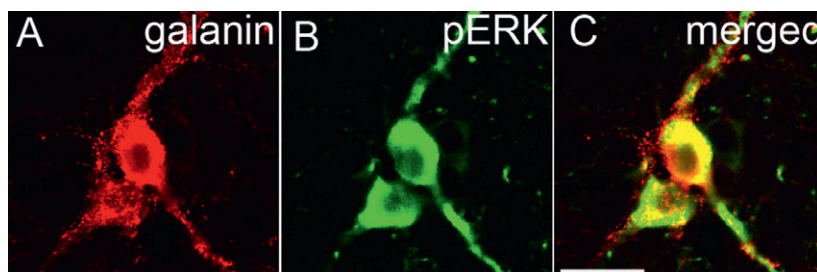
RC-3095 Did Not Affect DPN Stimulation-Induced pERK in LSt Cells

In order to verify that GRP antagonist RC-3095 blocks BCM bursting via actions in LSt target areas rather than acting directly on LSt cells, DPN stimulation-induced neural activation of LSt cells was examined. In both antagonist-treated and control males, DPN stimulation induced pERK in $97 \pm 0.03\%$ of LSt cells, similar to the activation reported previously in control males [22]. Thus, GRP receptor antagonist treatment did not affect the activation of LSt cells in response to DPN stimulation (Figure 6).

GRP^{20–29} Agonist Facilitated BCM Activity

During the first testing trial (control trial), there was an overall effect of DPN stimulation parameters on the number of BCM events ($F(4, 121) = 28.878$; $P < 0.001$) and bursts ($F(4, 121) = 54.775$; $P < 0.001$). Post hoc tests showed that following 30 Hz DPN stimulation, all treatment groups had significantly increased numbers of BCM events (compared to infusion, 5 Hz, or 10 Hz, $P < 0.001$; Figure 7C), and bursts (compared to infusion, 5 Hz, or 10 Hz, $P < 0.001$; Figure 7E). DPN stimulation at 60 Hz also increased numbers of BCM events and bursts (compared to infusion, 5 Hz, or 10 Hz, $P < 0.001$; Figure 7C, E). These data confirm that 30 and 60 Hz stimulations reliably trigger BCM bursting while 5 and 10 Hz represent sub-threshold levels of stimulation. Moreover, saline infusions alone (without DPN stimulation) did not trigger any BCM activity (Figures 7C, E, and 8E). Finally, during control trial 1, there were no differences between treatment groups in the numbers of BCM events nor bursts (Figure 7C, E), demonstrating

Figure 6 Confocal images (1 μm optical sections) demonstrating pERK expression (B) in LSt cells (A) induced by DPN-stimulation (merged image in C). The pattern of pERK labeling is representative of all antagonist- and control-treated animals. Scale bar indicates 20 μm .



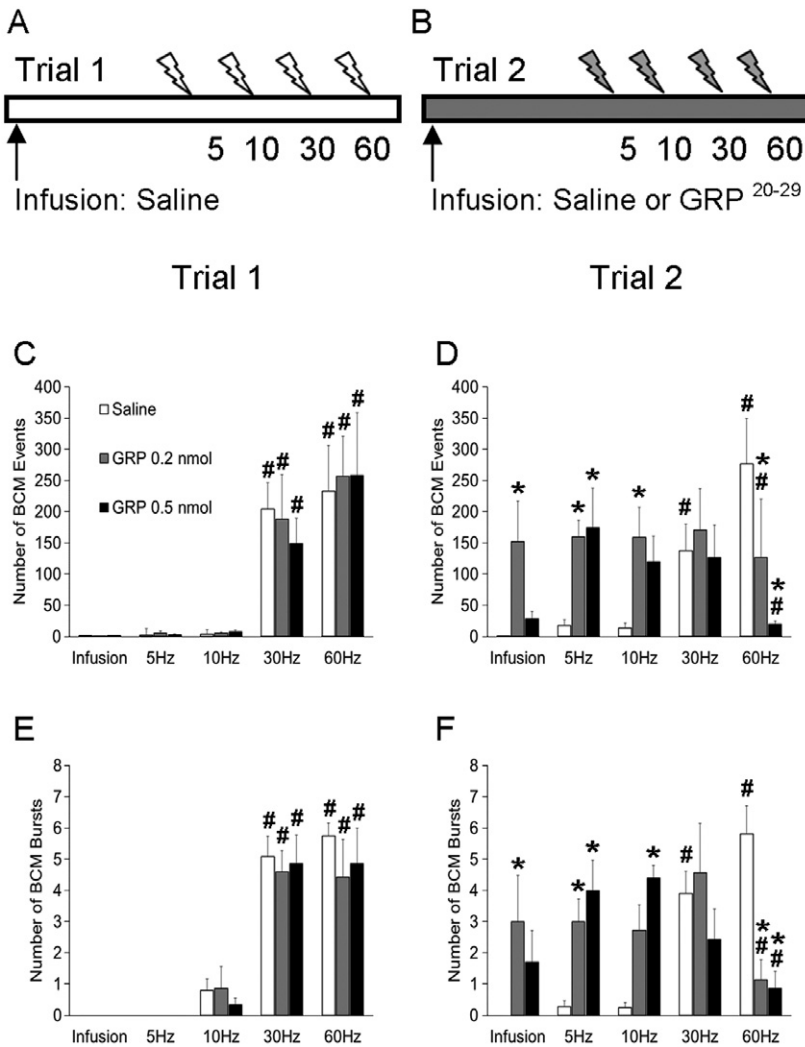


Figure 7 Quantitative analysis of BCM events and bursts following infusions with GRP²⁰⁻²⁹. During Trial 1 (A; control trial) all groups received intrathecal saline (arrow) followed by four DPN stimulations (bolt arrows) at 5–60 Hz (in counterbalanced manner). During Trial 2 (B; drug trial), all groups received intrathecal GRP²⁰⁻²⁹ (10 or 20 nmol; arrow) again followed by four DPN stimulations at 5–60 Hz (in counterbalanced manner). Numbers of events are shown in (C) (Trial 1) and (D) (Trial 2), while numbers of bursts are shown in (E) (Trial 1) and (F) (Trial 2). # denotes significant differences from BCM activity following infusion, 5 Hz, and 10 Hz within the same treatment group and trial, * indicates significant differences from the saline control group within the same DPN stimulation and trial.

that there were no initial group differences in BCM bursting responses and that repeated DPN stimulations in the randomized order did not affect BCM responses to subsequent stimulations.

During the second testing trial (drug trial), there were overall effects of DPN stimulations on BCM bursts ($F(4,120) = 2.795$, $P = 0.031$) and there were significant interactions between stimulations and drug treatments for events ($F(8, 119) = 5.048$; $P < 0.001$) and bursts ($F(8,120) = 8.053$; $P < 0.001$). First, the GRP²⁰⁻²⁹ receptor agonist significantly triggered BCM activity in the absence of DPN stimulation in a subset of animals (43% of 0.2 nmol and 43% of 0.5 nmol; Figures 7D, F and 8F). Latencies to BCM activity were 149 ± 13.66 (0.2 nmol) and 148 ± 5.69 (0.5 nmol) seconds post infusion. Comparisons of numbers of BCM events and bursts showed a significant increase in BCM activity in animals treated with the low dose of

GRP²⁰⁻²⁹ (0.2 nmol) compared to saline controls (events: $P = 0.015$; bursts: $P = 0.008$) and a trend toward significance compared to animals treated with the high dose of GRP²⁰⁻²⁹ (0.5 nmol; events: $P = 0.07$; Figure 7D, F). These results indicate that in a portion of the animals, the low dose of GRP²⁰⁻²⁹ was sufficient for triggering ejaculatory reflexes in the absence of sensory stimulation.

Furthermore, intrathecal infusions of GRP²⁰⁻²⁹ facilitated ejaculatory reflexes in response to subthreshold levels of DPN stimulation (5–10 Hz) as evidenced by the increased numbers of events and bursts (Figures 7D, F, and 8D). Post hoc analyses revealed significantly increased BCM events in response to DPN stimulation in animals treated with low dose of GRP²⁰⁻²⁹ (0.2 nmol) at 5 Hz ($P = 0.022$) and 10 Hz ($P = 0.042$) compared to saline-treated control animals (Figure 7D). Similarly, animals treated with the high dose of GRP²⁰⁻²⁹

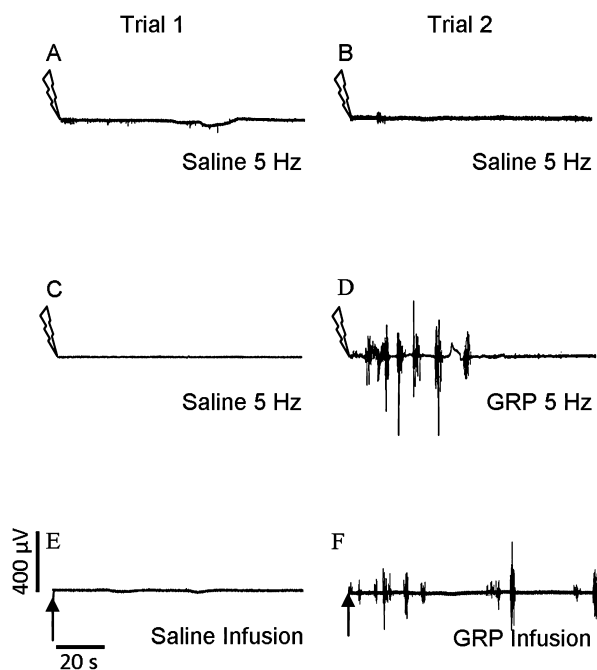


Figure 8 Representative EMG recording traces showing BCM activity induced by subthreshold stimulation of the DPN (5 Hz; indicated by bolt arrows) following intrathecal infusion of saline (A–C) or GRP^{20–29} (D). Subthreshold DPN stimulation did not induce BCM activity following intrathecal infusions of saline in trial 1 (A, C), or following saline in trial 2 (B; same animal as A). In contrast, subthreshold DPN stimulation following intrathecal infusion of GRP^{20–29} (0.2 nmol) did induce BCM activity (D; same animal as C). Infusion of GRP^{20–29} (0.2 nmol) (F) but not saline (E; same animal as F) also induced BCM activity without DPN stimulation. Each trace represents 90 seconds of BCM activity.

(0.5 nmol) demonstrated significantly increased BCM events in response to 5 Hz DPN stimulation ($P = 0.012$; Figure 7D). Animals treated with the low dose of GRP^{20–29} (0.2 nmol) also had significantly more BCM bursts in response to 5 Hz DPN stimulation ($P = 0.016$) and a trend to significance at 10 Hz ($P = 0.041$) compared to saline-treated animals (Figure 7F). Similarly, animals treated with the high dose of GRP^{20–29} (0.5 nmol) demonstrated significantly increased BCM bursts after 5 Hz ($P = 0.001$) and 10 Hz ($P = 0.012$) DPN stimulation compared to saline controls (Figure 7F).

GRP^{20–29} treatment did not affect BCM activity at 30 Hz stimulation. But, unexpectedly, GRP agonist treatment significantly decreased BCM activity in response to high levels of DPN stimulation (60 Hz) at both the lower dose (events: $P = 0.016$; bursts $P < 0.001$) and the higher dose (events: $P < 0.001$; bursts: $P < 0.001$) of GRP^{20–29} compared to saline-treated control animals (Figure 7D–F). Finally, saline treatment did not

affect increased BCM events and bursts following 30 Hz (Events; infusion: $P = 0.007$, 5 Hz: $P = 0.017$, 10 Hz: $P = 0.037$; Bursts; infusion: $P < 0.001$, 5 Hz: $P < 0.001$, 10 Hz: $P = 0.001$) or 60 Hz (Events; infusion, 5 Hz, 10 Hz: $P < 0.001$, 30 Hz: $P = 0.006$; Bursts; infusion, 5 Hz, 10 Hz: $P < 0.001$) DPN stimulation, confirming that there was no effect of repeated DPN stimulations (Figure 7D–F).

GRP Mediates SVP Increases Following DPN Stimulation

The findings described above confirm a role for GRP in the expulsion phase of ejaculatory reflex. Next, we determined the role of GRP for increases in SVP, indicative of the emission phase. Since effects of DPN stimulation on SVP have not yet been documented, first saline-treated controls were analyzed. DPN stimulation triggered SVP increases that were concurrent with BCM EMG bursts in saline treated animals (Figure 9). There were no significant differences in the numbers of SVP increases following DPN stimulation between trial 1 and trial 2 in animals treated with saline (at 5, 10, 30, or 60 Hz; Table 1), showing lack of effect of repeated DPN stimulation. Similarly, and in concordance to the results described above, there were no significant differences in the numbers of BCM bursts induced by DPN stimulation between trial 1 and trial 2 in saline treated animals (Table 1). The frequency of SVP increases was closely coupled to the numbers of BCM bursts. Each SVP increase was accompanied by a BCM burst, although a number of DPN-stimulation induced BCM bursts occurred in the absence of SVP increases, in animals that received 30 or 60 Hz DPN stimulation. In the absence of BCM activity following subthreshold levels of DPN stimulation (Trial 1: control trial; saline infusion, 5 Hz, and 10 Hz) there was a similar absence of SVP increases (Table 1).

GRP appears to be critically involved in the regulation of both BCM EMG activity and SVP increases (Table 1, Figure 10). Indeed, RC-3095 (20 nmol) treatment completely blocked both BCM EMG bursts (confirming results described above) and SVP increases in response to 30 Hz (BCM: $P < 0.001$; SVP: $P = 0.03$) and 60 Hz (BCM: $P = 0.001$; SVP: $P = 0.052$) DPN stimulation (Table 1). Moreover, in 2/3 animals (66%), GRP^{20–29} triggered BCM bursts and SVP increases in the absence of DPN stimulation (Table 1, Figure 10). In addition, GRP^{20–29} facilitated BCM bursts and SVP increases in response to subthreshold levels: 5 Hz (BCM: $P =$

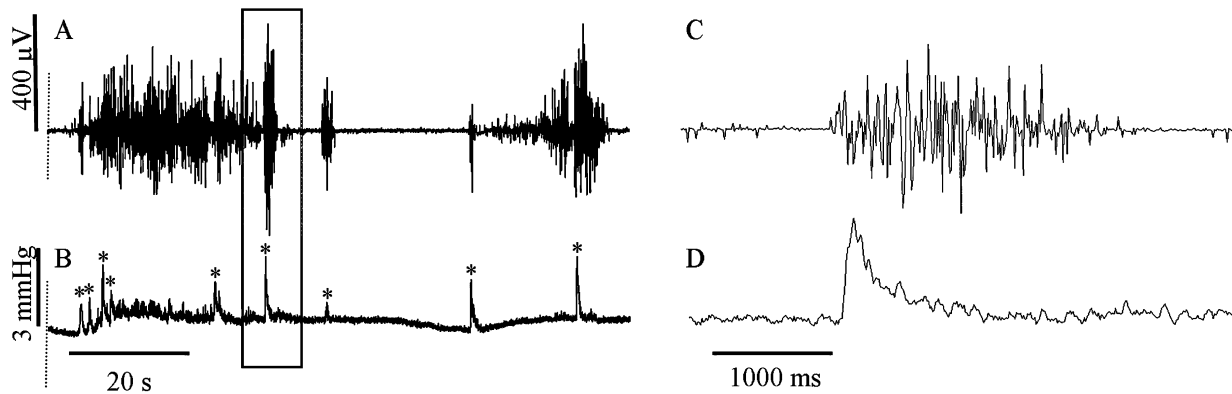


Figure 9 A representative example of a simultaneous recording of BCM activity and SVP (90 seconds duration) after 60 Hz DPN stimulation 10 minutes following intrathecal saline infusion. The dotted lines in A and B indicate the end of DPN stimulation. Stars in (B) indicate SVP increases. A fragment of the trace indicated in the box area in A and B is enlarged in C and D to clearly show a BCM burst (C) coupled with a single increase in SVP (D) over 4 seconds of recording.

0.004; SVP: $P = 0.005$) and 10 Hz (BCM: $P = 0.005$; SVP: $P = 0.013$) of DPN stimulation (Table 1).

Discussion

The findings of this set of studies support the hypothesis that GRP plays a critical role in the control of ejaculation by acting in autonomic and motor areas targeted by LSt axons. GRP was found to be present in all LSt cells and LSt axon projections within the lumbosacral spinal cord. As predicted, intrathecal administration of GRP

receptor antagonist RC-3095 severely disrupted both the emission and expulsion phases of ejaculation in response to sensory stimulation, reflected in the reduced numbers of SVP increases and BCM activity in response to DPN stimulation in RC-3095-treated male rats. In contrast, intrathecal infusion of GRP²⁰⁻²⁹ facilitated both the emission and expulsion phases of ejaculation, as evidenced by the surge in the numbers of SVP increases and BCM bursts respectively in male rats treated with GRP²⁰⁻²⁹. In particular, GRP²⁰⁻²⁹ was most effective in facilitating ejaculation after sub-

Table 1 Numbers of seminal vesicle pressure (SVP) increases and BCM bursts in animals treated with saline-saline (N = 3), saline-RC3095 (20 nmol; N = 3) or saline-GRP (0.2 nmol; N = 3) in trial 1-trial 2, respectively, and with DPN stimulation at 0, 5, 30, or 60 Hz frequencies

Saline-saline (N = 3)	Trial 1: Numbers of SVP	Trial 2: Numbers of SVP	Trial 1: Numbers of BCM bursts	Trial 2: Numbers of BCM bursts
Infusion	0	0	0	0
5 Hz	0	0	0	0
10 Hz	0	0	0	0
30 Hz	3.33 ± 1.43	2.33 ± 0.94	8 ± 1.08	8.33 ± 0.62
60 Hz	4 ± 0.71	2.33 ± 1.03	8.33 ± 0.24	9.33 ± 0.62
Saline-RC3095 20 nmol (N = 3)	Trial 1: Numbers of SVP	Trial 2: Numbers of SVP	Trial 1: Numbers of BCM bursts	Trial 2: Numbers of BCM bursts
30 Hz	4.66 ± 1.86	0*	6.66 ± 0.47	0*
60 Hz	3.66 ± 2.03	0 ($P = 0.052$)	8 ± 0.71	0*
Saline-GRP 0.2 nmol (N = 3)	Trial 1: Numbers of SVP	Trial 2: Numbers of SVP	Trial 1: Numbers of BCM bursts	Trial 2: Numbers of BCM bursts
Infusion	0	3.66 ± 1.89	0	5 ± 2.86
5 Hz	0	1.33 ± 0.24*	0	4.33 ± 0.62*
10 Hz	0	2 ± 0.41*	0	4 ± 0.71*
30 Hz	1 ± 0.41	3.33 ± 0.47*	7.33 ± 0.62	6.66 ± 0.62
60 Hz	4.33 ± 2.09	2 ± 0.41	8 ± 0.41	6.33 ± 0.62

*Indicates significant difference from trial 1 within the same treatment group and stimulation parameter

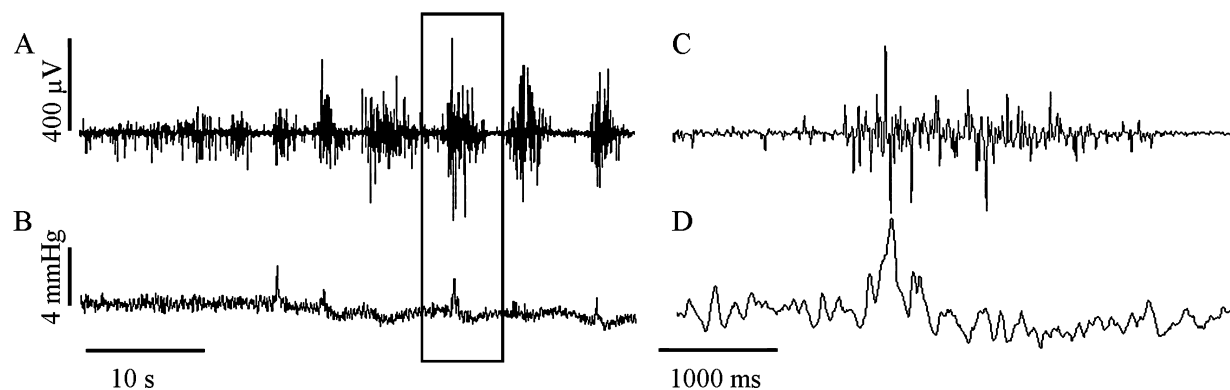


Figure 10 Simultaneous recording of BCM activity (A) and SVP (B) in one animal (45 seconds of EMG recording) 145 seconds after an intrathecal infusion of GRP²⁰⁻²⁹ (0.2 nmol). The traces in A and B are magnified to show one BCM burst (C) occurring simultaneously with an increase in SVP (D).

threshold sensory stimulation (5–10 Hz), signifying the presence of a cumulative effect of GRP receptor activation by intrathecal infusion of GRP and the release of endogenous GRP or other neuropeptides in response to stimulation of the DPN.

GRP is a bombesin-like peptide that is widely distributed in the central nervous system of mammals [33] and is involved in many physiological processes including the suppression of feeding behavior [34], circadian rhythms [35–37] and itching behavior [38]. In the lumbosacral spinal cord, GRP was shown here to be exclusively expressed by LSt cells, as 100% of LSt neurons, identified by immunoreactivity for galanin, co-expressed GRP and vice versa. Moreover, virtually all galanin-IR LSt axons in the autonomic and motor areas co-expressed GRP. This finding is in slight contrast with a previous report of a subpopulation of GRP-IR neurons that do not co-express galanin dorsal to the central canal in the lumbar spinal cord [18]. The discrepancy is possible due to differences in the sensitivity of immunohistochemical detections: in the present study, two different immunohistochemistry protocols were performed in which either one of the neuropeptide signals were amplified for optimal sensitivity of peptide detection. Results from both protocols were identical and complete colocalization of galanin- and GRP-ir was found in soma and axon terminals. Moreover, the present study utilized spinal cord tissues from both spinal intact and spinal transected male rats, thereby correcting for the possibility of supraspinal influences on galanin or GRP expression. A few GRP-IR fibers that did not co-express galanin-IR were observed in the region of and dorsal to the SPN, suggesting that the SPN receives inputs from GRP

neurons outside of the spinal ejaculation generator and the lumbosacral spinal cord. Sources of GRP indeed exist outside of the spinal ejaculatory network [33]. The SPN is known for its role in erectile function [39–41] and emission [26,42] and destruction of sacral autonomic centers abolishes reflex erections [43] but a specific role for SPN in the expulsion phase of ejaculation has not been documented. Therefore, it is likely that GRP axons located dorsally to the SPN are predominantly involved in erectile function and emission, but not expulsion [31,41,44]. Indeed, one of the sources of GRP outside of the spinal ejaculation is the paraventricular nucleus of the hypothalamus [33], an area well known to be involved in erectile function [45,46] and to project to the lumbosacral spinal cord [41,47]. It is currently unknown if GRP in the PVN or its projections mediate erection, and this possibility can be tested in future studies.

The expression of GRP in LST cells and projections is consistent with the proposed role of GRP in control of ejaculation via action in autonomic and motor areas in the lumbosacral spinal cord (see Figure 3). Moreover, intrathecal injections of GRP receptor antagonist RC-3095 blocked BCM bursting in response to DPN stimulation. It is assumed that RC-3095 acted primarily in the areas of the spinal cord that are located adjacent to the pia-arachnoid space, including all LSt target areas. Indeed, GRP receptor expression has been reported in the SPN and SNB of the lumbosacral spinal cord [18], however its expression in other LSt target regions, including the CAN and IML requires further investigation. Another possibility is that GRP or GRP antagonist treatment acts directly on the LSt cells. Galanin- and GRP-ir axon terminals arising from LSt cells that make putative

contacts with LSt cells have previously been observed (see Figure 5; [17]) and thus GRP may be released directly onto LSt cells to control ejaculation. However, RC-3095 treatment did not decrease neural activity of LSt cells, indicated by pERK expression, substantiating that RC-3095 likely blocks the ejaculatory reflex by acting on GRP receptors downstream of the LSt cells, rather than directly disrupting activity of LSt cells.

In a portion of the animals (43–66%), GRP^{20–29} was sufficient in triggering ejaculatory reflexes in the absence of DPN stimulation. It is possible that the differences in the ability of GRP to induce ejaculatory reflexes between groups of rats are due to differences in receptor densities for neuropeptides or receptors expressed in the spinal ejaculation generator, including GRP. In addition, GRP^{20–29} was not sufficient to trigger ejaculatory reflexes in all the animals indicating that a higher dose of GRP agonist may have been necessary to trigger BCM bursting and SVP increases in some animals. Alternatively, the lack of responses to GRP^{20–29} in all animals suggests that other neuropeptides expressed in LSt cells and potentially released in LSt target areas may act in concert with GRP to control ejaculation. These neuropeptides include galanin [25], cholecystinin [14], and enkephalin [20]. An unexpected finding was that the higher dosages of GRP^{20–29} caused significant suppression of BCM activity induced by the highest stimulation parameters. A similar finding is that stimulation of the DPN at higher frequencies (such as 120 Hz) does not trigger ejaculatory reflexes (unpublished personal observations). Hence, such stimulation by agonists and high frequency may represent a super physiological manipulation that leads to dysfunction or possible muscle fatigue.

An advantage of the current study of the role of GRP in ejaculation is the use of the DPN stimulation paradigm which utilizes anesthetized and spinalized male rats and elicits rhythmic contractions of the BCM and concurrent SVP increases, akin to those observed in freely-moving animals during mating [4,5,8,22,23,48]. Thus, complete transection of the spinal cord permits the study of ejaculation as a reflex response in the absence of supraspinal influences [49–51]. The DPN is the primary afferent that transmits sensory information related to the summation of mating to the spinal ejaculation generator and triggers ejaculation [52,53]. Indeed, bilateral transection of the DPN blocks ejaculation in mating rats [54]. Although the pathways whereby sensory information is transmitted from the DPN to the LSt cells

are currently unknown, it has been shown that DPN nerve fibers terminate in the lower lumbar and upper sacral levels of the spinal cord in the general vicinity of the LSt cells, dorsal horn and dorsal grey commissure (DCG) [31,52,55]. Furthermore, it has previously been shown that DPN stimulation activates LSt cells in a similar manner as ejaculatory behavior in freely moving animals [22,23]. The mechanism for LSt cell activation is through NMDA receptors [23] and map kinase signaling [22]. Moreover, this activation is required as pharmacological blockade of either of these components results in the blockade of DPN stimulation-induced ejaculatory reflexes in male rats [22,23]. Therefore, the activation of LSt cells is essential for the transmission of sensory information from the DPN to the outputs of the spinal cord ejaculation generator [56]. A previous study demonstrated effects of GRP treatment on erectile and ejaculatory reflexes using an *ex copula* paradigm in spinal intact rats [18]. However, in this paradigm, it is not possible to eliminate actions of GRP on the supraspinal areas that control ejaculation from the spinal ejaculation generator, including the nucleus of the paragigantocellularis (nPGi) [57–59], the medial preoptic area (MPOA) [60] and the paraventricular nucleus of the hypothalamus [58,61–63]. Moreover, emissions or ejaculations as observed in the *ex-copula* paradigm are not dependent on the spinal ejaculation generator [56] as lesions of the LSt cells did not block sexual reflexes using this paradigm. While, in contrast, intact LSt cells were pivotal for ejaculatory reflexes as induced in three accepted paradigms for ejaculation [56], including DPN or urethro-genital stimulation, and pharmacological activation of D3 receptors in spinal transected anesthetized male rats [56] as well as for ejaculatory behavior in freely mating male rats [13].

In conclusion, these data confirm that GRP receptor activation in the lumbosacral spinal cord is required for sensory stimulation-induced ejaculation reflex in anesthetized, spinalized male rats. Furthermore, GRP appears to act by activating receptors in the outputs of the LSt cells. However, GRP receptor expression in the target regions of the LSt cells remains to be elucidated. In addition, the roles of other neuropeptides expressed in the LSt axons, including enkephalin, galanin and cholecystinin in the control of ejaculatory behavior and reflexes remain to be investigated.

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Statement of Authorship

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