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Adapted Technological Platform for Screening and Identifying Compounds Capable of *In vivo* Spinal Network-mediated Reflex Ejaculation in Non-anesthetized, Chronic Paraplegic Mice: Evidence of Clonidine-elicited Seminal Emission

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Authors' contributions

This work was carried out in collaboration between all authors. Author PAG designed the study and wrote the protocol. Author IS wrote the first draft of the manuscript. Author PAG managed the literature searches. Authors IS and PR conducted analyses and all experiments. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Several drugs have been shown to facilitate locomotor rhythmogenesis and treadmill training after Spinal Cord Injury (SCI). Clonidine, an alpha-adrenoceptor agonist, is of particular interest given its well-known effects on facilitation of reflex-induced spinal stepping in acute or chronic complete low-thoracic spinal cord-transected (Tx) cats. Since locomotion and ejaculation are controlled by neuronal networks located in the same area of the spinal cord (i.e., upper- to mid-lumbar segments), we hypothesized that clonidine may have comparable effects on reflex ejaculation in

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low-thoracic Tx animals. To achieve that, the main aim was to adapt a technological platform developed initially for in vivo testing of pro-locomotor compounds in order to establish and validate an approach for assessing both behaviourally and quantitatively, drug-induced reflex ejaculation ex copula in an animal model of SCI. At 6 or 7 days post-Tx, male mice received a single injection of clonidine (0.005-2.5 mg/kg, i.p.). At doses ranging between 0.1 and 2.5 mg/kg, clonidine acutely induced, in 88% of cases (35/40 mice), seminal emissions as fluids or plugs (*in-urethra*) with no erection nor expulsion. Given that clonidine is a partial alpha-2 agonist, the results suggest that alpha-2 adrenoceptor activation is associated with seminal emission in non-copulating and non-stimulated (e.g., manually or electrically) chronic paraplegic mice. To our knowledge, this is the first report of alpha-2-mediated, clonidine-induced seminal emission.

Keywords: Paraplegia; spinal cord injury; transection; reproduction; anejaculation; rodents; mice.

ABBREVIATIONS

SGE *Spinal Generator of Ejaculation*
Tx *Spinal cord-transected*
SCI *Spinal Cord Injury*
s.c. *Subcutaneous*
i.p. *Intraperitoneal*
T9/10 *Thoracic segments 9 and 10.*

1. INTRODUCTION

Spinal Cord Injury (SCI) generally leads to an immediate and irreversible loss of sensation and voluntary motor control below the level of injury as well as to a rapid development of severe health problems such as osteoporosis, muscle atrophy, immune deficiency, hormonal dysregulation, infertility, autonomic dysreflexia, diabetes, obesity, cardiovascular complications, bladder problems, and sexual dysfunction [1-3].

It is well-established that most SCI patients are young men (80-85%) in their reproductive years [4], which may explain why compromised ejaculation and infertility are important issues [5]. Although, erection may be restored to some extent with electrostimulation or vibrostimulation, ejaculation remains particularly affected– indeed, less than 50% have reported successful ejaculation with these methods [5]. Thus, the development of innovative solutions dedicated specifically at restoring ejaculation *per se* would fulfill a poorly addressed medical need.

It has been clearly shown that ejaculation is controlled in part by a network of spinal neurons called SGE (i.e., spinal generator for ejaculation) located at the mid-lumbar level [6]. This network is believed to normally integrate sensory inputs and, once activated, to trigger ejaculation (two phases- emission and expulsion) by coordinating the sympathetic, parasympathetic and somatic outflow.

Given that a comparable network involved in the generation of locomotor movements in localized in the same area of the spinal cord in rodents [7], we hypothesized that families of molecules such as alpha-adrenergic agonists known to display modulation actions upon spinal-mediated locomotion [8] may also perhaps alter spinal reflex ejaculation. To study the effects of clonidine on SGE activation, we adapted a technological platform initially developed to study drug-elicited spinal stepping in rodents [9]. The same approach was used (low-thoracic mice, drug-tested at 1 week post-Tx, single test with systemic injection) but with quantitative assays specifically developed to measure in vivo ejaculatory and erectile motor behaviors. The main aim was to establish and validate an approach for assessing both behaviourally and quantitatively, drug-induced reflex ejaculation ex copula in an animal model of SCI.

Intraperitoneal administration of 0.005 - 2.5 mg/kg clonidine in freely moving (unanaesthetized) male mice low-thoracic Tx (Th9/10) 6 days prior to testing. Incidences of seminal emission (fluids or plugs), erection, and ejaculation (expulsion) were examined.

This standardized simple and reliable approach may ease comparison of drug-induced effects on spinal-mediated mechanisms of ejaculation in animal models of SCI. This may facilitate and accelerate basic research, drug screening experiments and the development of new potent treatments, aimed at treating reproductive dysfunction in males with a SCI or comparable neurological disorders.

2. MATERIALS AND METHODS

2.1 Animal Model and Surgical Procedures

All experimental procedures were conducted in accordance with the Canadian Council for Animal Care guidelines and accepted by the Laval

University Animal Care and Use Committee. A total of fifty-four (54) male mice (Charles River, Montreal, QC) initially weighing 30-40 g prior to surgery were used in this study. Preoperative care included lactate-Ringer's solution (1 ml, s.c.), analgesic (buprenorphine; 0.1 mg/kg, s.c., Schering-Plough, Pointe-Claire, QC), and antibiotic (Baytril; 5 mg/kg, s.c., Bayer, Toronto, ON). Spinal transection at the low-thoracic level was performed under complete anesthesia with 2.5% isoflurane. The spinal cord was completely transected intervertebrally using microscissors (no. 15000-08, Fine Science Tools, North Vancouver, B.C.) inserted between the 9th and 10th thoracic vertebrae [10]. To ensure that complete transection was achieved, the inner vertebral walls were explored and entirely scraped several times with scissor tips in order to disrupt any small fibres which had not been previously severed. Opened skin areas were sutured and animals were placed for a few hours on heating pads. Postoperative care included administration of lactate-Ringer's solution (2 x 1 ml/day s.c.), buprenorphine (0.2 mg/kg/day, s.c.) and Baytril (5 mg/kg/day) for four consecutive days. Bladders were emptied manually also for four days or until a spontaneous return of micturition. Animals were left in their cage with food and water *ad libitum*. Complete Tx was confirmed by 1) mainly flaccid hindlimbs and 2) post-mortem examination of the lesioned area using either coronal or longitudinal sections of the spinal cord stained with luxol blue and cresyl violet.

2.2 Drug Administration and Protocol

All mice were tested on the 6th or 7th day post-Tx. The effects of administration of 0.005 – 2.5 mg/kg, i.p. clonidine (Sigma, St-Louis, MO, USA) were examined after a single treatment to prevent cumulative effects or drug-induced plasticity at the sublesional spinal level [11]. Two (2) mice received 0.005 mg/kg of clonidine whereas 4, 8, 8, 8, 8, 8, 8 mice received 0.01, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 mg/kg of clonidine, respectively. Prior to drug administration, bladders were manually voided to eliminate possible contributions (afferent input-induced) to neuronal network activation. Penises were examined immediately prior to testing to confirm the absence of seminal fluids or plugs.

2.3 Quantitative Assays

Animals were tested separately. Upon injection (vehicle in this case was sterile water), each animal was placed in the place in a Plexiglas arena (circular shape, diameter of 60 cm) for 60 minutes for observation and data collection. After drug administration, penises were examined visually (no manipulations of the penises were performed) every 10 min for 1 hour. Given that ejaculation consists of two phases, emission (secretion and movement of seminal fluids to the urethra) and expulsion (forceful ejection of urethral contents), we separately assessed these events. We assessed the incidence (number of animals tested in which the specific drug-induced event assessed is found) of seminal emissions either as fluids or plugs (coagulated form), inside or outside the urethra. Seminal plugs, when found, were also weighed.

2.4 Statistical Analysis

ANOVAs following with *Bonferroni* post-hoc tests, when significant, were performed using SPSS. Results were reported either as percentages (incidences) or as means ± SE (weights). *P* values <0.05 were considered statistically significant.

3. RESULTS

Fig. 1 shows the acute effects of clonidine in adult male mice spinal cord Tx 6 or 7 days prior to testing (n=54). Administration of vehicle (control – sterile water) or 0.005 mg/kg clonidine failed to induce seminal emission. In turn, administration of 0.01 - 2.5 mg/kg induced seminal emissions as fluid in 14 animals and as plug in 30 other animals (see also Fig. 2). In other words, at doses above 0.01 mg/kg, 81% of the animals displayed clonidine-induced seminal emissions. At doses above 0.1 mg/kg, the success rate (incidence of seminal emissions) reached 88% (35/40 mice). However, in none of the animals, did clonidine evoked copulatory-like behaviours, erections or full ejaculations. Seminal emissions as fluids were typically released slowly (passively) outside the urethra whereas seminal emissions as plugs rapidly coagulated in-urethra where it remained until taken out by the experimenter. Seminal plug weights ranged between 5.3 mg and 48.7 mg. The graph bar in Fig. 1 clearly shows a lack of clonidine-induced effect (i.e., no seminal emission) at 0.005 mg/kg and a plateau-like effect (incidences of seminal emission between 75% and 100%) at doses ranging between 0.01 and 2.5 mg/kg. Thus, it is clear that seminal emission was not dose-dependent. In turn, the

proportion of plugs (white bars) vs. fluids (black bars) apparently dose-dependently increased. In fact, only plugs with no fluids were found at 2.5 mg/kg (N=8/8, Fig. 1). Fig. 2 reveals an

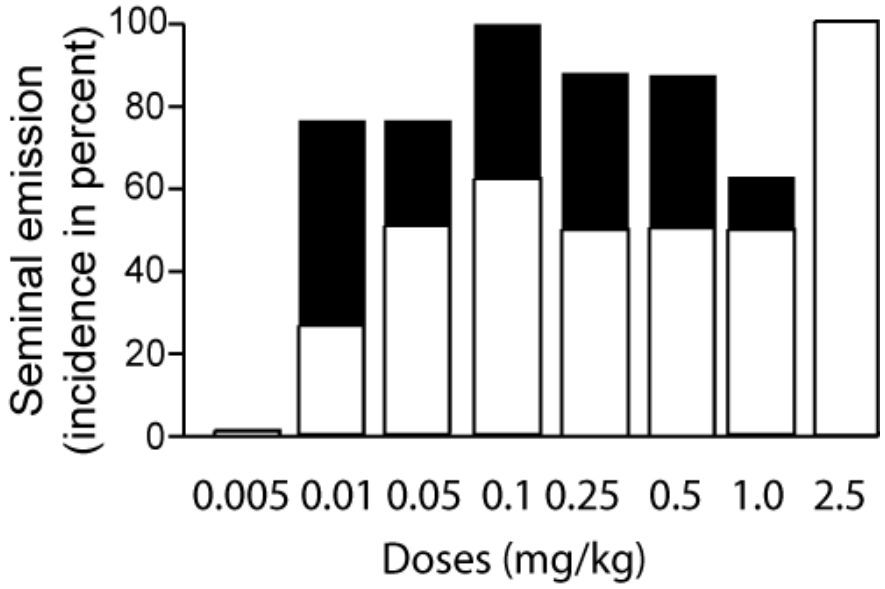

Fig. 1. Dose-response effect on the incidence of seminal emission. Black bars are seminal fluids whereas white bars are seminal plugs. There combinations constitute the overall incidence of seminal emissions (fluids and plugs)

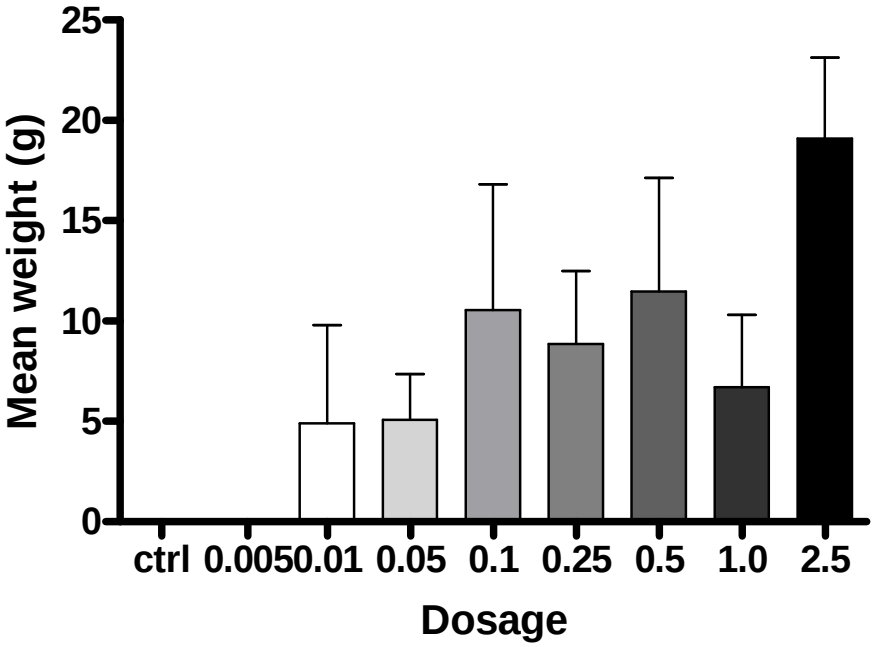

Fig. 2. Dose-response effect on seminal plug weights. Weights expressed in mg were measured and reported for each dose at which plugs were found. Note that no plugs were found at 0.005 mg/kg and only plugs (no seminal fluid) were found at 2.5 mg/kg clonidine

apparent dose-dependent increase in plug weights although non-significantly statistical levels were reached. Nonetheless, the heaviest plugs were found following administration of 2.5 mg/kg clonidine.

4. DISCUSSION

One of our main findings is that clonidine, at doses ranging between 0.1 and 2.5 mg/kg induced seminal fluid emissions in 88% of the animals. No significant dose-dependent changes in plug weight or in seminal emission incidence were found although greater changes were found at the highest dose tested (2.5 mg/kg).

Results of this study are in contrast with those that have been reported elsewhere [12,15]. Indeed, in other studies, it is generally shown that alpha-1 adrenoceptor agonists, but not alpha-2 agonists, promote sexual functions in normal subjects [12]. For instance, alpha-1 agonists or alpha-2 antagonists administered intraperitoneally 10 minutes prior to mating tests were found in other studies to reduce the ejaculatory threshold, evidenced by a decrease in the number of intromissions preceding ejaculation [12,13]. The same group of researchers has reported that, conversely, alpha-2 adrenoceptor agonists or alpha-1 antagonists generally suppress sexual functions given that pre-treated male rats drastically reduced the number intromissions and ejaculations in mating tests [12-14]. Comparable results have been reported by Carro-Juarez and Rodriguez-Manzo in an animal model of SCI [15]. They have shown that acute administration of alpha-1 agonists, but not alpha-2 agonists, can induce ejaculation in mid-thoracic (T6 level) completely Tx rats [15]. In those studies, the clonidine was found to inhibit the spontaneous *ex-copula* fictive ejaculatory behaviour displayed immediately after spinal cord transection in an anesthetized rat preparation [15]. Consequently, this was taken as evidence that alpha-2 adrenoceptor activation inhibits sexual functions and, specifically ejaculation in Tx animals as well as in non-SCI animals.

Several reasons may explain this discrepancy between their studies and ours. Indeed, in non-Tx preparations, clonidine can be mediated at all levels of the central nervous system (i.e., both brain and spinal cord). The physiological outcome of systemically administered compounds such as clonidine will be the sum of its brain-mediated and spinal-mediated actions and interactions. In turn, in spinal cord Tx models, clonidine-induced effects on lower body functions (e.g., locomotion, micturition, ejaculation) would normally be attributed mainly to spinal-mediated actions. Supporting this, differences in drug-induced effects between non-Tx and Tx models have already been reported in cats [16,17]. Different drug-induced effects among the many existing SCI preparations are also possible. For instance, in the Carro-Juarez's study, they used an adult anesthetized rat acutely spinal cord Tx at the mid-thoracic level (T6) whereas, in this study, we used a freely moving (unanaesthetized) adult mouse chronically spinal cord Tx at the low-thoracic level (T9/10). Consequently, differences in clonidine-induced effects could depend upon the species, lesion level (mid- vs. low-thoracic), state of anaesthesia (with or without anaesthetics) or time post-lesion (acute vs. chronic). Compelling evidence exists also that sublesional spinal networks undergo significant plasticity changes and reorganization post-Tx which is imperative to consider when attempts are made to identify drug treatments for chronic SCI cases [11]. Moreover, drug-induced responses have also been shown to drastically change in anesthetized vs. unanaesthetized preparations where, for instance, MK-801 was found, respectively, to inhibit or facilitate micturition [18].

It is important to point out that no acclimation was conducted and no restrainer tube was used. Therefore, parasympathetic mechanisms may indirectly have contributed to these rather high incidence values. Moreover, freely-moving animals may have licked themselves from time to time and may have had their belly and penises rubbing against the floor of the Plexiglas chamber.

5. CONCLUSION

In conclusion, approaches, animal model and quantitative assays used to conduct these experiments led to identify spinally-mediated reflex ejaculation (mainly seminal emission and passive expulsion) in a murine model of chronic SCI. This platform may ease standardized comparisons of drug-induced effects on SGE activation and, hence, accelerate the identification and development of potent and safe SGE-activating drugs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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