Ana Rita Pimentel Mendes

UNRAVELLING THE CIRCUITS INVOLVED IN EJACULATION IN MICE (*MUS MUSCULUS*)

Dissertação de Mestrado em Investigação Biomédica, orientada pela Doutora Susana Q. Lima e pela Doutora Cláudia Maria
Fragão Pereira e apresentada à Faculdade de Medicina da Universidade de Coimbra

Julho de 2017

UNIVERSIDADE DE COIMBRA

Agradecimentos /Acknowledgements

I would like to start by thanking to my supervisor, Susana Lima, for the opportunity to work in her lab, for all the support and for allowing me to pursuit my goals. Without you this would not have been possible.

To the Masters in Biomedical Research, I would like to thank to Henrique Girão, for believing in me and let me chase my neurobiology dream. Your patience, friendship and advices makes this Masters the best. To all the MIBs from my year, thank you for your companionship, for all the dinners and the support, we were a hell of a year!

To all the members of the Lima Lab, I would like to let you know that you are amazing. The friendship and family environment that you gave me made my year! Francisco, thank you for all the help in my work, all the discussions and the support you gave me. Without you it would have been much harder. Margarida, thanks for all the help, all the talks and all the funny videos that would always make me happier. You are an amazing person. Inês and Diogo, thanks for the friendship, all the incentive talks and the shared snacks. Keep as genuine and kind as always. Sue and Banas, thanks for all the work tips and help, but even more important, thanks for all the companionship in the past year. António and Luís, thanks for the entertaining times, for making fun of me at all possible opportunities and for all the help. Basma, Baylor and Lourenço, without you this would not be the same, thanks for everything. Constanze, even though you have only came to the lab very recently, I would like to thank you for all the help with my thesis writing. I will try to make you feel as welcome to the lab as I felt.

To the CCU, thank you for your constant availability and presence. The working environment here is amazing thanks to that! To Nuno and André a special thanks for all the support, without you it would not be the same.

A todos os meus amigos, um enorme obrigado por estes últimos 5 anos. Sem vocês a vida era uma tristeza! À Bea por toda a amizade, companheirismo e carinho, sabes que foram essenciais. À Joana pela constante presença na minha vida, por todo o sushi e por toda a amizade. À Ana Tomé e à Carolina por estarem sempre lá quando precisava. Aos rapazes, José, Bernardo, Valério, Areal, Tozé, Santinha, Bidarra e Piedade, pelos momentos de pura diversão. O slogan “Ana Rita e os Rapazes” é para a vida! Ao pessoal de Química, obrigada por me acolherem quando as coisas não estavam tão fáceis, significou muito. Ao pessoal

de Condeixa, obrigada por todos os cafés de fim-de-semana e por todo o carinho. A todos os meus outros amigos, um muito obrigada por tudo, a vida é feita de pessoas como vocês. Às minhas amigas da melhor casa de Lisboa, Magda e Rita, obrigada por terem estado sempre lá quando precisava. Foi um ano intenso mas com vocês passou mais rápido. Ao Manel por fazer desta casa um lugar ainda mais divertido.

Por último, à minha família, agradeço pelo apoio constante e por serem os melhores de todos. Aos meus avós, Avô Manel, Avó Lucinda e Avó Maria por sempre terem cuidado de mim, pela comidinha boa e mais especialmente por todo o amor. Aos meus tios, Tio João e Didi, por serem os maiores companheiros e amigos, por todos os jantares, os jogos de cartas e as conversas, não podia pedir melhor! Ao meu irmão, João Pedro, por estar sempre presente e pronto a ajudar, és um homem cinco estrelas! À minha irmã, Maria Inês por todo o amor e amizade, por poder partilhar tudo com ela e porque nunca vou duvidar que estarás sempre lá. Aos meus pais, por todas as oportunidades que me deram na vida e que me fizeram chegar aqui, pelo amor incondicional e por todo o carinho, o meu maior obrigado!

Contents

List of figures

List of tables

Resumo

Em humanos, doenças relacionadas com a ejaculação, como a ejaculação precoce, têm um grande impacto no bem-estar dos pacientes. A ejaculação é um processo biológico inato essencial para a perpetuação da maioria das espécies. Em ratazanas e humanos já foi proposto um gerador central de padrões para a ejaculação Este encontra-se na medula espinal, sendo normalmente designado por gerador espinal para a ejaculação. Este gerador encontra-se situado nos segmentos lombares 3 e 4 da medula espinal, nas lâminas X e VII. É composto por interneurónios ricos em galanina que projetam diretamente para o cérebro, mais especificamente, para o núcleo talâmico subparafascicular. A sua localização na espinal medula e a projeção talâmica conferem a este grupo de interneurónios o nome de células lombares espinotalâmicas. No entanto, sabe-se muito pouco sobre como é que a informação sensorial proveniente dos genitais, recebida durante o coito, e a atividade no cérebro, controlam este gerador. Para estudar os circuitos neuronais que controlam o processo ejaculatório, utilizámos o murganho (*Mus musculus* Linnaeus, 1758) como modelo animal. Esta escolha deve-se ao facto desta espécie permitir usar diversas ferramentas genéticas que não existem noutros modelos e que ajudarão a perceber os mecanismos envolvidos neste processo. Em murganho, o processo ejaculatório é pouco conhecido, sendo ainda desconhecida a localização do gerador e o tipo de células presentes no mesmo. Assim sendo, com este trabalho propusemo-nos tentar determinar quais os circuitos neuronais que controlam a ejaculação em murganhos. Injetámos estes animais com um vírus que mimetiza o vírus da raiva (Ka-gEI-mCherry) e que apresenta a capacidade de infetar neurónios no sentido retrógrado, tendo como locais de injeção o músculo bolboesponjoso e o pénis. O padrão de marcação foi avaliado na medula espinal e nos cérebros destes murganhos. Esta marcação encontra-se de acordo com os resultados obtidos para humanos e ratos. Observámos sinal desde os segmentos T8-S4 da espinal medula, com maiores níveis de expressão particularmente entre os segmentos T11 e S4. A localização do sinal foi consistente com a localização dos núcleos simpáticos e

parassimpáticos nos segmentos T13-L2 e L5-S1, respetivamente. Nos segmentos lombares 3 e 4 o sinal era maioritariamente confinado às lâminas X e VII, corroborando dados obtidos para a localização do gerador em ratazana. Adicionalmente, foi realizada uma marcação por imunohistoquímica contra a galanina, para determinar-se se os neurónios infetados com o vírus seriam semelhantes aos interneurónios descritos anteriormente. Apesar do protocolo ter sido realizado em medulas espinais de animais que não foram injetados com vírus, a localização do sinal para a galanina é semelhante à marcação obtida com o vírus. Relativamente à marcação no cérebro, os núcleos Paraventricular, Gigantocelular e Rafe estavam uniformemente marcados nos seis animais que foram sujeitos a análise. Em suma, estes resultados sugerem que a ejaculação em murganho é controlada por um gerador que se situa nos segmentos lombares 3 e 4.

Palavras-chave: Ejaculação, Pénis, Músculo Bulboesponjoso, Circuitos Neuronais, Rastreamento Neuronal.

Abstract

In humans, ejaculatory disorders such as premature ejaculation have a severe impact on the well-being of the patient. Ejaculation is an innate biological process essential for the perpetuation of most sexually reproducing species. There is evidence from rats to humans that this process depends on a spinal control centre named spinal ejaculation generator (SEG). The SEG is composed of interneurons located around the central canal in laminae X and VII, which express molecular markers such as galanin, cholecystokinin and encephalin. These interneurons are also referred to as lumbar spinothalamic (LSt) cells due to their direct projections to the thalamus. However, very little is known about how genital sensory information received during copulation, and descending input from the brain, control ejaculation. To study this problem at the circuit level, we used the mouse as model system, since we have access to several genetically based tools that allow us to label and manipulate specific population of cells and understand the process at the mechanistic level. However, ejaculation in mice is poorly understood: we do not know where the spinal ejaculation generator is located, nor the types of cells involved in the process. In this work, we intended to understand the mechanisms involved in the ejaculatory process in mice. To do so, we used adult male mice (*Mus musculus* Linnaeus, 1758), which were injected with a retrograde pseudorabies virus carrying a fluorescent protein (Ka-gEI-mCherry) in the bulbospongiosus muscle (the muscle whose rhythmic contraction is necessary for ejaculation to occur) or in the penis. The presence of fluorescent labelled neurons was evaluated in the spinal cord and brain of the injected animals, revealing that the signal was in accordance to what has been described for rats and humans. We observed fluorescent labelled neurons in the spinal segments T8-S4, with higher expression between T11 and S4. Moreover, the spinal location of the signal was consistent with the sympathetic and parasympathetic nuclei in the T13-L2 and L5-S1 segments, respectively. In the lumbar segments 3 and 4, the signal was high specifically in lamina X and VII, which is the location for the putative SEG in rat. Additionally, an immunohistochemical

detection of galanin was performed to investigate if these labelled neurons could be the LSt cells. The signal obtained was present in similar areas to animals previously injected with the pseudorabies virus. Regarding the location of fluorescent labelled neurons in the brain, several regions were positive, such as the Paraventricular, Gigantocellular and the Raphe nucleus, which are known to affect sexual behaviour in mammals. These data suggest that ejaculation in mice is controlled by a SEG located in the spinal lumbar segments 3 and 4.

Keywords: Ejaculation, Penis, Bulbospongiosus Muscle, Neuronal Circuits, Neuronal Tracing

Introduction

1. Sexual Behaviour

Sex is an essential behaviour for the continuation of species and is usually associated with a pleasurable feeling. Sexual dysfunctions, (like sexual desire disorder, anorgasmia, and premature ejaculation), the perception that the sexual experience was unsatisfying or that the performance was inadequate can lead to problems in performing the behaviour. Taking this into account, it is understandable why sexual behaviour in humans has much more implications on the individuals' life than the simple purpose of reproduction, leading to several social and psychological consequences[1]. Sexual behaviour is believed to be highly regulated by a neuronal network that is able to integrate several signals, from the emotional state to the motor outputs necessary to perform the behaviour. So, studying the circuitry associated with sexual activity is essential to better understand it.

1.1. Sexual Behaviour has a cycle of events

Sexual behaviour can be seen as a cycle (Figure 1) of events and behaviours divided in:

1. Excitement - characterized by genital arousal and emotional desire;
2. A plateau phase - consisting in physical sexual activity and bodily and genital arousal;
3. Orgasm;
4. Refractive period.

This cycle can be compared with other pleasure cycles and, because of it, can also be expressed in terms of motivation – consummation – satiety or wanting – liking – inhibition[1,2]. However, sexual behaviour is usually divided in only two phases, the appetitive and the consummatory, being the first one prior to copulation and the second during and after it.

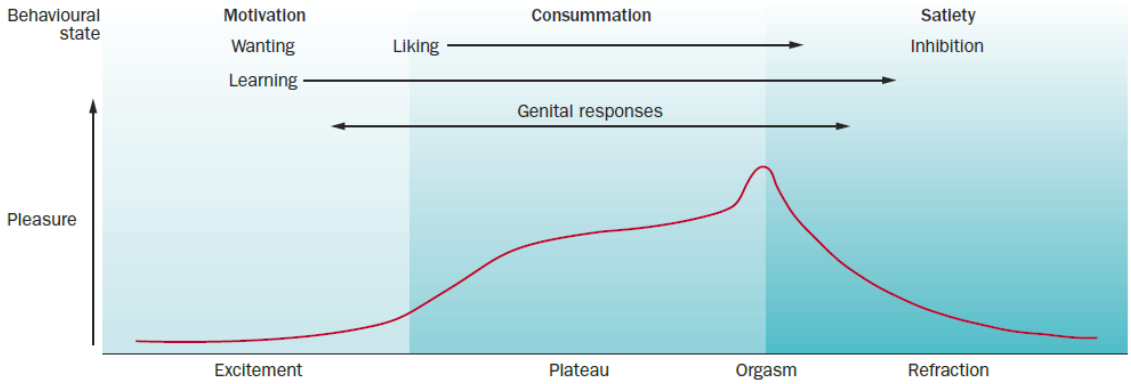

Figure 1. Sexual Pleasure Cycle. The sexual behaviour cycle comprises the excitement, plateau, orgasm and refraction phases but can also be divided in wanting, liking and inhibition stages. The orgasm phase usually overlaps with the end of the sexual liking stage and the beginning of the inhibition stage where the individual is not able to enter in a new cycle. Adapted from[1].

1.2. Sexual behaviour in humans and rodents

Sexual behaviour in humans and rodents has common characteristics. In the appetitive phase both humans and rats have sexual desire or willingness to engage in the behaviour and perform solicitations towards a possible sexual partner. However, in the consummatory step, there are few common aspects, essentially because the behaviour itself is very different. In humans, once the action starts, the two individuals stay together until ejaculation and/or the orgasm is reached; this is in contrast with rodents, where the animals engage in cycles of mounting and dismounting, until ejaculation occurs[2].

More specifically, the human sexual behaviour can be described has in the cycle mentioned above. First, there is a motivation phase that is usually associated with sexual desire. However, this desire may not be the first step since it can also be sensed as a response to a sexual stimulus[3]. Then the cycle continues with physical sexual activity and body and genital stimulation that eventually leads to ejaculation and/or orgasm[1]. Afterwards the post ejaculatory refractory period (PERP) begins and the individuals are not able to copulate for a certain period of time (usually 20min)[4]. After ceasing of the PERP this cycle can be partially or completely repeated during that sexual encounter. The capacity of this repetitions is dependent on, among other things, age, relationship duration, the individual's mental health and the relationship's happiness. Ultimately, sexual behaviour in humans is linked with a reward feeling[3].

In the case of the rats, the male rat begins the sexual activity by investigating the anogenital region and the face of the female. Then, the male mounts the female and performs several rapid shallow thrusts until he detects the vagina. The male gives a deeper thrust and introduces his penis into the female's vagina for 200-300 ms (this is called an intromission). Subsequently, he leaves the female, grooms his genitals and, after 1 to 2 min, performs another intromission. After 7 to 10 intromissions, the ejaculation occurs being characterized by a longer intromission and slower dismount. The male grooms himself again and after a PERP of 6 to 10 min he restarts mating, repeating this process until he reaches 7 to 8 ejaculations.

Normally, the male will not copulate again for 1 to 3 days[5]. Once more, the behaviour is different from humans, obviously, however, we can find some similarities like the capacity to ejaculate several in the same sexual encounter. This does not happen in the mice sexual behaviour, for example.

1.2.1. Sexual behaviour in mice

The male mouse starts by exploring the anogenital region of the female. Afterwards, the male does some pelvic thrusts by pressing his forepaws against the female's flanks. Once his penis enters the female's vagina the mounts start to be slower and deeper becoming, what is called, intromissions. After a certain number of intromissions the male ejaculates and usually falls to the side and stays in that position for several seconds[5]. Then, the male loses interest in performing sexual behaviour for at least 24h, entering in PERP (personal communication from the lab). However, if a new female is presented, after two or three hours the male can copulate again.[5,6] This behaviour is very different from the two described above where the male can ejaculate several times with the same female in the same sexual encounter. Therefore, it is important to study sexual behaviour in mice, more specifically the ejaculatory process and the PERP, because such differences in the behaviour may reflect differences in the neuronal circuit itself.

Furthermore, different strains of mice have distinct behaviours. McGill (1962) studied the differences in the sexual behaviour of three strains of mice, C57BL, BALB/c and DAB/2. In this study C57BL mice were the fastest to achieve a mount with intromission, spent less time between intromissions and to reach ejaculation. Contrarily, the DAB/2 strain took longer to reach the first mount with intromission, had longer inter-intromission intervals and intromission latencies when compared to the C57BL and BALB/c strains. However, the DAB/2 males need fewer intromissions to ejaculate. The BALB/c mice required the longest time to perform the behaviour with the greatest number of thrusts and intromissions before ejaculation[6]. Again, these differences in strains need to be better studied in order to understand what is behind such distinct behaviours.

1.2.2. Post ejaculatory refractory period (PERP)

The PERP is defined has the time after an ejaculation in which further erections or ejaculations are inhibited, and can be compared with the refractory period defined in Figure 1. It has been shown the existence of PERP in several species, including humans, mice and rats, however, it has different characteristics[3,4].

In humans, the PERP differs with age, being of approximately 20 min in young adults and increasing to hours in the elderly[4].

In rats, there is evidence that after mating there is a period when the male cannot feel aroused by the female present in the cage, entering in the PERP. Nonetheless, if a new receptive female is presented, the male rat can overcome this state and became sexually active towards this new female. This is called the Coolidge effect, which states that the presence of a novel female causes a new increase in the arousal reducing the PERP[7,8]. This behaviour might be explained in an evolutionary way, in which the male seeks different partners to allow the spreading of his genes. However, successive ejaculations showed less sperm in the female reproductive tract and the male epididymis and vas deferens, which goes against this evolutionary reason[8]. In humans the Coolidge effect cannot be assessed by obvious social and ethical concerns. Even tough, there are some indirect evidence that might indicate the existence of this effect in humans: after 2 years of marriage, there is a significant decrease in coital activity; men have a greater interest in seeking for different partners; when presented to pictures of males and females, males tend to prefer familiarity towards male pictures but not female pictures, suggesting again the preference for novelty in a possible sexual partner[7].

A question that still remains unanswered is what leads to the implementation of the PERP, the ejaculation itself or the orgasm? In rodents this is a subject difficult to assess considering that we cannot assess directly the emotional state associated with the orgasm, as we do in men. In humans, the problem remains in the fact that the orgasm and ejaculation (despite being different mechanisms) are coincident.[4,7] However, men that experience orgasm without ejaculation, do not enter a refractory period, suggesting that ejaculation is necessary for the establishment of an inhibitory

phase.

2. Ejaculation

2.1. Ejaculation can be divided in two phases: emission and expulsion

The ejaculatory process is composed by several complex steps that ultimately lead to the production and accumulation of sperm and its expulsion. This process can be divided in two major steps, emission and expulsion. The emission phase is the physiological process responsible for the accumulation of the sperm (spermatozoa and seminal fluids) in the prostatic urethra. The expulsion phase, as the name suggests, controls the expulsion of the sperm by the rhythmic contraction of the perineal muscles[9]. These two phases are controlled by different neuronal circuits. Regarding the emission phase, the organs involved in it are enervated by dense sympathetic and parasympathetic nerve fibres that form the pelvic plexus, also known as inferior hypogastric plexus in humans. In contrast, the expulsion phase is controlled by somatic motor components of the perineal branch of the pudendal nerve[10].

2.2. Ejaculation in humans and rodents

To understand the ejaculatory process in humans the division in emission and expulsion is essential, since different components of the pelvic structures are involved in different functions. Regarding the emission phase, the organs necessary are:

- The epididymis - final maturation of the spermatozoa and its consequent accumulation;

- The vas deferens – peristaltic contractions to move the spermatozoa to

the urethra;

- The seminal vesicles - secretion of 50%-80% of the final volume of the sperm;

- The prostate gland - secretion of 15%-30% of the ejaculatory product;

- The bulbourethral glands or Cowper's glands[9,11].

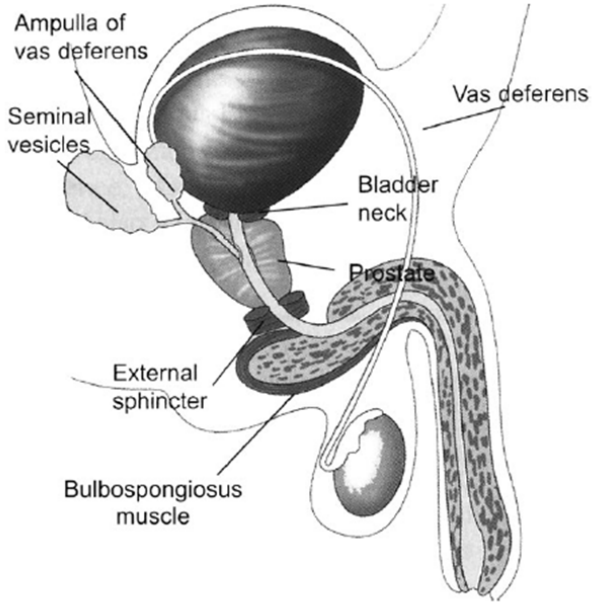

Figure 2. **Scheme of the human male reproductive system anatomy.** The picture depicts the several structures responsible for the two phases of ejaculation, emission and expulsion. Adapted from[9].

On the other hand, the expulsion phase needs the coordinated action of the bladder neck and urethra (essential for the ejaculatory reflex) and the rhythmical contraction of the perineal striated muscles, which include the bulbospongiosus, ischiocavernosus and levator ani muscles, being the main responsible for the ejection of the sperm[9,11]. The most important structures can be seen in Figure 2, where the male reproductive system is depicted. First, in the emission phase, the sympathetic nerves promote the closure of the bladder neck. Then, the prostate gland releases fluid that will be mixed with the spermatozoid rich fluid secreted by

the vas deferens. This liquid will accumulate in the prostatic urethra that, in turn, will receive seminal vesicle fluid that allows the accumulation of the final ejaculatory product. Then, the expulsion phase begins by promoting the ejection of the ejaculatory product from the urethra by the coordinated action of the bladder neck, the urethra and the perineal striated muscles[10].

All this organs are highly enervated by peripheral neuronal pathways, namely, through the autonomic nervous system, being controlled by several sympathetic and parasympathetic pathways. Regarding the sympathetic system, it has already been proven that it commands the contraction of the smooth muscle present in the accessory glands. However, the role of the parasympathetic system is still not understood[9]. Also, the action of somatic motoneurons is essential for the contraction of the perineal nerves and consequent expulsion of the sperm.

The rat, mouse and human male reproductive systems are very similar, making them appropriate models to study ejaculation. The major differences observed are the existence of two additional glands, the preputial and the ampullary glands, and the division of the prostate in lobes (three in the rat and four in the mice)[12,13]. The remaining structures are similar to the human reproductive system. The table in Figure 3 depicts the differences and similarities between mice and human urogenital system.

Tissue	Mouse	Human
Testes	Paired; present within scrotum; open inguinal canals	Paired; outside body, within scrotum
Epididymis	Head, body, tail; posterior aspect of each testis	Head, body, tail; posterior aspect of each testis
Vas deferens	Present	Present
Seminal vesicles	Attached to anterior lobe of prostate	Diverticuli of ductus deferens
Prostate	Divided into four lobes (anterior, dorsal, lateral, and ventral)	Single, alobular organ with central, peripheral, and transitional zones
Bulbourethral glands	Present	Present (Cowper's glands)
Ampullary glands	Glandular outpouchings of ductus deferens	Absent
Preputial glands	Modified sebaceous glands located in the caudal subcutaneous tissue lateral to the base of the penis	Absent
Penis	Present within prepuce; os penis	Present outside body
Urethra	Membranous, penile	Prostatic, penile

Figure 3.Table summarizing the differences and similarities between human and mouse male reproductive system. It is important to highlight the addition of two glands in the mouse reproductive system, the preputial and the ampullary glands. Another difference may be noted in the division of the prostate in four lobes contrarily to the single prostate present in humans. Adapted from[13].

2.3. The bulbospongiosus muscle role in the ejaculatory process

As already depicted above, the bulbospongiosus muscle (BSM) has a decisive role in the expulsion phase of the ejaculation. It is a striated muscle that belongs to the category of perineal or pelvic muscles. In humans, it is connected to the ischiocavernosus muscle forming one unit that has common functions and morphologies[14]. Furthermore, some authors believe that the BSM also has a connection with the external anal sphincter[15], but others consider that they are two independent structures and its connection might be due to an anogenital disorder[14]. As can be seen in figure 2, the BSM surrounds the base of the penis, forming an incomplete ring from the anterior part of the penis to the posterior portion of the anal canal[15]. This muscle is enervated by the pudendal nerve that arise from the sacral segments 2-4 anterior rami of the sacral plexus[15].

In the case of mice and rats, the perineal muscles location is very similar between them and comparing to the human positions. Depicted in Figure 4, the BSM muscle (BS in the picture) is located in bellow the penis and in close contact with the ischiocavernosus muscle (IC) and the external urethral sphincter (EUS)[16]. The location of the BSM bellow the penis is because this muscle surrounds the corpus spongiosum, whereas the ischiocavernosus muscle is in a rostral position because it is in contact with the crura of the corpus cavernosum[16]. In rat, the enervation of the BSM is also done trough the sacral or pelvic plexus, by the motor branch of the pudendal nerve that originates in the lumbar segments 5 and 6 more exactly in the Onuf's nucleus[11,16,17]. In mice, the information regarding the pelvic muscles is almost non-existent. As far as we know, there is no study characterizing the pelvic muscles in mice or the pudendal nerve anatomy. However, there are some studies that demonstrates the importance of this muscle in the ejaculatory process. Elmore et al. (1988) observed that in the house mouse, the excision of the BSM caused a significant increase in the amount of interruptions during a thrust with intromission. This suggest that the BSM is essential to maintain an erection that in turn allows the successful intromission pattern in the house mouse[18]. Allard et al. (2008), also saw

that electrostimulation of the dorsal penile nerve led to the erectile responses in the BSM. Furthermore, after bilateral transection of the sensory branch of the pudendal nerve, this response was gone[19]. These results support the role of the BSM in the ejaculatory process, however, they are focused on erection and not on ejaculation.

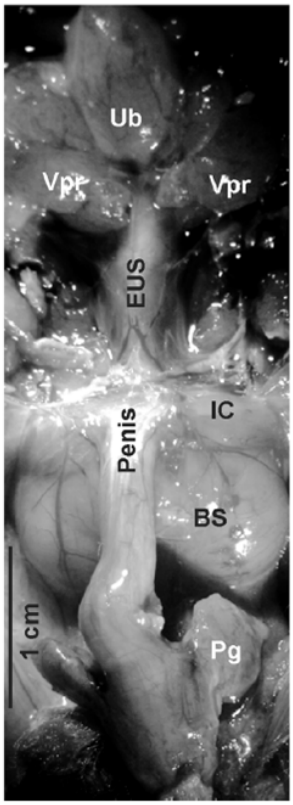

Figure 4. Scheme of the rat male reproductive system anatomy. The picture depicts the several structures responsible for the ejaculation, in rat. It is important to highlight the position of the BSM muscle (labelled as BS in the picture), in the base of the penis and in close contact with the ischiocavernosus muscle (IC). Ub – Urinary bladder; Vpr – Ventral prostate; EUS – External Urethral Sphincter. Adapted from[16].

2.4. Penis anatomy in humans and mice

In humans, the penis is divided in two different areas in a dorsal/ventral axis. Dorsally, it is composed by a pair of corpora cavernosa, whereas ventrally, it consists

in a single midline corpus spongiosum. Inside the corpora cavernosa, exists a structure named venous sinusoids that fill with blood during erection. The corpus spongiosum has the function of surrounding the penile urethra and, in the distal end, it forms the glans penis. In the end of the penis, surrounding the glans penis, it is the prepuce[20,21].

If we analyse the mouse penis anatomy, we can see several similarities with the human penis. However, there are some structures that are different. For example, mice have two prepuces, the internal and the external. The internal prepuce covers the glans when the penis is internalized and, after externalization, the internal prepuce retracts to expose the glans. The external prepuce is a skin covered in fur that protects the whole penis when it is internalized[22]. Comparing human and mice penis (Figure 5), the external prepuce is the equivalent of the human prepuce that protects the glans. Another difference between the two species is that the mouse penis ends with a salient cartilaginous element that is called the Male Urogenital Mating Protuberance (MUMP), which is surrounded by the MUMP ridge. This structure does not have an equivalent in the human penis[22]. Also, the mouse penis can be divided in two areas, the penile body, which is inside the mouse body, and the glans that is exposed when the penis is externalized. The penile body begins with a pair of corpora cavernosa (crura), similarly to humans. Then, these two structures fuse to form the corpus cavernosum that surrounds the urethra and connects to the glans in the distal part of the penile body. The corpus cavernosum can be compared to the corpus spongiosum described in humans[23].

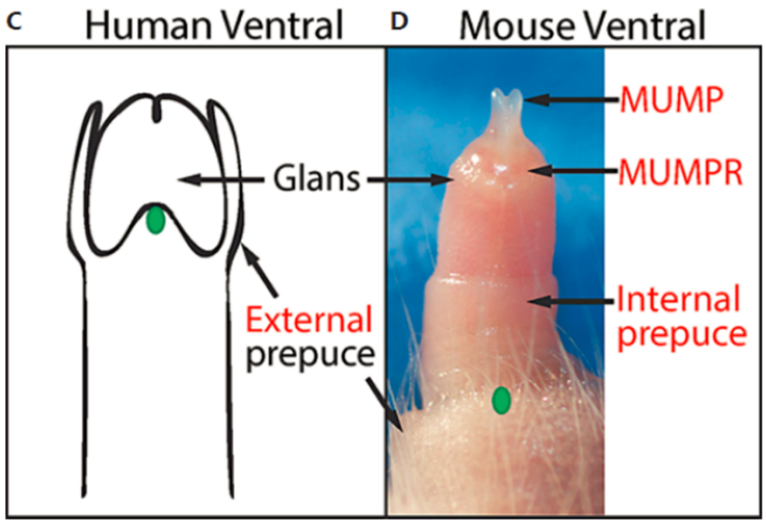

Figure 5. Comparison between the adult human penis and the mouse penis. We can see that the external prepuce in mice is the equivalent of the prepuce in humans. The internal prepuce is non-existent in humans. MUMP – Male Urogenital Mating Protuberance; MUMPR – MUMP Ridge. Adapted from[22].

3. Peripheral Nervous System and Ejaculation

The peripheral nervous system is an essential component of the neuronal control of ejaculation. Sensory inputs from the penis, namely through dorsal nerve of the penis that is a sensory branch of the pudendal nerve, allow the initiation of the ejaculatory process. The efferents of somatic motoneurons, that leave the spinal cord through the motor branch of the pudendal nerve, are responsible for the enervation of the pelvic muscles. Furthermore, the autonomic nervous system, by the coordination of the sympathetic and parasympathetic systems, controls the smooth muscle of the pelvic glands allowing the emission of the sperm[11].

3.1. Peripheral nerves controlling ejaculation

The spinal cord integrates input and outputs from the pelvic organs in order to promote ejaculation. The sensory inputs from the pelvic organs are made through the dorsal nerve of the penis, which is the sensory branch of the pudendal nerve,

and the lumbosacral trunk. On the other hand, the outputs from the spinal cord are sent by autonomic pathways and somatic motoneurons[24].

The function of the dorsal nerve of the penis in ejaculation was seen in rats, when, after the sectioning of this nerve, only 4 out of 12 male rats were able to ejaculate[25]. Also in rat, it was seen that activation of the dorsal nerve of the penis, in spinalized animals, promoted an expulsion reflex, similar to an ejaculation[26]. In humans, it was shown that the ejaculatory process was completely abolished after anesthetisia of the dorsal nerve of the penis. The patients regained the ability to ejaculation when the effect of the anesthetic was gone[27]. Based on these findings, there are already treatments for premature ejaculation based on the resection[28] or the cryoablation[29] of this nerve. Furthermore, the dorsal nerve of the penis has projections to the lumbosacral spinal cord, from segments L1 to S2. However, the largest number of projections was seen in the lumbar segments L4 to L6, which may correspond for the local in which the sensory information from the penis reaches the spinal cord and from there is integrated to induce the ejaculatory process[30].

The sacral plexus, is composed by two trunks, one arising from the spinal segments L5 to S1, and the other from the segments L3-L5 (corresponding to the lumbosacral trunk). The lumbosacral trunk, even if implicated in the innervation of the pelvic structures, is mainly associated with the sciatic nerve. On the other hand, the L5-S1 trunk is considered to be the pudendal nerve[31]. This nerve is one of the most important peripheral innervation to the pelvic organs. It was already referred that one branch of the pudendal nerve is the dorsal nerve of the penis. But the pudendal nerve has also a motor branch that innervates the pelvic muscles. This is way, it has been described as a mixed somatic nerve[30]. The importance of this nerve has been demonstrated in rats, when a transection of the pudendal nerve caused impairments in the animals capacity to perform sexual behaviour and, consequently ejaculate[32–34]. The pudendal motoneurons innervating the perineal muscles are located in the dorsomedial and dorsolateral nuclei in the lumbosacral spinal cord (L5-S1), which is the region of the Onuf's nucleus[31]. Injection of a pseudorabies virus (PRV), into the BSM or the ischiocavernosus muscle of male rat confirmed the

location of the motoneurons in the L5-S1 spinal segments, in the dorsomedial and the dorsolateral nuclei, respectively[35].

The autonomic pathways involved in the ejaculatory process, promote the emission phase and, at some extent, the expulsion phase. The sympathetic centre sends information through the hypogastric and the intermesenteric nerves, whereas, the parasympathetic centre uses the pelvic nerve. This two systems act synergistically to promote the emission of sperm by the several sexual glands[24]. Studies using horseradish peroxidase as a retrograde tracer, showed that sympathetic preganglionic neurons from the hypogastric nerve arise from the segments T12-L2; however, the biggest amount of cells were in the lumbar segments 1 and 2. The cells were mainly located in the dorsal gray commissure (while some were found in the dorsal intermediolateral area)[36–38]. Stimulation of the hypogastric nerve in rats showed the importance of this nerve for the closure of the bladder neck and contraction of the seminal vesicles and vas deferens in the emission of sperm and ejaculation occurrence[39,40]. Regarding the parasympathetic neurons, their role in ejaculation is still poorly understood. Hsieh (2013), saw that activation of the parasympathetic pathway, by administration of acetylcholine, originated a phasic contraction of the seminal vesicles in rats[41]. This phasic contraction is abolished by sectioning the pelvic nerve[42]. The preganglionic neurons of the parasympathetic pathway lay within the L5-S1 spinal cord segments. The location of these neurons, similar to the location of the sympathetic neurons, is along the intermediolateral area, the lateral zone of the dorsal horn and in the dorsal gray commissure[43].

4. Central Nervous System and Ejaculation

All the signals provided by the peripheral nervous system need to be integrated in the central nervous system in order to elicit ejaculation. Several brain area have been implicated in this process, however, the spinal cord seems to have

a major role in it. A central pattern generator has been described for the ejaculatory process, the spinal ejaculation generator (SEG)[44].

4.1. Spinal ejaculation generator (SEG)

The existence of an SEG has been proposed for humans[45] and rats[44]. It has been shown that the SEG is capable of triggering the ejaculatory process without direct influence of the brain. These evidence arise from studies in spinalized rats[46] and patients with spinal cord injury[45], where the stimulation of the SEG alone is capable of inducing an ejaculation.

Marson et al. (1996), injected a PRV from the Bartha strain in the BSM and the ischiocavernosus muscle of male rats. The authors discovered the presence of "putative spinal interneurons" from segments T13-S1, with a large number of neurons in the T13-L2 segments and L6-S1, where, respectively, the sympathetic and parasympathetic preganglionic neurons are located. However, the exact location of these neurons was not in the autonomic areas; instead, they were located around the central canal, at lamina X[35]. Another study, performed a double labelling experiment with one virus injected into the BSM and the other into the prostate. The signal of the PRVs was seen approximately in the same areas, with 60% of it being located in the medial part of the lumbar segments 3 and 4[47]. A more recent study, confirmed that the location of the signal was mainly medial, on the dorsal grey commissure, but also on the sympathetic and parasympathetic preganglionic neurons areas[48]. This approach of pseudorabies virus injection was also used to trace the circuits from the penis. The authors injected a Bartha strain PRV into the corpus cavernosus tissue. They observed the labelling of sympathetic and parasympathetic preganglionic neurons that were part of the major pelvic ganglia, and also, the labelling of putative interneurons. Again, these interneurons were preferentially located in the medial part of lumbosacral spinal cord. An important conclusion from this study was that, even though there are no autonomic efferents to the lumbar segments 3 and 4, the authors observed a massive amount of labeled

cells there. These cells had an interneuron-like morphology, which further supports the presence of the SEG in this location[49]. All these studies point for the existence of a spinal control centre, the SEG, in the medial area of the lumbosacral spinal cord, as described by Truitt et al. (2002)[44].

The SEG is located in the lumbar segments 3 and 4, lamina X and VII[44]. Also, it sends a direct projection to the thalamus, more specifically to the Subparafascicular Thalamic Nucleus[50]. For this reason, the cells present in the SEG are designed lumbar spinothalamic cells. It is believed that the SEG is composed by galanin, encephalin, neurokinin-1 receptor, cholecystokinin, substance P, calcitonin gene-related peptide positive cells[51–53].

4.2. The brain and the ejaculation

Even if some studies suggest that the SEG is capable of triggering ejaculation by itself[45,46], this spinal control centre is under inhibitory and excitatory control provided by the brain. Studies in the rat indicate that the medial preoptic area (MPOA), is the brain nuclei where sexual relevant stimuli are integrated and, from there, sexual responses are generated. This is mainly because the MPOA sends direct projections to several other nuclei in the brain that in turn control the spinal centres[54]. Other brain areas that can modulate the SEG are the paraventricular nucleus (PVN), the nucleus paragigantocellularis, the anterior lateral hypothalamus, the bed nucleus of the stria terminalis and the medial amygdala[55].

The PVN, a hypothalamic structure, was shown to have direct projections to the lumbar segments 5 and 6, specifically to the dorsomedial and dorsolateral nuclei, responsible for the innervation of the perineal muscles. Lesions to the PVN, impair male rat sexual behaviour[56], whereas, its activation promoted the ejaculatory response, by decreasing the intromissions latency and facilitating ejaculation[57]. Furthermore, this study showed that the activation of the PVN caused an increase in the lumbar sympathetic response, which is probably the mechanism by which the

PVN controls ejaculation[57].

The Gigantocellular Reticular Nucleus (Gi), receives inputs from the MPOA. Shen et al. (1990) saw that the Gi was labelled after injection of fluorogold in the spinal motor nucleus that innervates the BSM. This possibly indicates a direct projection from the Gi to the lumbar segments of the spinal cord[58]. Another study confirmed that there is a direct projection from the ventrolateral division of the Gi to the lumbar segments L2-L5 in the rat spinal cord[59]. This study also saw a direct projection from the caudal Raphe Nuclei, namely from the Raphe Magnus and the Raphe Pallidus[59]. Shen et al. (1990) similarly identified the presence of labelled cells in the Raphe Nuclei after the injection of fluorogold in the spinal cord[58].

5. Pseudorabies virus for tracing experiments

The use of a pseudorabies virus (PRV) for tracing experiments, in order to assess the neuronal circuit associated with the ejaculatory process in mice, is justifiable by the retrograde traveling capacity (across synaptically connected neurons) of these viruses.

PRV or suid herpesvirus is a pig pathogen that can infect several animals, however, higher primates and humans are resistant. This virus belongs to the subfamily of *Alphaherpesvirinae*, also known as *neurotropic herpesviruses*, being in the same category as herpes simplex virus type 1 and 2, two of the most common viruses of this subfamily[60]. This virus is capable of performing transsynaptic labeling of the cells, by passing trough the dendrites of neurons innervating the infected cells[61]. Several of the studies performed to unravel the ejaculatory circuit were made using PRVs[35,47–49]. These studies usually take 4 to 5 days until expression is detected in the brain of the rats. Smaller infection times usually label less cells, allowing to better understand the several steps of the circuit. It is expected that in mice approximately the same periods of time would lead to similar results.

6. Aims & Hypotheses

Considering the differences of mice sexual behaviour, when compared to other rodents, it is important to understand if the circuitry associated with the ejaculatory process is also different, or if it is conserved. There is evidence for the existence of a SEG in rats [1] and humans [2], however, there are no evidence in mice regarding this spinal control centre.

Therefore, our hypothesis are:

1. There is spinal cord control centre for the ejaculatory process in mice.
2. This SEG is capable of controlling the BSM and the penis of the mice through peripheral innervation.
3. The SEG is composed by galanin positive neurons.
4. The brain areas associated with this centre have a direct impact on the ejaculatory process in mice.

To prove these hypothesis, our aims are:

1. Perform retrograde tracing experiments using PRVs, injected into the BSM and the penis of male adult mice. Then, evaluate the expression of this PRVs in the mice spinal cord.
2. Execute an immunohistochemistry protocol for the labeling of galanin and correlate its location to the position of the PRV signal.
3. Assess the pattern of expression of the PRV signal in the brain of the mice four days after injection into the BSM and the Penis.

Materials and Methods

1. Animals

All procedures were submitted to the Portuguese National Authority for Animal Health (Direcção Geral de Alimentação e Veterinária; DGAV) and to the Commission for Experimentation and Animal Welfare of the Champalimaud Centre for the Unknown (Órgão para o Bem Estar Animal; ORBEA). Data was collected from male 37 C57BL/6 wild-type mice with 3-6 months. Animals were kept in a 12:12 hour light/dark cycle with the darkness phase set from 8am to 8pm, and with a controlled temperature of 23 ± 1 °C. After weaning at 20-21 days of age, mice were housed in groups of 3 to 5 animals in standard cages (1284L, Techniplast, 365 x 207 x 140 mm and 1145T, Techniplast, 369 x 165 x 132 mm) being single-housed after the surgical procedure. All animals had access to food and water (Global Diet 2914, Mucedola s.r.l) ad libitum. For environmental enrichment, cotton (cocoon cylinders, LBS) and paper houses (GLP Des Res Mice Dome Home, LBS) were provided.

2. Pseudorabies Virus for Tracing Studies

Four different PRVs were injected in the primary somatosensory area, barrel field (SSp-bf) to access it expression patterns. The following virus were used:

- Ba-gG-mRFP
- Ba-ASP-EYFP
- Ka-gEI-VampGFP
- Ka-gEI-mCherry

The chosen PRVs were from two strains, Bartha (Ba) and Kaplan (Ka), being the later more infective. The gG, gEI and ASP represents the viral genes where the DNA of the fluorescent protein was inserted, which leads to the deletion of these genes from the PRV genome. gG and gEI stands for type I transmembrane glycoproteins G, E and I. These proteins are essential for the PRV capacity of passing from presynaptic to postsynaptic neurons, anterograde movement. However, the deletion of these glycoproteins, allow the PRV to gain the ability of moving retrogradely, passing from postsynaptic to presynaptic neurons and allowing retrograde tracing studies. The Antisense Promoter (ASP), is presumed to control the expression of an antisense transcript which function it is not yet known. Even though, a mutation in this gene is associated with a decrease in PRV virulence. The fluorescent proteins inserted in the four different virus were the monomeric Red Fluorescent Protein (mRFP), the Enhanced Yellow Fluorescent Protein (EYFP), the Green Fluorescent Protein (GFP) and mCherry. Finally, VampGFP denotes for the link of the fluorescent protein GFP with a synaptic vesicle associated membrane protein (VAMP) also called synaptobrevin[60,61].

3. Surgical Procedures

3.1. Brain Injection Surgery

In order to assess the viral expression of the four PRVs, a brain injection

surgery was performed. The primary somatosensory cortex, barrel field (S1BF) was chosen as the injection site because of its superficial location, simplifying the procedure. Two animals were used to test each virus, and the presence or absence of the virus was assessed. The intensity and type of signal were also considered.

Mice were anesthetized with isoflurane (Vetflurane, 575837-4, Virbac) and placed in a stereotaxic apparatus (Model 963 Ultra Precise Small Animal Stereotaxic, KOPF®), with continuous flow of oxygen and isoflurane, allowing head fixation. The animals' eyes were covered with ointment (Clorocil, 150626, Edol), to prevent drying, and the head was shaved and disinfected with Betadine® (MEDAPharma) and 70% ethanol (Aga - Álcool e Géneros Alimentares, S.A.). At this point, an analgesic, rimadyl (5mg/kg, Carprofen, Zoetis), was administered intramuscularly to be in its maximum effect at the end of the surgery when the animal awakes. The skin was cut, starting between the eyes and moving caudally as needed. Two soaked cotton balls were placed under the skin to keep the skull uncovered. Using a tester needle in the stereotaxic left arm, the bregma and lambda coordinates were determined and the two points were aligned on the rostral/caudal (R/C) and dorsal/ventral (D/V) axis. The bregma coordinates were considered as the origin of the axis. Then, in the S1BF coordinates (R/C: - 2.46, Lateral: + 3.75, D/V: - 0.65), a craniotomy was performed to expose the brain. Meanwhile, one injection needle, attached to a Nanojet system (Nanoject II Auto-Nanoliter Injector Drummond Scientific), was loaded with approximately 1µL of one of the previously described PRVs. After inserting the needle in the brain and waiting 5min (to allow tissue adaptation) the Nanojet system was connected to a pulse generator and 4.6 nL of virus/pulse were delivered at a frequency of 0.1 Hz. The injection time was calculated to be of 8min to inject 220 nL of virus. After waiting 10min since the last pulse, the needle was pulled out slowly. Afterwards, the skin was sewed up and cleaned with Betadine® and the animal was single-housed. Three days of recuperation were allowed before perfusion.

3.2. Muscle Injection Surgery

After analysing the brain surgeries results, the Ka-gEI-mCherry PRV was chosen to continue with the tracing experiments. The BSM was chosen since it is the biggest muscle controlling erection and ejaculation, having a major role in each one of these processes[11]. In figure 6 the location of the BSM can be seen in an adult male rat. The location of this muscle is exactly the same in mice, the model used in this study.

Mice (n=12) were anesthetized with isoflurane (Vetflurane, 575837-4, Virbac) and placed in a stereotaxic apparatus (Model 963 Ultra Precise Small Animal Stereotaxic, KOPF®) only with the teeth fixed. Then, mice were turned to stay in a supine position and facilitate the access to the BSM. After shaving the anogenital area and cleaning with Betadine® (MEDAPharma) and 70% ethanol (Aga - Álcool e Géneros Alimentares, S.A.), a small cut was made near the scrotum. At this point, the analgesic rimadyl (5mg/kg, Carprofen, Zoetis) was administered intramuscularly in the posterior tight. The BSM was exposed after removing fat tissue in the cut area. An injection needle, attached to a Hamilton Syringe system (EQL00617, Harvard Apparatus), was loaded with approximately 1µL of virus. The Hamilton Syringe system was chosen because it uses metal needles that easily penetrate the muscle, contrarily to the glass needles used in the Nanojet system. After inserting the needle in the right side of the ventral part of the BSM and waiting 5min, the Hamilton Syringe system was set up to a frequency of 0.05 µL/min. Considering this, the injection time was calculated to be 20min to deliver 1 µL of virus. The needle was pulled out slowly, after waiting 10min since the end of the injection. The skin was sewed up and cleaned with Betadine®. The mice were single-housed and 4 days of recuperation were allowed.

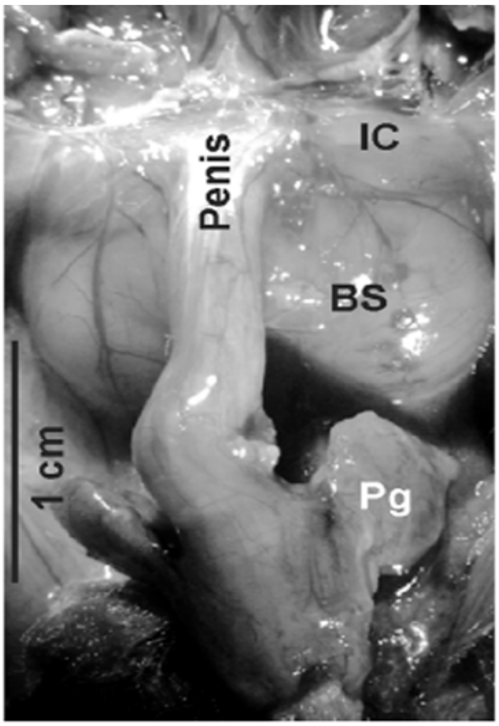

Figure 6. Photo depicting part of the adult male rat muscles controlling the ejaculatory process. The pelvic muscles are equal between rat and mice. Therefore, the localization of the BSM in mice is the same as the one evidenced in this picture. The injection was made in this muscle after a small incision in the mice scrotum. Adapted from [16]

BS – Bulbospongiosus Muscle; IC – Ischiocavernosus Muscle; PG – Preputial Gland

3.3. Penis Injection Surgery

As in the muscle, in the penis injection surgeries, the Ka-gEI-mCherry PRV was chosen to continue with the tracing experiments. Three zones in the mice penis were chosen to inject, the male urogenital mating protuberance (n=2), the glans (n=4) and the internal prepuce (n=8), and they are all depicted in figure 7. All these structures were elected considering the different tissues present in each one of them, which can facilitate or not, the penetration of the needle and consequent entrance of the virus.

Mice were anesthetized with isoflurane (Vetflurane, 575837-4, Virbac), placed in a stereotaxic apparatus (Model 963 Ultra Precise Small Animal Stereotaxic,

KOPF®) with the teeth fixed and turned to stay in a supine position to facilitate the access to the penis. Rymadil (5mg/kg, Carprofen, Zoetis), was administered intramuscularly. Then, the penis was exposed, by securing its base with a forcep, and cleaned with Betadine® (MEDAPharma) and 70% ethanol (Aga - Álcool e Géneros Alimentares, S.A.). Meanwhile, an injection needle, attached to an Hamilton Syringe system (EQL00617, Harvard Apparatus), was loaded with approximately 1µ of Ka-gEI-mCherry virus. After inserting the needle in the penis and waiting 5min, the Hamilton Syringe system was set up to a frequency of 0.05 µL/min. The injection time was calculated to be of 20min to deliver 1 µL of virus. After waiting 10min since the end of the injection, the needle was pulled out slowly and the skin was cleaned with Betadine®. Mice were single-housed and allowed 4 days of recuperation.

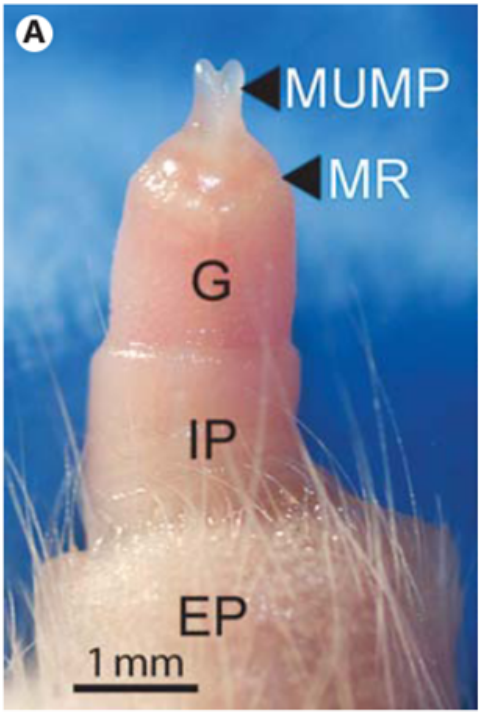

Figure 7. Photo depicting the exterior part of the adult mice penis. The penis can be divided in five areas, and three of them were chosen to be injected with the Ka-gEI-mCherry PRV, the glans, the internal prepuce and the male urogenital mating protuberance. All of them can be accessed without making any incision in the penis. G-Glans; EP – External Prepuce; IP – Internal Prepuce; MUMP – Male Urogenital Mating Protuberance; MR – Male Urogenital Mating Protuberance Ridge. Adapted fro

4. Histology

Two or four days after surgery, the animals were deeply anesthetized with a mixture of ketamine/xylazine [8% of ketamine (Imalgene 1000, 03661103001898, Merial) and 12% of xylazine (Rompun, (01)04007221017929, Bayer) in Saline Solution] and perfused transcardially first with Phosphate Buffered Saline 0.01 M (PBS) and finally with 4% paraformaldehyde (PFA) in PBS. The brains and the spinal cords were removed and postfixed in the 4% PFA solution at 4°C, overnight or for 4 hours (in the case of one immunohistochemistry experiment). Afterwards, the tissues were stored in 30% sucrose [30% sucrose (S0389, Sigma-Aldrich®) in Phosphate-Buffer 0.01M and 10% Sodium Azide (190381000, ACROS Organics™)] for at least one day to allow cryopreservation. Then, they were cut in 50-µm-thick sections in a cryostat (CM3050S, Leica), after embedding in optimum cutting temperature (OCT) compound (Tissue-Tek, 4583), and divided in two series. One series was subjected to NeuroTrace™ staining to identify the brain areas and spinal cord segments. The second was used for immunohistochemistry experiments. In each case, a Z1 AxioScan Microscope (Zeiss) with a 10x magnification objective, was used to acquire data. Later on, to have higher resolution images of some immunohistochemistry experiments, a confocal microscope was used, with a 10x magnification objective as well.

5. Nissl Staining

The slides were washed 2 times in PBS to remove the excess of OCT used in the cryostat. Then, the sections were rehydrated for 40 min in PBS 0.1M, pH 7.2 (PBS 10x) and permeabilized for 10min with 0.1% Triton-X (T9284-100ML, Sigma-Aldrich®) in PBS 10x. The tissue was washed 2 times 5min with PBS 10x. The staining solution was prepared by diluting the NeuroTrace™ (NeuroTrace™ 500/525 Green Fluorescent Nissl Stain, N21480, ThermoFisher Scientific; NeuroTrace™ 530/615 Red Fluorescent Nissl Stain, N21482, ThermoFisher Scientific;

NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain, N21479, ThermoFisher Scientific) 1:100 in PBS 10x and it was applied to the slides for 20 min. Then, the tissue was incubated with the 0.1% Triton-X solution used before for 10 min. After washing 2 times 5min with PBS 10x, the slides were rinsed with distilled water, allowed to dry and coverslipped.

6. Immunohistochemistry

Immunohistochemistry experiments were conducted in order to characterize the cells earlier identified in the tracing experiments. Four different protocols were used:

A) *Immunohistochemistry experiment, with tissue postfixed overnight.* First the slides were washed two times in PBS to clean the OCT. Then, the slices were incubated for 15min in 1% Sodium Borohydride (452882-25G, Sigma-Aldrich®) to diminish the tissue autofluorescence. The tissue was washed several times in PBS and incubated in 1% bovine serum albumin (BSA, A7906-100G, Sigma-Aldrich®), 0.3% Triton-X (T9284-100ML, Sigma-Aldrich®) in PBS (PBT-BSA) for 60min. The primary antibodies were diluted in a PBT-BSA solution with 0.1% Sodium Azide in the following concentrations: rabbit anti-Galanin (AB2233, Milipore) 1:5000; rabbit anti-Galanin 1:1000; rabbit anti-GFAP (G9269, Sigma-Aldrich®) 1:1000. GFAP was used as a positive control considering its high expression and a negative control was performed by not adding primary antibody. The slides were incubated with the primary antibodies for two overnights at 4°C. Afterwards, the tissue was washed in PBS and the secondary antibody, Alexa anti-Rabbit 488 (ab150077, Abcam) diluted at 1:1000 in PBT-BSA, was added for 3 hours at room temperature. After rinsing with PBS, a DAPI (4,6-Diamidine-2′-phenylindole dihydrochloride) solution was used as a counterstain. DAPI was diluted in PBS at 1:1000 and it was applied to the slides for 20min. Lastly, the tissue was washed with PBS and rinsed with distilled water. After being dried, the slides were coverslipped with *mounting media*.

B) *Tyramide SuperBoost Kit with Alexa Fluor Tyramides, with tissue postfixed overnight.* The Tyramide SuperBoost™ Kits with Alexa Fluor™ Tyramides (ThermoFisher Scientific) allow the amplification of signal by using secondary antibodies conjugated with poly-horseradish peroxidase (HRP) that react with the Alexa Fluor tyramides, to produce bright and photostable Alexa Fluor dyes around the protein of interest. To begin, the slides were washed with PBS and 1% Sodium Borohydride (452882-25G, Sigma-Aldrich®) in PBS as in the protocol described in A). Then, to quench endogenous peroxidase activity, a 3% Hydrogen Peroxide Solution was added for 60min at room temperature. After washing with PBS, a solution of 0.3% Tween (P9416-50ML, Sigma-Aldrich®) in PBS was added for 60min to increase tissue permeability. The slides were rinsed 3 times with PBS and incubated with the blocking buffer provided by the kit for 60min. Afterwards, the primary antibodies were diluted in the blocking buffer in the following concentrations: rabbit anti-Galanin (AB2233, Milipore) 1:5000; rabbit anti-Galanin 1:1000; rabbit anti-Choline Acetyltransferase (ChAT, AB143, Merck) 1:1000; and the tissue was incubated overnight at 4°C. After washing 3 times 10min in PBS, the poly-HRP-conjugated secondary antibody was added overnight at 4°C. Subsequently the slices were rinsed with PBS and the tyramide working solution was added to the slices for 0, 5 or 10min. The reaction between the HRP and the tyramides was stopped by adding the Stop Reagent Solution. Then the cells were counterstained with NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain as described above. After rinsing with distilled water the tissue was allowed to dry and it was coverslipped with *mounting media*.

C) *Tyramide SuperBoost Kit with Alexa Fluor Tyramides, with tissue postfixed for 4h.* This protocol was an adaptation of protocol B). The differences were: the time of tissue postfixation, that was only of 4h after perfusion; the concentration and type of detergent used to permeabilize the tissue, in this case 0.4% Triton-X (T9284-100ML, Sigma-Aldrich®) in PBS; the elimination of the blocking step with the blocking buffer provided by the kit; the use of the 0.4% Triton-X in PBS solution to

dilute the primary antibodies; the addition of a negative control by having slides that were incubated without primary antibody; and the elimination of the time step 0min with the tyramide working solution.

D) *VECTASTAIN Elite ABC HRP kit with tissue postfixed overnight*. The VECTASTAIN Elite ABC HRP kit (PK-6100, Vector Laboratories) uses biotinylated secondary antibodies that bind with the Avidin/Biotinylated Enzyme Complex and, after adding the substrate 3,3'-Diaminobenzidine (DAB), creates a brown precipitate that allows the visualization of proteins. To begin, the tissue was washed 3 times for 10min in Tris-buffered saline 0.05M, pH 7.6 (TBS). Then, an antigen retrieval step was performed by incubating the slices in Tris-HCl 0.01M, pH 10, for 5min at 90°C. After allowing the slices to reach room temperature, an incubation solution [0.3% Triton-X (T9284-100ML, Sigma-Aldrich®) and 0.25% BSA (A7906-100G, Sigma-Aldrich®) in TBS] was added to promote the permeabilization and blocking of the tissue. The endogenous peroxidases were blocked by adding a solution of 30% hydrogen peroxide (H1009-100ML, Sigma-Aldrich®) in methanol (34966-1L, Honeywell Fluka) and TBS for 10min. After washing with TBS, the primary antibody, [anti-Galanin (AB2233, Milipore) diluted 1:1000 in 2% goat serum in incubation solution] was added for 3 nights at 4°C. Subsequently, the tissue was rinsed 3 times for 10min with TBS and incubated with the biotinylated secondary antibody (anti-Rabbit diluted 1:200 in incubation solution) for 90min at room temperature. The A/B Vectastain Elite solution, made in incubation solution, was added for 90 min, allowing the binding of the Avidin/Biotinylated Enzyme Complex to the secondary antibody. The tissue was washed with TBS and a reaction with 3,3'-Diaminobenzidine (DAB) was allowed for 2 min. The DAB solution is composed of DAB, 1% Nickel and 30% Hydrogen Peroxidase in TBS. Finally, the slices were rinsed with TBS, dried and coverslipped.

7. Data Analysis

The presence, location and number of PRV-positive cells was assessed for each injected. Subjects without viral expression were excluded from the study. In the case of the spinal cord images, the analysis of the location was made considering two parameters:

- An anatomical characterization based on the laminae division represented in the Paxinos mouse spinal cord atlas[62].
- A functional distinction dependent on the type of neurons present in that area (Figure 8). The dorsal part of the spinal cord is associated with sensory neurons, therefore it was considered the sensory area. The ventral zone is linked with motor functions, being composed by motor neurons, so it was considered the motor area. Finally, the medial part of the spinal cord is usually composed by interneurons, therefore the medial area was considered independently of the other two areas.

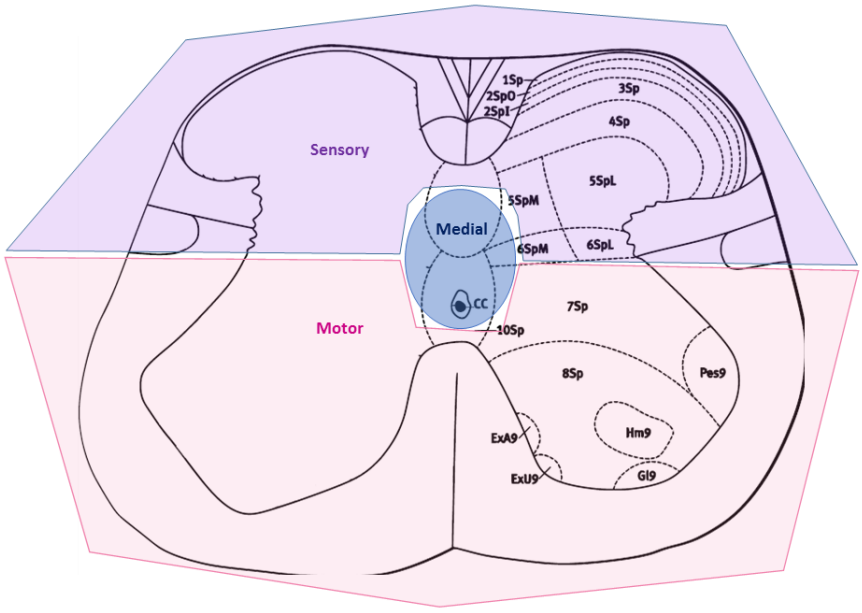

Figure 8. Scheme highlighting the division in laminae made in the Paxinos mouse spinal cord atlas (on the right) and the division in functional areas. In purple is depicted the sensory area, in pink the motor area and in blue the medial. Adapted from[62].

The number of infected cells was assessed using the ImageJ software. Three regions of interest (ROIs) were chosen based on the functional division described above. After setting the threshold (different between animals, because of differences in tissue characteristics), the size (0.001 to 0.075 inch^2) and the circularity (maximal) of the particles, the "Analyse Particles" option was chosen to count the number of cells present in each area. A simple ImageJ Macro program was written to automatize this process (Figure 9). Afterwards, a division in spinal cord segments intervals was made. The intervals chosen were: T8-T10; T11-T13; L1-L2; L3-L4; L5-L6; S1-S4. This segmentation was chosen considering the distribution of different cell types described in the rat: the final thoracic segments and beginning of lumbar segments are mainly composed of sensory and medial cells; the lumbar segments are essentially rich in medial cells (location of the putative SEG); and the final lumbar segments and sacral segments have more motor cells, even if rich in sensory and medial cells too[63]. The results were then summarized using the GraphPad Prism 7 Software.

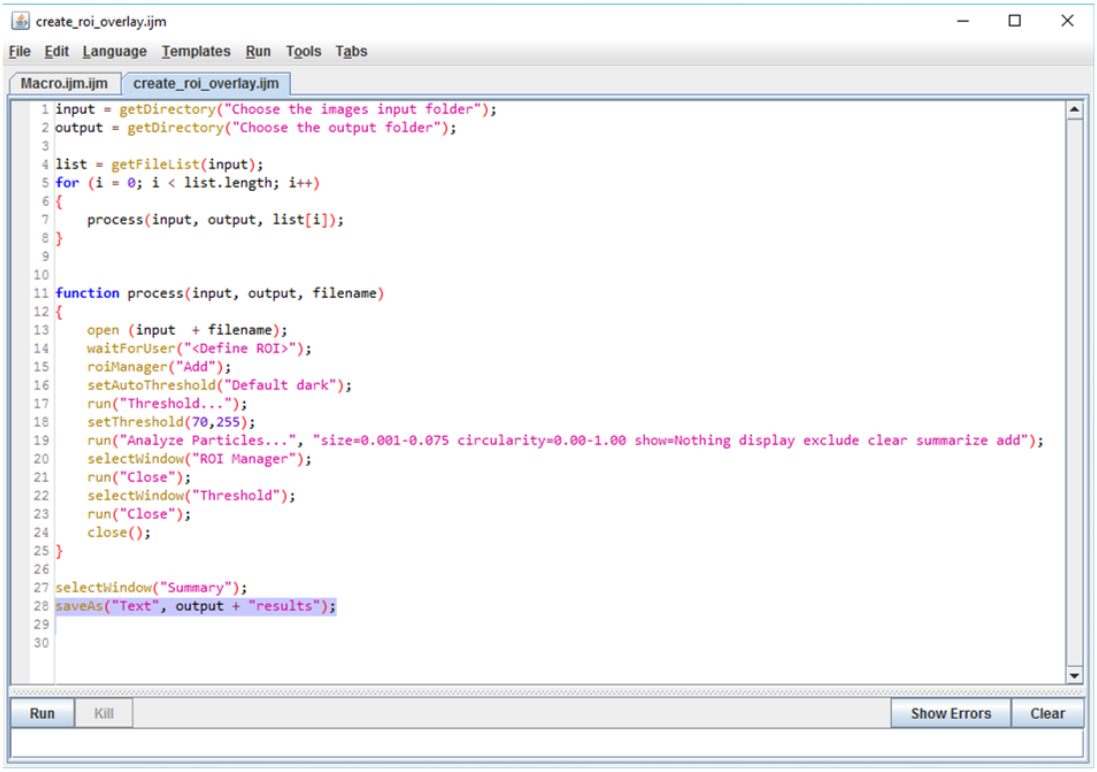

Figure 9. ImageJ macro for particle analyses.

For the immunohistochemistry results, the only analysis made was the location of the signal, based on the rules set for the PRV signal.

In the case of the brain, the analysis of the PRV-positive cells position was made based on the Paxinos mouse brain atlas[64] division and nomenclature.

Results

In this work, we assessed the neural circuitry between the penis/BSM and the central nervous system (spinal cord and brain) in order to start establishing the existence of a SEG in the mouse. To do so, we performed PRV injections into the BSM or the penis of C57BL/6 wild-type mice, allowing to map the potential neuronal circuitry controlling ejaculation. Specifically, we related our results to the findings in rats, where an SEG was already reported. Labelling with galanin helped us to strengthen our identification of an existing SEG in mice, since this neurotransmitter has been found in ejaculation promoting cells in the rat spinal cord. Finally, our study enabled us to further analyse the network labelled with the PRV in order to understand how ejaculation is possibly controlled in mice.

1. Evaluation of the most potent PRV: Ka-gEI-mCherry leads to the highest viral expression and distribution

Initially, we evaluated the viral expression of four different PRVs: Ba-gG-mRFP, Ba-ASP-EYFP, Ka-gEI-VampGFP and Ka-gEI-mCherry. Injecting the viruses into the S1BF and allowing tracer transport for 3 days, resulted in no signal in the case of the Ba-gG-mRFP and Ba-ASP-EYFP viruses. However, injections of the Ka-gEI-VampGFP and Ka-gEI-mCherry, led to strong fluorescent signal in the brain but with different characteristics. The Ka-gEI-mCherry signal is stronger, brighter and more abundant in comparison to the Ka-gEI-VampGFP signal (Figure 10). Furthermore, the Ka-gEI-mCherry PRV spreads throughout the brain and is concentrated in the somata. The expression of Ka-gEI-VampGFP signal is less intense, less distributed and seems to surround the cell body. Based on these findings, we chose the Ka-gEI-mCherry PRV for our subsequent experiments, which should unravel the rough neural circuitry of ejaculation.

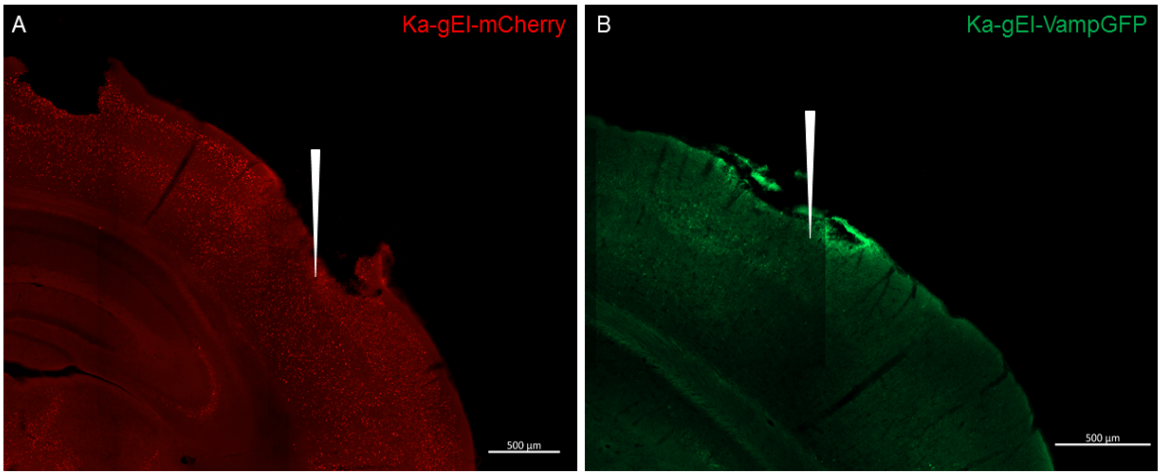

Figure 10. Pseudorabies virus expression following the injection of Ka-gEI-mCherry (A) and Ka-gEI-VampGFP (B) into the S1BF of C57BL/6 wild-type mice. Expression of Ka-gEI-mCherry (red) is abundant and highly spread in the brain, contrarily to the expression of Ka-gEI-VampGFP which shows a weak labelling. **Injection sites are marked with white arrows. Scale bars 500µm.**

2. Muscle injections of Ka-gEI-mCherry PRV resulted in labeling of several cell types in the mice spinal cord

The injection of the Ka-gEI-mCherry PRV in the mice BSM allowed the identification of several spinal cord areas and cell types related with the control of this muscle and, consequently, the control of ejaculation in mice. The PRV signal ranged from segment T8 until segment S4 (n=3). The signal is conserved across animals.

Figure 11 shows labelling at different spinal cord areas for three injected animals (PRV M1-M3). In A.1, A.2 and A.3 labelling of sensory cells in the dorsal part of the spinal cord can be observed. E.1, E.2 and E.3 shows in addition to labelling of sensory cells, signal in motor cells in the ventral part of the spinal cord. In the lumbar segments 3 and 4 (Images in C.1, C.2 and C.3), the Ka-gEI-mCherry PRV signal is mainly located around the central canal in the laminae X and VII. This pattern was already associated with the presence of an SEG in the rat (Truitt et al. 2002)[44]. The images represented in B, D and F result from the merge with the NeuroTrace® signal, which allows us the correct identification of the spinal cord segment. This identification is represented in each picture and is essential to understand the pattern of labelling. Thus, we can see that the signal in the medial part of the spinal cord is mainly in L3 and L4 segments of lamina X and VII, as already described. The sensory signal was seen in at the final thoracic and beginning of the lumbar segments (until L2) and at the sacral and the end of the lumbar segments (L6 and S1 mainly). These are the equivalent locations of the sympathetic and parasympathetic centres, respectively[54]. The motor signal is mainly seen in the L6 and S1 segments in the dorsomedial nucleus, however, some cells are also spread in the whole ventral part of the spinal cord.

Taken together, our results match with the findings reported for rats[44]. Hence, we speculate that we found a SEG in the lumbar segments L3 and L4 of the mouse spinal cord.

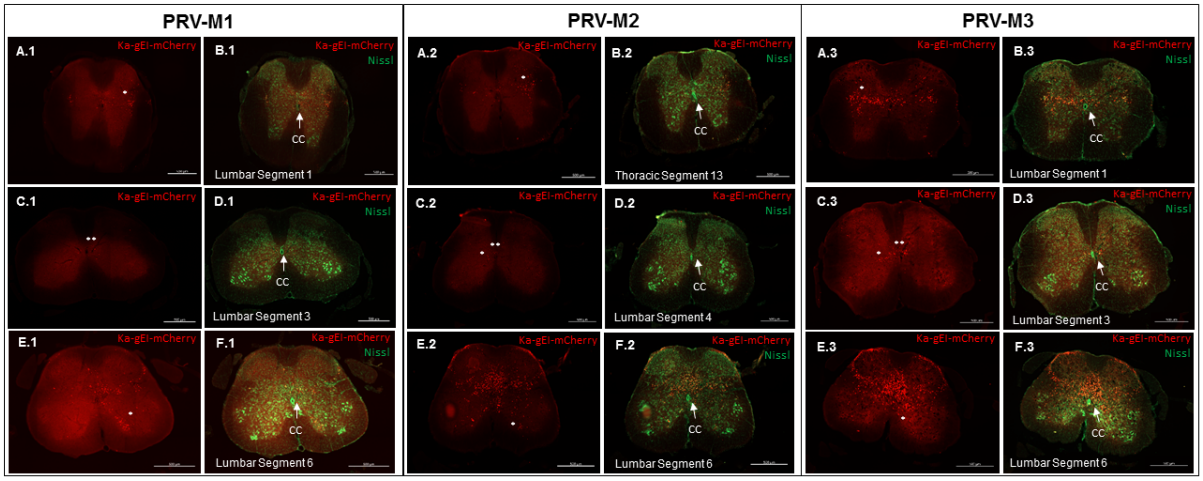

Figure 11. Coronal sections of the spinal cord revealing conserved labelling after injections of Ka-gEI-mCherry PRV into the BSM of three mice. PRV-M1, PRV-M2 and PRV-M3 stands for the three mice injected in the muscle, which had signal in the spinal cord. The first row (A and B) of every animal is composed by spinal cord segments that better represent the pattern of labelling of sensory cells. The second row (C and D) is a representation of the cells labeled on the medial part of each segment and may show the putative location of the mice SEG. Finally, the third row (E and F) shows the motor nuclei in the ventral part of the spinal cord. In A and E the sensory and motor nuclei are evidence by an *. In C the medial cells are evidenced by a * if they are located on lamina VII and ** if in lamina X. The images B, D and F represent the same segment has in A, C and E, respectively but, in this case, the image also has the NeuroTrace® signal in green. The NeuroTrace® staining is essential to identify the spinal segment. CC – Central Canal. Images acquired in the Z1 AxioScan Microscope with 10x magnification objective.

3. Penis injections of Ka-gEI-mCherry PRV resulted in similar pattern of labeling as in the muscle injections

Penis injections led to very similar labelling patterns when comparing to BMS injections. The Ka-gEI-mCherry PRV signal was observed in the same spinal cord areas, which might indicate the labelling of the same cell type. The location of the labelled neurons per segment was similar, too, ranging from segment T4 until segment S4 (n=5). Again, this distribution is conserved over animals (figure 12). The images depicted in A.1, A.2, A.3, A.4 and A.5 show the location of the possible sensory cells involved in the ejaculation circuitry, whereas the E.1, E.2, E.3, E.4 and E.5 illustrate the motor areas. C.1, C.2, C.3, C.4 and C.5 highlight the cells in the medial part of the segments L3 and L4 (lamina X and VII), possibly representing the

presence of a putative SEG in mice.

In summary, the labelling pattern following PRV penis injections is similar to the one observed when injections were made into the BSM. The same result was reported for rats [3]. This points to:

(1) A main location of sensory cells associated with the ejaculatory process in the final thoracic, the sacral and the beginning and last parts of lumbar segments

(2) A position of the motor signal, mainly seen in the L6 and sacral segments, in the dorsomedial nucleus.

(3) A putative location of the SEG in the lumbar segments L3 and L4.

Again, our results support the idea of an existent SEG in the mice spinal cord, and its capacity for controlling different components of ejaculation in mice.

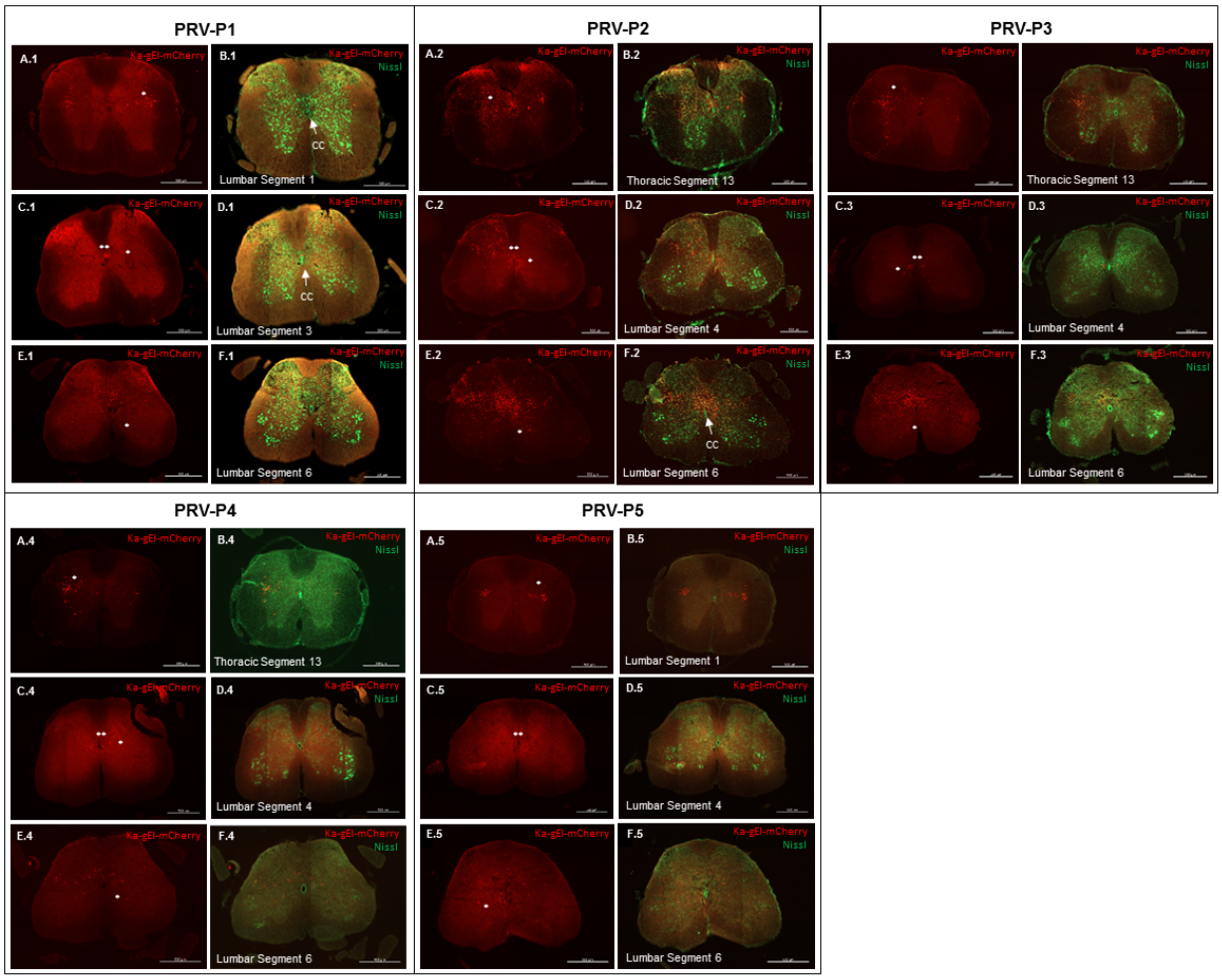

Figure 12. Spinal cord labelling four days after the injection of Ka-gEl-mCherry PRV into the penis of five mice. PRV-P1, PRV-P2, PRV-P3, PRV-P4 and PRV-P5 stands for the five mice injected in the penis, which had signal in the spinal cord. The first row (A and B) of every animal is composed by spinal cord coronal sections that better represent the pattern of labelling of sensory cells. The second row (C and D) is a representation of the cells labeled on the medial part of each segment and may show the putative location of the mice SEG. Finally, the third row (E and F) shows the motor nuclei in the ventral part of the spinal cord. In A and E, the sensory and motor nuclei are evidence by an *. In C, the medial cells are evidenced by a * if they are located on lamina VII and ** if in lamina X. The images B, D and F represent the same segment has in A, C and E, respectively, but, in this case, the image also has the NeuroTrace® signal in green. The NeuroTrace® staining is essential to identify the spinal segment. CC – Central Canal. Images acquired in the Z1 AxioScan Microscope with 10x magnification objective.

4. Amount of cells labelled in different spinal cord segments: Medial cells are the most prominent type

Figure 13 summarizes the amount of labelled cells observed in each animal which received either injections into the BSM (A) or directly into the penis (B). Each dot represents the average cell number normalized for the region of interest (ROI) in cm^2, of one animal.

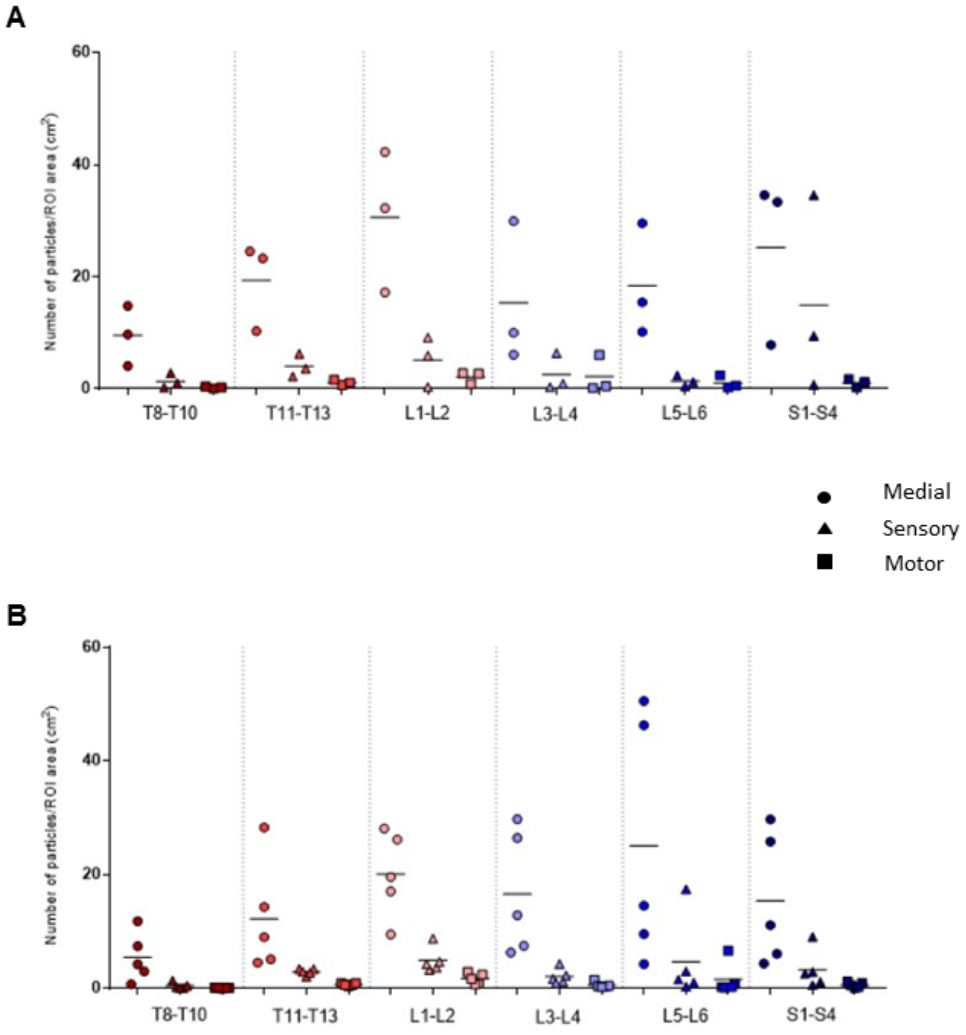

Figure 13. **Summary of the Ka-gEI-mCherry PRV signal of each animal, divided by segment interval and type of cell.** Each dot corresponds to the average of the cells for each animal, in each

condition. The black bar is the mean for each condition. The segment intervals chosen were from T8-T10, T11-T13, L1-L2, L3-L4, L5-L6 and S1-S4. The different cell types are represented by different geometric symbols (circle for medial cells, triangle for sensory cells and squares for motor cells). Graphic A summarizes the 3 animals injected in the muscle, with signal, whereas graphic B summarizes the five animals injected in the penis.

Note that, the distribution of signal is very similar across animals injected in the muscle or in the penis (as shown by immunostaining in Figures XX). Interestingly, the ratio of medial cells is larger in all segments, when compared to the sensory or motor cells. Overall, the ratio of motor cells represent the smallest population or is very similar to the ratio of sensory cells. Looking at the cell distribution over spinal cord segments, reveals that the lumbar segments contain a larger amount of medial cells, whereas the sacral segments have the ratio of medial and sensory cells closer. The segments T8-T10 possess very few labelled cells in comparison to the other spinal cord segments.

5. Galanin is expressed in the same areas labelled with the Ka-gEl-mCherry PRV

Since previous work[52-54] showed that the ejaculation controlling neurons are galanin positive, we wanted to counterstain our brains and spinal cords with antibodies for this marker. We also counterstained for ChAT, to try to identify the motoneurons, since ChAT is one of the enzymes responsible for the production of acetylcholine, the neurotransmitter used by these neurons. Therefore we evaluated four different immunohistochemistry protocols (see Material and Method section). Whereas all staining techniques did not lead to positive results regarding the ChAT antibody, galanin signal could be observed with the two last protocols described in our methods part.

In the protocol described in C), the galanin signal was strong, however, the

ChAT signal was not observed.

From Figure 14 we can conclude that galanin signal is located approximately in the same areas as the ones labelled with the Ka-gEI-mCherry PRV. More precisely, the coronal sections of a representative spinal cord in panel A and E reveals the signal at the medial and sensory part of the spinal cord. These are the segments where we observed the most potent signal in the PRV injected animals. Furthermore, we observed galanin signal at lamina X in the medial part of the spinal cord in lumbar segment 4, where the putative rat SEG is located[44]. Unfortunately, this spinal cord did not came from an animal previously injected with the PRV. This spinal cord is not originated from an animal previously injected with the PRV. This happened because, while optimizing the immunohistochemistry protocol for the TSA Method, we tried to use spinal cords that were post fixed for only 4h, contrarily to the overnight post fixation used in the spinal cords from injected animals. We believe that this change in the post fixation time with PFA allowed the observation of signal in this animal.

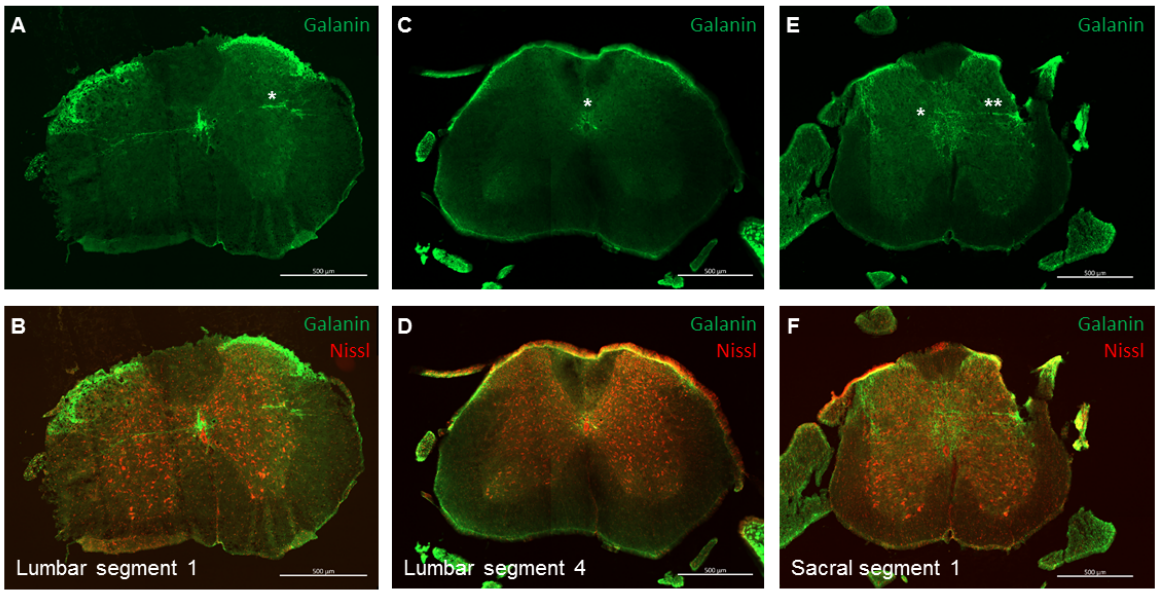

Figure 14. Galanin signal in mice spinal cord is approximately in the same areas observed in for the Ka-gEI-mCherry PRV signal. The first row, A, C and E, is composed by an image of the galanin signal. In the second row, B, D and F, is represent the same segment has in A, C and E, respectively, but, in this case, the image also has the NeuroTrace® signal in red. The NeuroTrace®

staining is essential to identify the spinal segment. Images acquired in the Z1 AxioScan Microscope with 10x magnification objective.

In an attempt to use the spinal cords from animals that were injected with the Ka-gEI-mCherry PRV, we performed a DAB based immunohistochemistry protocol, as described in section D in immunohistochemistry protocols in the Materials and Methods. A preliminary analysis confirmed the presence of signal for Galanin in some of the slides (data not shown) although the results are still being evaluated.

6. Labelled brain areas following injections into the BSM or penis are similar

We analysed the brains of three animals where we injected either PRV into the BSM or into the penis. This led to the identification of several brain areas that showed labelling in all six animals. We observed signal from medullary to thalamic structures. In tables I and II, we identified, (common in animals), the following areas: Raphe Obscurus Nucleus (ROb); Raphe Pallidus Nucleus (RPa); Raphe Magnus Nucleus (RMg); Medullary Reticular Nucleus, Ventral Part (MdV); Gigantocellular Reticular Nucleus (Gi); Gigantocellular Reticular Nucleus, Alpha Part (GiA); Gigantocellular Reticular Nucleus, Ventral Part (GiV); Dorsal Paragigantocellular Nucleus (DPGi); Lateral Paragigantocellular Nucleus (LPGi); Lateral Paragigantocellular Nucleus, External Part (LPGiE); Pontine Reticular Nucleus, Oral Part (PnO); Barrington's Nucleus (Bar); Paraventricular Hypothalamic Nucleus, Dorsal cap (PaDC); Paraventricular Hypothalamic Nucleus, Ventral Part (PaV).

Even though labelling in most of the brain areas was observed throughout animals regardless of the location of injection, some areas showed labelling dependent on the injection site. In the muscle injected animals, we also observed signal in the following structures: Nucleus Of The Solitary Tract, Commissural Part (SolC); Paramedian Reticular Nucleus (PMn); Pontine Reticular Nucleus, Caudal Part (PnC); and Lateral Reticular Nucleus (LRt). In the penis injected animals we

also saw labelling in these areas: Solitary Nucleus, Central Part (SolCe); Solitary Nucleus, Medial Part (SolM); Ventral Spinocerebellar Tract (vsc); Lateral Superior Olive (LSO); Medioventral Periolivary Nucleus (MVPO); Locus Coeruleus (LC); Subcoeruleus Nucleus, Dorsal Part (SubCD); Subcoeruleus Nucleus, Ventral Part (SubCV); Laterodorsal Tegmental Nucleus (LDtg); Red Nucleus, Magnocellular Part (RMC); Lateral Hypothalamic Area (LH); Peduncular Part Of Lateral Hypothalamus (PLH); Paraventricular Hypothalamic Nucleus, Posterior Part (PaPo); Paraventricular Hypothalamic Nucleus, Medial Parvicellular Part (PaMP).

Furthermore, there are some areas that were only labelled in two out of three animals. From this category, we emphasize the labelling of diverse Solitary Nuclei, other parts of the Hypothalamus and the Paraventricular Nuclei, different Pontine Reticular and Subcoeruleus Nuclei and, finally, the presence of signal in the Somatosensory Cortex.

Most of our observed areas are known to be involved in the processing of social or sexual stimuli.

Table 1 Brain areas labelled after injection of Ka-gEI-mCherry PRV in the BSM muscle of adult mice. Abbreviation list in annex I.

Brain areas	ROb	RPa	RMg	RIP	DRL	PDR	MdV	MdD	SolC	SolM	SolVL	SolDL	SolV	PSol	Gi	LPGi	GiV	LPGiE	DPGi	GiA	vsc	ts	rs	PMn	PMnR	PnC	PnV	PnO	IRt	LRt	PCRtA	CVL	RVL	A5	A7
PRV-M1	x	x	x				x		x						x	x	x	x	x	x		x		x		x		x		x						
PRV-M2	x	x	x				x		x	x	x	x	x	x	x	x	x	x	x	x	x		x	x		x	x	x	x	x			x		x
PRV-M3	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x		x	x

Brain areas	Bar	IOD	LSO	SPO	DPO	MVPO	MVe	MVeMC	MVePC	SpVe	SuVe	LVe	LC	SubCV	SubCA	SubCD	Su5	5n	Pr5	DMtg	Ptg	LDtg	VTg	VLPAG	LPAG	DLPAG	DMPAG	p1PAG	RMC	RPC	mRT	RLi	EW	RMM
PRV-M1	x		x	x				x				x																						
PRV-M2	x	x	x	x	x		x		x		x			x	x	x	x	x				x	x	x	x	x			x					
PRV-M3	x					x	x			x	x		x	x	x	x	x		x	x	x	x		x	x		x	x	x	x	x	x	x	x

Brain areas	InC	ArcMP	LH	PH	SPF	PsTh	PLH	DM	DMD	DA	VMH	CM	AHA	PMV	ns	MPtA	S1HL	PaDC	PaPo	PaMP	PaV	PaLM	MPA	MPOM	VLPO	VMPO
PRV-M1																		x			x	x				
PRV-M2							x	x		x	x		x		x	x	x	x	x	x	x					
PRV-M3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Table 2 Brain areas labelled after injection of Ka-gEI-mCherry PRV in the penis of adult mice. Abbreviation list in annex I.

Brain areas	ROb	RPa	RMg	DRV	MdV	MdD	SolCe	SolVL	SolV	SolM	PSol	Gi	LPGi	GiV	LPGiE	DPGi	GiA	vsc	rs	PMn	PnC	PnV	PnO	IRt	LRt	PCRtA	CVL	A5	A7
PRV-P1	x	x	x	x	x		x		x	x		x	x	x	x	x		x		x		x	x	x	x		x	x	
PRV-P4	x	x	x		x		x		x	x		x	x	x	x	x	x	x	x		x		x	x					x
PRV-P5	x	x	x		x		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x			

Brain areas	Bar	LSO	LVPO	MVPO	MVeMC	MVePC	SuVe	LC	SubCV	SubCD	Su5	DMTg	LDTg	VLPAG	LPAG	DLPAG	p1PAG	RMC	RPC	mRT	RLi	Arc	LH	PH	PsTh	PLH	DM
PRV-P1	x	x		x			x	x	x	x			x	x	x			x				x	x	x	x	x	x
PRV-P4	x	x		x				x	x	x	x	x	x					x					x	x		x	x
PRV-P5	x	x	x	x	x	x		x	x	x	x		x		x	x	x	x		x	x		x			x	

Brain areas	DMD	DA	VMH	AHP	ns	MPtA	LPtA	S1BF	S1HL	PaDC	PaPo	PaMP	PaV	VLL	C3
PRV-P1			x						x	x	x	x	x		
PRV-P4				x	x					x	x	x	x	x	
PRV-P5	x	x	x		x	x	x	x	x	x	x	x	x		x

Discussion

Our objective was to understand the neuronal circuitry that controls ejaculation in mice. Ejaculation has been studied in rats and humans, and the results point to the existence of a SEG that controls this process. Also, it has been shown that the SEG is capable of triggering the ejaculatory process without the influence of the brain. These evidence arise from studies in spinalized rats[46] and patients with spinal cord injury[45], where the stimulation of the SEG alone is capable of inducing an ejaculation. The SEG is composed of LSt cells, rich in galanin, that are located in lamina X and VII of the lumbar segments 3 and 4[44]. Furthermore, ejaculation depends on the coordinated action of the sympathetic, parasympathetic and somatic systems, as well as on the action of the central nervous system[11].

1. Ka-gEI-mCherry PRV being from the Kaplan strain has a higher infective capacity

In the first experiment, we demonstrated that, from four different viruses, the Ka-gEI-mCherry was the one with the better signal. This might be explained by the original strain from which this virus is derived. As already referred, the Ka-gEI-mCherry PRV results from the modification of a virus from the Kaplan strain. This strain, when compared to the Bartha strain (the original strain for two other viruses tested), is much more infective[60]. Therefore, it was expected that the Kaplan derived viruses would have a better signal when compared to the Bartha derived ones. That is probably why that after 3 days of viral infection we could only observe the signal for the Ka-gEI-VampGFP and Ka-gEI-mCherry viruses. Probably, if we had given more time for viral replication, we would have also observed signal for the Ba-gG-mRFP and Ba-ASP-EYFP viruses.

Comparing the Ka-gEI-VampGFP and Ka-gEI-mCherry PRVs, we observed that the last one had a stronger and brighter signal. This might be due to the fact that the Ka-gEI-VampGFP virus has the GFP protein linked a synaptic vesicle associated membrane protein and, this way, the expression of the fluorescent protein will be confined to those membranes. Therefore, the signal will be more specific, but also less strong because the amount of vesicles is a limiting factor for the expression of the virus. The fact that the Ka-gEI-mCherry is not linked to any external protein allows a higher expression of the virus and consequent stronger signal[60].

2. Muscle injections results suggest the existence of a SEG in mice

One of the first studies that used a PRV to try to understand the ejaculatory process in rat was made by Marson et al. (1996), and they injected a PRV from the Bartha strain in the BSM and the ischiocavernosus muscle of the animals. In this study, the authors discovered the presence of “putative spinal interneurons” from segments T13-S1, with a large number of neurons in the T13-L2 segments and L6-

S1, where, respectively, the sympathetic (hypogastric nerve) and parasympathetic (pelvic nerve) preganglionic neurons are located. However, the location of these neurons was not in accordance with the location of the autonomic system nuclei instead, they were around the central canal. Furthermore, they confirmed the location of the BSM motoneurons in the dorsomedial nucleus of the spinal segments L5 and L6[35]. Xu et al. (2006), performed a double label experiment with two different Bartha strain PRVs, one injected in the BSM and the other in the prostate. The signal of the PRVs was seen approximately in the same areas, with 60% of it being located in the medial part of the lumbar segments 3 and 4[47]. In a more recent study, Dobberfuhl et al. (2014), confirmed the location of Bartha strain PRV infected cells (after injection in the BSM of pre-adolescent rats) in the segments T11-S1. Again, the location of the signal was mainly medial, on the dorsal grey commissure, but also on the nuclei of the sympathetic and parasympathetic preganglionic neurons. It is important to highlight that most of the PRV-labelled cells were seen in the segments L5-S1, whereas, less amount of cells was in the segments L3-L4[48]. All of these studies point to the existence of a spinal control centre, the SEG, in the medial area of the lumbarsacral spinal cord that has been described by Truitt et al. (2002). As described by these authors, the SEG is located in the lumbar segments 3 and 4, lamina X and VII and is composed by galanin, cholecystokinin, encephalin and neurokinin-1 receptor positive cells with a direct projection to the thalamus, reason why they were designated lumbar spinothalamic cells. In this study, the authors used a toxin with high affinity for the neurokinin-1 receptor, causing the specific lesion of the LSt cells, and proving their importance for ejaculation, since lesioned rats had a completely disruption of the ejaculatory behaviour display[44].

In our study, we were able to identify PRV labelled cells in areas similar to the ones identified in the studies described above. We saw Ka-gEI-mCherry positive cells from segments T8-S4. However, in segments T8-T10 and the final sacral segments, the amount of labelled cells was considerably less, therefore our results are in accordance with the studies performed in rat, where the signal ranged from segments T11-S1. Furthermore, the location of the PRV positive cells was also

consistent with what is described in the literature, being essentially in the medial part of the spinal segments (lamina X and VII), but also, in the sympathetic and parasympathetic nuclei, equivalent to the preganglionic neurons from the hypogastric and pelvic nerves.

The amount of cells located in the sensory area, measured in this work, reflect the labelling of the sympathetic and parasympathetic nuclei. Has we can see in the quantification, there is a slightly bigger number of sensory cells in the segments corresponding to the autonomic centres (Figure 11). However, the difference is not that obvious and further analysis needs to be done. For example, by defining new intervals for quantification where the intermediolateral cell column (sympathetic preganglionic neurons from T13-L2 and parasympathetic preganglionic neurons from L5-S2) and the dorsal central autonomic nucleus (sympathetic nuclei) are considered separately from the sensory area[55].

Regarding the motoneurons, interestingly, we do not see a high amount of labelling. This is unexpected considering that the injection on the BSM should heavily label the motoneurons nuclei in the segment L5-S1[35]. However, by carefully analysing the images, we were able to see that, when there are motoneurons, they are mainly located in the dorsomedial nucleus and, sometimes, in the dorsolateral nuclei. Therefore, even if the quantification of the cells was not according to the expected, their location was. The labelling of the dorsolateral nuclei might be from an error in the injection, and some virus was uptaken by the ischiocavernosus muscle, that has its motoneurons in this part of the spinal cord[35]. Again, it is important to redo the quantification analysis and considerer these two spinal nuclei separately.

Finally, regarding the location of the putative SEG in rat, we could see a parallelism with the labelling in mice. In our study we were able to discriminate signal specifically in lamina X and VII of the lumbar segments 3 and 4, which is in accordance with what has been described for rat. Furthermore, the quantification analysis supports this finding, by clearly showing higher amount of cells per ROI area in the medial part of the spinal cord. Moreover, by carefully analysing the images resented in Figure 11, in segments L3 and L4, the specificity of the signal for

the medial area is evident.

3. Penis injections results further support the idea for the presence of a SEG in mice

Another study performed by Marson et al. (1993), used the same approach described for the BSM, but this time, the authors injected a Bartha strain PRV into the corpus cavernosus tissue of the penis of rats. They observed the labelling of sympathetic and parasympathetic preganglionic neurons that were part of the major pelvic ganglia, and also, the labelling of putative interneurons, that were preferentially located in the medial part of lumbosacral spinal cord. Signal ranged from segments C1 to S4, however, the higher amount was from T13 to S1 segments, where the preganglionic neurons from the hypogastric and pelvic nerve are located, as already referred. They never saw the presence of motoneurons. Nonetheless, an important conclusion from this study was that, even though there are no autonomic efferent to the lumbar segments 3 and 4, the authors observed a massive amount of labeled cells there. These cells had an interneuron-like morphology, which further support the presence of the SEG in this location[49].

Regarding our results, the same kind of signal was observed for the expression of the Ka-gEI-mCherry PRV. We observed PRV-positive cells from segments T8-S4, with a higher amount of medial and sensory cells. The location of this cells was consistent with the location of the sympathetic and parasympathetic centres in the spinal cord, as described above. The presence of a higher amount of medial cells on the L3 and L4 segments was observed, which further supports the theory for the location of the SEG in this location. Again, in these segments the signal was highly confined to lamina X and VII, excluding the possibility of being autonomic preganglionic neurons.

An unpredicted result was the labelling of neurons in the motor area in some of the segments. However, the location of these cells was not specific for the BSM or the ischiocavernosus motoneuron nuclei, the dorsomedial and the dorsolateral

nuclei respectively, as it can be seen in the Figure 12. Instead, most of them are close to the sensory and medial areas, which might mean that they are still sensory or interneurons, correspondingly. Furthermore, the spinal cord intrinsic connectivity is still highly unknown, therefore, considering that the Ka-gEI-mCherry PRV is a transsynaptic travelling virus able to cross any synapse connecting to the neuron where it was uptaken, this PRV positive cells might be from other pathways that also dependent on the spinal cord. This other pathways might be from other perineal muscles, like the levator ani, or from muscles innervating the external anal sphincter or the external urethral sphincter, which motoneurons are also present in the upper sacral segments[62].

4. Muscle and penis injections show very similar results

As we have been describing, the spinal cord signal in the muscle injected animals is very similar to the signal observed in the spinal cord of the penis injected mice. The labelling of the sympathetic and parasympathetic centres is consistent in the two cases. Also, the presence of essentially medial cells in the lumbar segments 3 and 4 that might represent the putative interneurons of the SEG is present in all the animals. However, the labelling of the motoneurons is not consistent, which makes sense considering that the penis injection should not label motoneurons. All these results points for the existence of a mouse SEG that is able to control several structures of the ejaculatory process, in our case the BSM and the penis.

5. Galanin positive neurons might be the same as the ones labelled with the Ka-gEI-mCherry PRV

Galanin is a neuropeptide widely expressed in the brain and spinal cord. Ju et al. (1987) published two works regarding the expression of several neuropeptides in the spinal cord of the rat, namely galanin. These studies were the first to relate

the presence of galanin in the lumbar spinal cord of rats, and its co-localization with other neuropeptides[51,52]. Galanin is present in the lumbar ganglionic cells and co-localizes with substance P, calcitonin gene-related peptide and cholecystokinin[52]. Also, Ju et al. showed that the galanin-positive cells from the lumbar segments had projections to the thalamus (spinothalamic tract)[52]. Furthermore, Xu et al. (2005), confirmed that this galanin-positive cells located in the lumbar segments of the rat are lumbar spinothalamic cells and that they are involved in the control of the BSM and the prostate[65].

In this work, we were unable to have signal for galanin in animals that were previously injected with the Ka-gEI-mCherry PRV. This was probably because of the PFA post fixation protocol. In the injected animals, the spinal cords were maintained in PFA overnight and the over fixation of the tissue might be impairing the penetration of the antibody and consequently the signal quality. However, when the spinal cords were maintained in PFA for only 4h, galanin signal was present. Therefore, we were only capable of showing the results for the immunohistochemistry protocol done in animals that were not subjected to surgery. Nevertheless, this result indicates that the galanin signal is approximately in the same location has the PRV signal. As we can see in figure 14, the galanin-positive cells are present in the area of sympathetic and parasympathetic centres, which might indicate that this signal is from preganglionic neurons. Furthermore, we can clearly see galanin-positive cells around the central canal (lamina X) in the lumbar segments 3 and 4. Again, this indicates that the galanin is in close contact with the putative LSt cells present in this area. These results tend to support the role of galanin in the control of ejaculation that has it has been proposed for rat, even if we cannot know for sure if the galanin-positive cells are the same as the PRV-positive cells seen in the rest of the work. Further experiences are necessary.

6. The brain areas labelled with the Ka-gEI-mCherry PRV are related with social and sexual behaviour

Even if an actual SEG exists in mice, this spinal control centre must be under inhibitory and excitatory control provided by the brain. Studies in rat indicate that the medial preoptic area (MPOA), is one of the most important brain areas where sexual relevant stimuli are integrated and, from there, sexual responses are generated. This is mainly because the MPOA sends direct projections to several other nuclei in the brain that in turn control the spinal centres[54].

One of this areas is the paraventricular nucleus (PVN), a hypothalamic structure. Wagner et al. (1991), showed that the PVN has direct projections to the lumbar segments 5 and 6, specifically to the dorsomedial and dorsolateral nuclei, responsible for the innervation of the perineal muscles[66]. A few years later, Liu et al. (1997), observed that lesions in the PVN, by application of a high dose of N-methyl-D-aspartic acid (NMDA) or radiofrequency impaired male rat sexual behaviour[56]. More recently, Xia et al. (2017), proved that activation of the NMDA receptors in the male rat PVN promoted the ejaculatory response, by decreasing the intromissions latency and facilitating ejaculation. Furthermore, this study saw that the activation of the PVN caused an increase in the lumbar sympathetic response, which is probably the mechanism by which the PVN controls ejaculation[57].

Another brain area that receives inputs from the MPOA is the Gigantocellular Reticular Nucleus (Gi). Shen et al. (1990) injected fluorogold in the spinal motor nucleus that innervates the BSM, the dorsomedial nucleus, and saw retrograde labelling in the Gi, indicating, possibly, a direct projection from the Gi to the lumbar segments of the spinal cord[58]. Facchinetti et al. (2014) showed that indeed there is a direct projection from the ventrolateral division of the Gi to the lumbar segments L2-L5 in the rat spinal cord[59]. Furthermore, this study also saw a direct projection from the caudal Raphe Nuclei, namely from the Raphe Magnus and the Raphe Pallidus[59]. Shen et al. (1990) similarly identified the presence of labelled cells in the Raphe Nuclei after the injection of fluorogold in the spinal cord[58]. Yamanouchi et al. (1992), observed that lesions in the Raphe Obscurus supressed the ejaculatory

behaviour, whereas lesion on Raphe Magnus had no significant effect on ejaculation[67]. However, Kondo et al. (1997), saw that lesions on the medullary Raphe Nucleus (Obscurus, Magnus and Pallidus), facilitated the ejaculatory behaviour in rats[68]. This means that the serotonergic signal that the Raphe nucleus sends to the spinal cord has opposite effects depending on each nucleus is sending the information[67,68].

All these areas, seem to be directly controlling the ejaculation in rat, through the putative SEG existent in the lumbar segments. In our work, the PVN, the Gi and the Raphe Nucleus were labelled in all the six animals analysed. Either the muscle or penis injection of the Ka-gEI-mCherry PRV produced labelling of this three areas (in several of their subgroups) has it can be seen in the Tables I and II. Therefore, our results are in agreement with what has been seen for rats and this further supports our hypothesis for the existence of a SEG in mice.

7. Future Directions

This work allowed the identification of a putative circuit, dependent on a SEG, for the ejaculatory process in mice. However, these results are preliminary and further experiments must be done. In the near future we intend to:

1. Perform a time step experiment where the animals are sacrificed 2, 3, and 5 days after the injection on the BSM or the penis. This way, we will be able to understand more about the order by which the neurons are labelled and, consequently, the direct connections within the spinal cord and to the brain.

2. Optimize the immunohistochemistry protocol and mark for other SEG markers like cholecystokinin, or encephalin.

3. Execute an anterograde tracing experiment. Through this experiment, we will be able to identify the direct projections from the SEG to the rest of the spinal cord or the brain. If we observe signal in the thalamus, we will be one step closer to

assume that the cells found in this work are the LSt cells described for the SEG in rat.

For long term experiments, we plan to:

1. Optogenetically activate the SEG in transgenic anesthetized mice, infected with cre-dependent Channelrhodopsin-2 (ChR2) expressing viruses. We will use ejaculation and BSM contractions as readout. This experiment will allow us to establish which stimulation protocol leads to ejaculation.

2. Develop a paradigm for spinal cord optogenetic manipulation of SEG in awake sexually behaving mice to interfere with ejaculation/sexual behaviour.

References

1. Georgiadis, J. R., Kringelbach, M. L. & Pfaus, J. G. Sex for fun: a synthesis of human and animal neurobiology. *Nat. Rev. Urol.* **9,** 486–498 (2012).

2. Pfaus, J. G. Pathways of sexual desire. *J. Sex. Med.* **6,** 1506–1533 (2009).

3. *Handbook of Clinical Neurology: Autonomic Nervous System*. **117,** (Elsevier B.V., 2013).

4. Wibowo, E. & Wassersug, R. J. Multiple Orgasms in Men—What We Know So Far. *Sex. Med. Rev.* **4,** 136–148 (2016).

5. Hull, E. M. & Dominguez, J. M. Sexual behavior in male rodents. *Horm. Behav.* **52,** 45–55 (2007).

6. McGill, T. Sexual behaviour in three inbred strain of mice. *Behaviour* **19,** 341–350 (1961).

7. Levin, R. J. Revisiting Post-Ejaculation Refractory Time—What We Know and What We Do Not Know in Males and in Females. *J. Sex. Med.* **6,** 2376–2389 (2009).

8. Tlachi-López, J. L., Eguibar, J. R., Fernández-Guasti, A. & Lucio, R. A. Copulation and ejaculation in male rats under sexual satiety and the Coolidge effect. *Physiol. Behav.* **106,** 626–30 (2012).

9. Clement, P. & Giuliano, F. Physiology and Pharmacology of Ejaculation. *Basic Clin. Pharmacol. Toxicol.* **119,** 18–25 (2016).

10. Sheu, Geraldin, Revenig, Louis, Hsiao, W. *Physiology of ejaculation*. (Springer New York, 2014). doi:10.1007/978-1-4939-0425-9

11. Giuliano, F. & Clement, P. Neuroanatomy and physiology of ejaculation. *Annu. Rev. Sex Res.* **16,** 190–216 (2005).

12. Jesik, C. J., Holland, J. M. & Lee, C. An anatomic and histologic study of the rat prostate. *Prostate* **3,** 81–97 (1982).

13. Knoblaugh, S. & True, L. in *Comparative Anatomy and Histology* 285–308 (Elsevier, 2012). doi:10.1016/B978-0-12-381361-9.00018-4

14. Peikert, K., Platzek, I., Bessède, T. & May, C. A. The Male Bulbospongiosus Muscle and its Relation to the External Anal Sphincter. *J. Urol.* **193,** 1433–1440 (2015).

15. Plochocki, J. H., Rodriguez-Sosa, J. R., Adrian, B., Ruiz, S. A. & Hall, M. I. A functional and clinical reinterpretation of human perineal neuromuscular anatomy: Application to sexual function and continence. *Clin. Anat.* **29,** 1053–1058 (2016).

16. Ju_arez, Rauls, Cruz, Y. Urinary and ejaculatory dysfunction induced by denervation of specific striated muscles anatomically related to the urethra in male rats. *Neurourol. Urodyn.* **33,** 437–442 (2014).

17. Tang, Y., Rampin, O., Giuliano, F. & Ugolini, G. Spinal and brain circuits to motoneurons of the bulbospongiosus muscle: retrograde transneuronal tracing with rabies virus. *J. Comp. Neurol.* **414,** 167–92 (1999).

18. Elmore, L. A. & Sachs, B. D. Role of the bulbospongiosus muscles in sexual behavior and fertility in the house mouse. *Physiol. Behav.* **44,** 125–9 (1988).

19. Allard, J. & Edmunds, N. J. Reflex penile erection in anesthetized mice: An exploratory study. *Neuroscience* **155,** 283–290 (2008).

20. Parker, R. A. *et al.* MR Imaging of the Penis and Scrotum. *RadioGraphics* **35,** 1033–1050 (2015).

21. Hsieh, C. *et al.* Advances in understanding of mammalian penile evolution, human penile anatomy and human erection physiology: clinical implications for physicians and surgeons. *Med. Sci. Monit.* **18,** RA118-25 (2012).

22. Phillips, T. R., Wright, D. K., Gradie, P. E., Johnston, L. A. & Pask, A. J. A Comprehensive Atlas of the Adult Mouse Penis. *Sex Dev.* **9,** 162–72 (2015).

23. Rodriguez, E. *et al.* New insights on the morphology of adult mouse penis. *Biol. Reprod.* **85,** 1216–21 (2011).

24. Allard, J., Truitt, W. A., McKenna, K. E. & Coolen, L. M. Spinal cord control of ejaculation. *World J. Urol.* **23,** 119–26 (2005).

25. Larsson, K. & Sodersten, P. Mating in male rats after section of the dorsal penile nerve. *Physiol. Behav.* **10,** 567–571 (1973).

26. Pescatori, E. S., Artibani, W., Pagano, F., Triban, C., Italiano, G. (1993). Electrical stimulation of the dorsal nerve of the penis evokes reflex tonic erections of the penile body and reflex ejaculatory responses in the spinal rat. *J. Urol.* **149,** 1993 (1993).

27. Wieder, J. A., Brackett, N. L., Lynne, C. M., Green, J. T. & Aballa, T. C. Anesthetic block of the dorsal penile nerve inhibits vibratory-Induced ejaculation in men with spinal cord injuries. *Urology* **55,** 915–917 (2000).

28. Zhang, G. X., Yu, L. P., Bai, W. J. & Wang, X. F. Selective resection of dorsal nerves of penis for premature ejaculation. *Int. J. Androl.* **35,** 873–879 (2012).

29. David Prologo, J. *et al.* Percutaneous CT-guided cryoablation of the dorsal penile nerve for treatment of symptomatic premature ejaculation. *J. Vasc. Interv. Radiol.* **24,** 214–219 (2013).

30. Núñez, R., Gross, G. H. & Sachs, B. D. Origin and central projections of rat dorsal penile nerve: Possible direct projection to autonomic and somatic neurons by primary afferents of nonmuscle origin. *J. Comp. Neurol.* **247,** 417–429 (1986).

31. McKenna, K. E. & Nadelhaft, I. The organization of the pudendal nerve in the male and female rat. *J. Comp. Neurol.* **248,** 532–549 (1986).

32. GOSTA DAHLOF, L. & Larsson, K. Interactional effects of pudendal nerve section and social restriction on male rat sexual behavior. *Physiol. Behav.* **16,** 757–762 (1976).

33. Lodder, J. & Zeilmaker, G. H. Effects of pelvic nerve and pudendal nerve transection on mating behavior in the male rat. *Physiol. Behav.* **16,** 745–51 (1976).

34. Sachs, B. D. & Liu, Y. C. Copulatory behavior and reflexive penile erection in rats after section of the pudendal and genitofemoral nerves. *Physiol. Behav.* **51,** 673–680 (1992).

35. Marson, L. & McKenna, K. E. CNS cell groups involved in the control of the ischiocavernosus and bulbospongiosus muscles: a transneuronal tracing study using pseudorabies virus. *J. Comp. Neurol.* **374,** 161–79 (1996).

36. Hancock, M. B. & Peveto, C. A. A preganglionic autonomic nucleus in the dorsal gray commissure of the lumbar spinal cord of the rat. *J. Comp. Neurol.* **183,** 65–72 (1979).

37. Nadklhaft, I. & McKenna, K. E. Sexual dimorphism in sympathetic preganglionic neurons of the rat hypogastric nerve. *J. Comp. Neurol.* **256,** 308–15 (1987).

38. Baron, R. & Janig, W. Afferent and sympathetic neurons projecting into lumbar visceral nerves of the male rat. *J. Comp. Neurol.* **314,** 429–436 (1991).

39. Giuliano, F., Bernabe, J., Droupy, S., Alexandre, L. & Allard, J. A comparison of the effects of tamsulosin and alfuzosin on neurally evoked increases in bladder neck and seminal vesicle pressure in rats. *BJU Int.* **93,** 605–608 (2004).

40. Kim, S. W., Lee, S. H. & Paick, J.-S. In vivo rat model to measure hypogastric nerve stimulation-induced seminal vesicle and vasal pressure responses simultaneously. *Int. J. Impot. Res.* **16,** 427–432 (2004).

41. Hsieh, J. T. *et al.* The role of sympathetic and parasympathetic nerve systems on the smooth muscle of rat seminal vesicles - experimental results and speculation for physiological implication on ejaculation. *Andrology* **2,** 59–64 (2014).

42. Clement, P., HOSSEIN K. KIA, STEPHANE DROUPY, J. B. & LAURENT ALEXANDRE, PIERRE DENYS, A. F. G. Role of Peripheral Innervation in P-Chloroamphetamine-Induced Ejaculation in Anesthetized Rats. *J. Androl.* **27,** 381–389 (2006).

43. Nadelhaft, I. & Booth, A. M. The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: A horseradish peroxidase study. *J. Comp. Neurol.* **226,** 238–245 (1984).

44. Truitt, W. A. & Coolen, L. M. Identification of a potential ejaculation generator in the spinal cord. *Science* **297,** 1566–9 (2002).

45. Chéhensse, C. *et al.* Human spinal ejaculation generator. *Ann. Neurol.* **81,** 35–45 (2017).

46. Chéhensse, C., Clément, P., Joussain, C., Bernabé, J. & Giuliano, F. The spinal generator of ejaculation: Functional consequences of chronic spinalization and effect of substance P in anesthetized rats. *Neuroscience*

336, 12–19 (2016).

47. Xu, C. *et al.* Identification of lumbar spinal neurons controlling simultaneously the prostate and the bulbospongiosus muscles in the rat. *Neuroscience* **138,** 561–73 (2006).

48. Dobberfuhl, A. D., Oti, T., Sakamoto, H. & Marson, L. Identification of CNS Neurons Innervating the Levator Ani and Ventral Bulbospongiosus Muscles in Male Rats. *J. Sex. Med.* **11,** 664–677 (2014).

49. Marson, L., Platt, K. B. & McKenna, K. E. Central nervous system innervation of the penis as revealed by the transneuronal transport of pseudorabies virus. *Neuroscience* **55,** 263–80 (1993).

50. Coolen, L. M., Veening, J. G., Petersen, D. W. & Shipley, M. T. Parvocellular subparafascicular thalamic nucleus in the rat: anatomical and functional compartmentalization. *J. Comp. Neurol.* **463,** 117–31 (2003).

51. Ju, G. *et al.* Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res.* **247,** 417–31 (1987).

52. Ju, G., Melander, T., Ceccatelli, S., Hökfelt, T. & Frey, P. Immunohistochemical evidence for a spinothalamic pathway co-containing cholecystokinin- and galanin-like immunoreactivities in the rat. *Neuroscience* **20,** 439–456 (1987).

53. Kozyrev, N., Lehman, M. N. & Coolen, L. M. Activation of gastrin-releasing peptide receptors in the lumbosacral spinal cord is required for ejaculation in male rats. *J. Sex. Med.* **9,** 1303–18 (2012).

54. Giuliano, F. Neurophysiology of erection and ejaculation. *J. Sex. Med.* **8,** 310–315 (2011).

55. Coolen, L. M., Allard, J., Truitt, W. A. & McKenna, K. E. Central regulation of ejaculation. *Physiol. Behav.* **83,** 203–15 (2004).

56. Liu, Y. C., Salamone, J. D. & Sachs, B. D. Impaired sexual response after lesions of the paraventricular nucleus of the hypothalamus in male rats. *Behav. Neurosci.* **111,** 1361–7 (1997).

57. Xia, J. D. *et al.* Centrally mediated ejaculatory response via sympathetic outflow in rats: role of N-methyl-D-aspartic acid receptors in paraventricular nucleus. *Andrology* **5,** 153–159 (2017).

58. Shen, P., Arnold, A. P., Micevych, A. M. P. E. & Al, P. S. E. T. Supraspinal Projections to the Ventromedial Lumbar Spinal Cord i n Adult MaleRaks. **272,** 263–272 (1990).

59. Facchinetti, P., Giuliano, F., Laurin, M., Bernabé, J. & Clément, P. Direct brain projections onto the spinal generator of ejaculation in the rat. *Neuroscience* **272,** 207–216 (2014).

60. Tóth, J. Analysis of Mutant Pseudorabies Virus Strains. (University of Szeged, 2010).

61. Boldogkői, Z. *et al.* Genetically timed, activity-sensor and rainbow transsynaptic viral tools. *Nat. Methods* **6,** 127–130 (2009).

62. Watson, Charles, Paxinos, George, Kayalioglu, G. *The spinal cord*. (2009).

63. Coolen, L. M. Neural control of ejaculation. *J. Comp. Neurol.* **493,** 39–45 (2005).

64. Paxinos, George, Franklin, K. B. J. *The mouse brain in stereotaxic coordinates*. *Academic Press* (2001). doi:10.1038/npp.2014.336

65. Xu, C. *et al.* Galanin and neurokinin-1 receptor immunoreactive [corrected] spinal neurons controlling the prostate and the bulbospongiosus muscle identified by transsynaptic labeling in the rat. *Neuroscience* **134,** 1325–41

(2005).

66. Wagner, C. K. & Clemens, L. G. Projections of the paraventricular nucleus of the hypothalamus to the sexually dimorphic lumbosacral region of the spinal cord. *Brain Res.* **539,** 254–262 (1991).

67. Yamanouchi, K. & Kakeyama, M. Effect of medullary raphe lesions on sexual behavior in male rats with or without treatments of p-chlorophenylalanine. *Physiol. Behav.* **51,** 575–579 (1992).

68. Kondo, Y. & Yamanouchi, K. Potentiation of ejaculatory activity by median raphe nucleus lesions in male rats: effect of p-chlorophenylalanine. *Endocr. J.* **44,** 873–879 (1997).

Annexes

1. Annex I – List of abbreviations from Table I and II

5n – Motor Trigeminal Nucleus

A5 - A5 Noradrenaline Cells

A7 - A7 Noradrenaline Cells

AHA - Anterior Hypothalamic Area, Anterior Part

AHP - Anterior Hypothalamic Area, Posterior Part

Arc - Arcuate Hypothalamic Nucleus

ArcMP - Arcuate Hypothalamic Nucleus, Medial Posterior Part

Bar - Barrington's Nucleus

C3 - C3 Adrenaline Cells

CM - Central Medial Thalamic Nucleus

CVL - Caudoventrolateral Reticular Nucleus

DA – Dorsal Hypothalamic Area

DLPAG - Dorsolateral Periaqueductal Gray

DM - Dorsomedial Hypothalamic Nucleus

DMD - Dorsomedial Hypothalamic Nucleus, Dorsal Part

DMPAG - Dorsomedial Periaqueductal Gray

DMtg - Dorsomedial Tegmental Area

DPGi - Dorsal Paragigantocellular Nucleus

DPO - Dorsal Periolivary Region

DRL – Dorsal Raphe Nucleus, Lateral Part

DRV - Dorsal Raphe Nucleus, Ventral Part

EW - Edinger-Westphal Nucleus

Gi - Gigantocellular Reticular Nucleus

GiA - Gigantocellular Reticular Nucleus, Alpha Part

GiV - Gigantocellular Reticular Nucleus, Ventral Part

InC - Interstitial Nucleus Of Cajal

IOD - Inferior Olive, Dorsal Nucleus

IRt - Intermediate Reticular Nucleus

LC - Locus Coeruleus

LDtg - Laterodorsal Tegmental Nucleus

LH - Lateral Hypothalamic Area

LPAG - Lateral Periaqueductal Gray

LPGi - Lateral Paragigantocellular Nucleus

LPGiE - Lateral Paragigantocellular Nucleus, External Part

LPtA - Lateral Parietal Association Cortex

LRt - Lateral Reticular Nucleus

LSO - Lateral Superior Olive

LVe - Lateral Vestibular Nucleus

LVPO - Lateroventral Periolivary Nucleus

MdD - Medullary Reticular Nucleus, Dorsal Part

MdV - Medullary Reticular Nucleus, Ventral Part

MPA - Medial Preoptic Area

MPOM - Medial Preoptic Nucleus, Medial Part

MPtA - Medial Parietal Association Cortex

mRT – Mesencephalic Reticular Formation

MVe - Medial Vestibular Nucleus

MVeMC - Medial Vestibular Nucleus, Magnocellular Part

MVePC - Medial Vestibular Nucleus, Parvicellular Part

MVPO - Medioventral Periolivary Nucleus

ns - Nigrostriatal Bundle

p1PAG – p1 Periaqueductal Gray

PaDC - Paraventricular Hypothalamic Nucleus, Dorsal Cap

PaLM - Paraventricular Hypothalamic Nucleus, Lateral Magnocellular Part

PaMP - Paraventricular Hypothalamic Nucleus, Medial Parvicellular Part

PaPo - Paraventricular Hypothalamic Nucleus, Posterior Part

PaV - Paraventricular Hypothalamic Nucleus, Ventral Part

PCRtA - Parvicellular Reticular Nucleus, Alpha Part

PDR – Posterodorsal Raphe Nucleus

PH - Posterior Hypothalamic Area

PLH – Peduncular Part Of Lateral Hypothalamus

PMn - Paramedian Reticular Nucleus

PMnR - Paramedian Raphe Nucleus

PMV - Premammillary Nucleus, Ventral Part

PnC - Pontine Reticular Nucleus, Caudal Part

PnO - Pontine Reticular Nucleus, Oral Part

PnV - Pontine Reticular Nucleus, Ventral Part

Pr5 - Principal Sensory Trigeminal Nucleus

PSol - Parasolitary Nucleus

PsTh - Parasubthalamic Nucleus

Ptg – Pedunculotegmental Nucleus

RIP – Raphe Interpositus Nucleus

RLi - Rostral Linear Nucleus Of The Raphe

RMC - Red Nucleus, Magnocellular Part

RMg - Raphe Magnus Nucleus

RMM – Retromammillary Nucleus, Medial Part

ROb - Raphe Obscurus Nucleus

RPa - Raphe Pallidus Nucleus

RPC - Red Nucleus, Parvicellular Part

rs - Rubrospinal Tract

RVL - Rostroventrolateral Reticular Nucleus

S1BF - Primary Somatosensory Cortex, Barrel Field

S1HL - Primary Somatosensory Cortex, Hindlimb Region

SolC - Solitary Nucleus, Commissural Part

SolCe - Solitary Nucleus, Central Part

SolDL - Solitary Nucleus, Dorsolateral Part

SolL - Solitary Nucleus, Lateral Part

SolM - Solitary Nucleus, Medial Part

SolV - Solitary Nucleus, Ventral Part

SolVL - Solitary Nucleus, Ventrolateral Part

SPF - Subparafascicular Thalamic Nucleus

SPO - Superior Paraolivary Nucleus

SpVe - Spinal Vestibular Nucleus

Su5 - Supratrigeminal Nucleus

SubCA - Subcoeruleus Nucleus, Alpha Part

SubCD - Subcoeruleus Nucleus, Dorsal Part

SubCV - Subcoeruleus Nucleus, Ventral Part

SuVe - Superior Vestibular Nucleus

ts - Tectospinal Tract

VLL - Ventral Nucleus of The Lateral Lemniscus

VLPAG - Ventrolateral Periaqueductal Gray

VLPO - Ventrolateral Preoptic Nucleus

VMH - Ventromedial Hypothalamic Nucleus

VMPO - Ventromedial Preoptic Nucleus

vsc - Ventral Spinocerebellar Tract

VTg - Ventral Tegmental Nucleus