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Research article

Separate urinary bladder and prostate neurons in the central nervous system of the rat: simultaneous labeling with two immunohistochemically distinguishable pseudorabies viruses

Irving Nadelhaft*[1,2,3], Alejandro J Miranda-Sousa[3] and Pedro L Vera[1,3]

Address: [1]Veteran's Administration Medical Center, Bay Pines, FL 33744, [2]University of South Florida, Dept of Pharmacology, Tampa, FL. 33612 and [3]University of South Florida, Dept of Surgery, Urology Division, Tampa, Florida 33612

E-mail: Irving Nadelhaft* - inadelha@hsc.usf.edu; Alejandro J Miranda-Sousa - amiranda@hsc.usf.edu; Pedro L Vera - pvera@hsc.usf.edu

*Corresponding author

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Abstract

Background: This work examines the central nervous system distribution of virus-labeled neurons from the rat urinary bladder and the prostate simultaneously within the same tissue sections. Two immunohistochemically distinct pseudorabies virus strains were simultaneously injected into male Sprague Dawley rats (~280 gm). One virus was injected into the bladder and the other into the prostate. After incubation intervals of 2.25, 2.5, 2.75, 3 and 4 days, sections from the spinal cord and brain were processed immunohistochemically to detect cells, within a single section, which were labeled separately by each virus or were labeled by both viruses.

Results: Each strain of virus labeled a separate population of neurons and some neurons were labeled by both strains. The majority of neurons labeled by virus from the urinary bladder were found in the L6-S1 spinal cord segments within the dorsal gray commissure, the intermediolateral area and the superficial dorsal horn. Neurons labeled by virus from the prostate were mainly found in the L1-L2 spinal cord segments in the dorsal gray commissure and the intermediolateral areas. Double-labeled interneurons in L1-L2 were mainly located in the intermediolateral area. In L6-S1 they were divided between the dorsal gray commissure and the intermediolateral area.

Conclusions: Spinal neurons innervating the bladder are clearly separate and different from those innervating the prostate. This difference also persists in the brain. In disagreement with previous reports, no direct anatomical evidence of parasympathetic innervation of the prostate was observed.

Background

The prostate gland is part of the male genitalia and its function is to secrete fluid that is released with the ejaculate during copulation. The prostate surrounds the urethra just distal to the urinary bladder and proximal to the external urethral sphincter [1] and is composed of smooth muscle and secretory alveoli. Nervous innervation of the prostate is believed to be mainly sympathetic [2]. These nerves descend through the hypogastric nerves and synapse on postganglionic neurons in the pelvic plexus (major pelvic ganglion in the rat) from which the prostate receives direct innervation. The sympathetic nervous innervation is split into two portions: postganglionic adrenergic nerves that synapse on blood vessels and the smooth

muscle that surround the alveoli, and sympathetic cholinergic nerves that innervate the glandular epithelium. Thus control of prostatic secretion is mediated by a sympathetic cholinergic pathway. Evidence for parasympathetic innervation of the prostate is present but its function is largely unknown. Speculation suggests some form of control of the volume or composition of the secretion [3]. There is also evidence for the presence of periprostatic ganglia and capsular ganglia respectively outside and inside the prostatic capsule [4] but not much is known about their pharmacology or function.

The urinary bladder functions to store and expel urine [5]. It has both sympathetic and parasympathetic innervation. The parasympathetic innervation arises from preganglionic neurons located in the distal lumbar and proximal sacral spinal cord (segments L6 and S1 in the rat [6,7]) and whose axons are found in the pelvic nerve and synapse on postganglionic neurons in the pelvic plexus. Parasympathetic impulses are the main excitatory influence and contract the bladder causing emptying. The sympathetic innervation is mainly concerned with the storage function of the bladder [8].

Thus there is major and fundamentally different innervation of the bladder and the prostate. However in humans the prostate can affect the micturition function since when a man ages and the prostate enlarges it constricts the urethral lumen. This condition is commonly known as benign prostatic hypertrophy and seriously affects the micturition function of the bladder. Although the effect of prostatic enlargement on the bladder is primarily mechanical it is possible that in time it might also alter the neuronal circuitry that mediates micturition. In view of the aforementioned considerations and despite prior knowledge about the separate functions and innervation of these two organs we thought it worthwhile to examine the neuroanatomical organization of the bladder and the prostate simultaneously within a single venue. We undertook this study to answer questions not previously addressed by other investigations, namely: (1) Are the lumbosacral parasympathetic and sympathetic preganglionic neurons that innervate the bladder different from those that innervate the prostate, or to what extent do they dually innervate these organs? (2) Are the lumbosacral interneurons that are part of the pathways innervating these two organs separate groups of interneurons or do they participate in the innervation of the two organs? (3) Are the supraspinal areas involved in innervating these organs separate or do they contribute to the innervation of these two organs? To do this we have employed the unique technique of two immunohistochemically distinguishable pseudorabies viruses, which will simultaneously be injected, one to each organ, as neuroanatomical tracers to determine each of the central nervous system distributions [9,10].

Results

Major pelvic ganglion

The major pelvic ganglion contains the postganglionic neurons that innervate both the urinary bladder and the prostate. Therefore these neurons are the first neurons to be labeled by virus arriving from either of the injected organs. Figure 1 depicts bladder and prostate neurons in the major pelvic ganglion three days post infection. The neurons labeled by virus from the prostate were larger than those that were labeled by virus from the bladder. Also shown are adrenergic prostate neurons that were stained by the antiserum to tyrosine hydroxylase as well as prostate neurons that are not adrenergic. A count of these prostate neurons revealed that only 77% were adrenergic.

Spinal cord

The different categories of labeled neurons were: B: neurons labeled by virus injected into the urinary bladder; P: neurons labeled by virus injected into the prostate; BChAT (or BCh): neurons labeled by virus injected into the urinary bladder that were also cholinergic; PChAT (or PCh): neurons labeled by virus injected into the prostate that were also cholinergic; DBL: neurons labeled by both viruses (no double-labeled neurons were also cholinergic). The areas in which these different categories of neurons were located are: IML: intermediolateral area; DGC: dorsal gray commissure; SDH: superficial dorsal horn. Note that all cholinergic neurons (BChAT and PChAT) were considered to be preganglionic neurons. Virus-labeled neurons that were not also cholinergic were, by definition, interneurons.

Virus-labeled neurons in lumbosacral spinal cord first appeared 2.25 days post-infection. Table 1 and Figures 2, 3 and 4 display the means and standard errors of numbers of labeled neurons found in the L1-L2 and L6-S1 segments of the spinal cord as a function of incubation time. The table and figures demonstrate the following points:

1) There were many more neurons labeled by the virus from the bladder than by the virus from the prostate. Virus from the bladder labeled a mean total of 3629 neurons. The majority of these (3507) were found in L6-S1. Virus from the prostate labeled a total of 368 neurons. The majority of these (294) were found in L1-L2.

2) There were few double-labeled neurons (57). They were found after 2.75 days in L1-L2 and L6-S1 with the majority in L6-S1. They were located in the dorsal gray commissure (DGC) and the intermediolateral area (IML) with the majority in DGC. The doubles were exclusively interneurons. No preganglionic neurons were doubles.

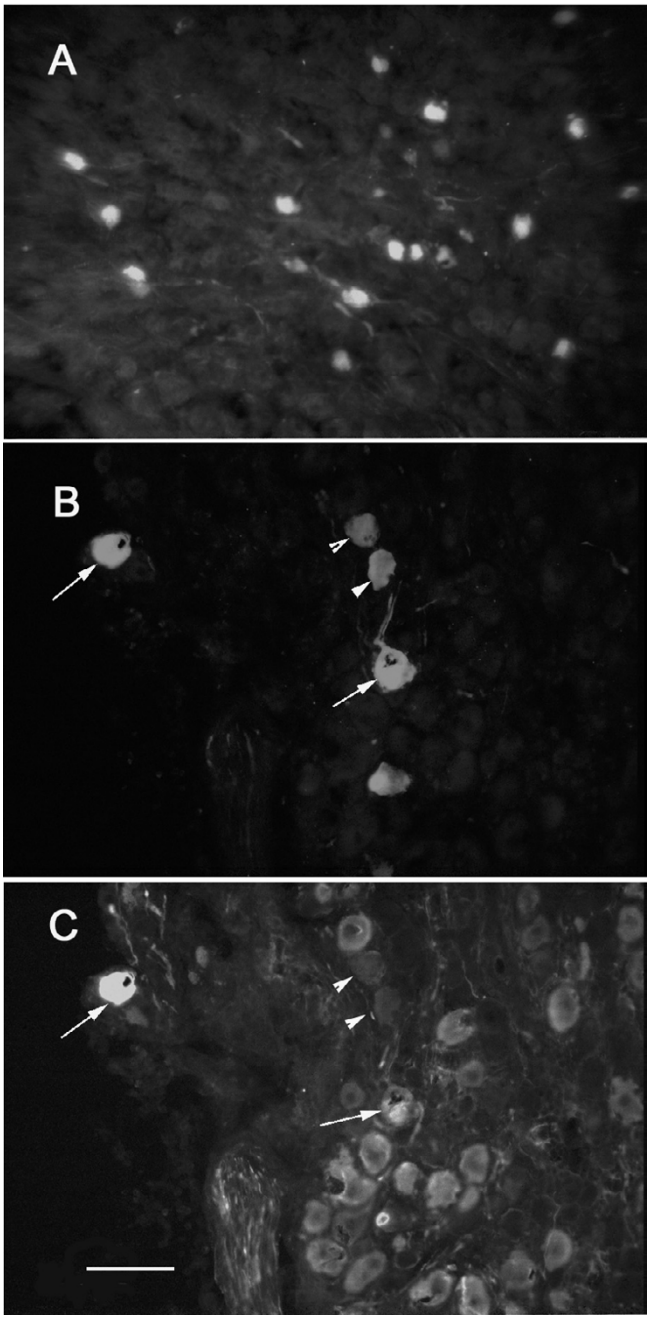

Figure 1
Virus-labeled neurons in the major pelvic ganglion A: Neurons labeled by virus injected into the bladder. **B:** Neurons labeled by virus injected into the prostate. **C:** Tyrosine hydroxylase-labeled neurons. In B and C, prostate neurons that are adrenergic are marked with arrows; prostate neurons that are not adrenergic are marked with arrowheads. Note that the prostate neurons are much larger than the bladder neurons. Bar: 100 μ.

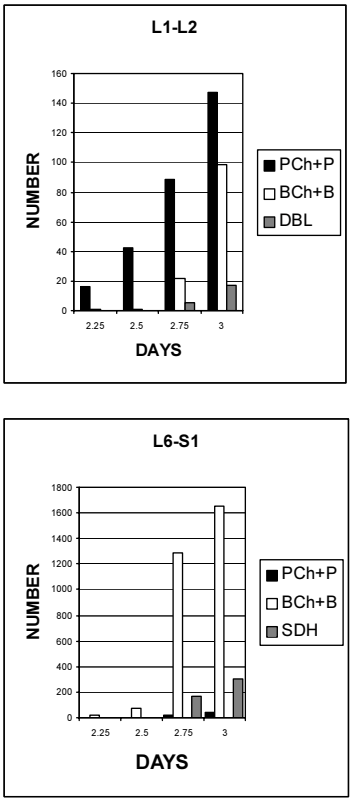

Figure 2
Prostate and bladder neurons in the lumbosacral cord as a function of incubation time Neurons labeled from the prostate (PCh+P) are found mainly in L1-L2 whereas neurons labeled from the bladder (BCh+B) are found mainly in L6-S1. Doubles are located in L1-L2 mainly in the dorsal gray commissure. Superficial dorsal horn neurons are found only in L6-S1. Note that: 1) the numbers of both prostate neurons and bladder neurons increase significantly at longer incubation times and: 2) the total number of bladder neurons is at least a factor of ten greater than the total number of prostate neurons.

3) Neurons located in the superficial dorsal horn (475) were only labeled from injections into the bladder and were only found in L6-S1 (Figure 5A) and only after 2.75 days.

4) With increasing incubation time more neurons of all types were labeled (Table 1 and Figures 3 and 4). These increases occurred mainly through the labeling of larger numbers of interneurons. For example, in L1-L2 the number of interneurons (prostate + bladder) at 2.25 days was 2.67. The number of interneurons at 3 days was 172.25, an increase by a factor of about 60. These are to be compared to the numbers of preganglionic cholinergic neurons (prostate + bladder) of 14.33 at 2.25 days and 74

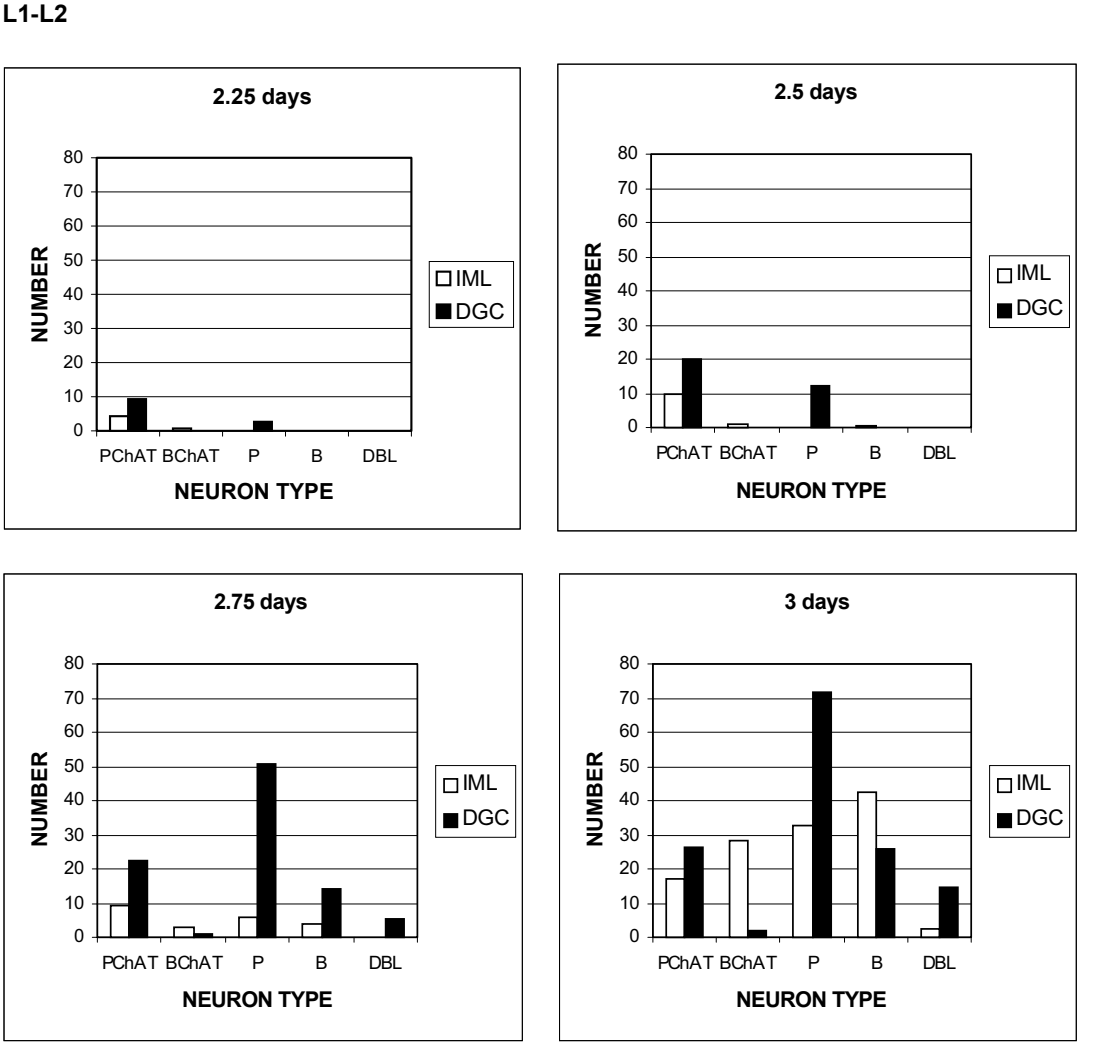

Figure 3
Types of neurons in the L1-L2 spinal cord segments as a function of incubation time The number of neurons of each type and at each location in the spinal cord section increases with incubation time. Virus from the prostate (types P, PChAT and DBL) labels the majority of the neurons. Cholinergic prostate neurons (PChAT) are located in the dorsal gray commissure and the intermediolateral area. Their number is approximately constant as a function of time. Cholinergic bladder neurons (BchAT) are found only after three days incubation and these are mainly located in the intermediolateral area. After three days, large numbers of prostate (P) and bladder (B) non-cholinergic neurons (presumably interneurons) are found in the dorsal gray commissure and the intermediolateral area. Double-labeled neurons are confined to the dorsal gray commissure.

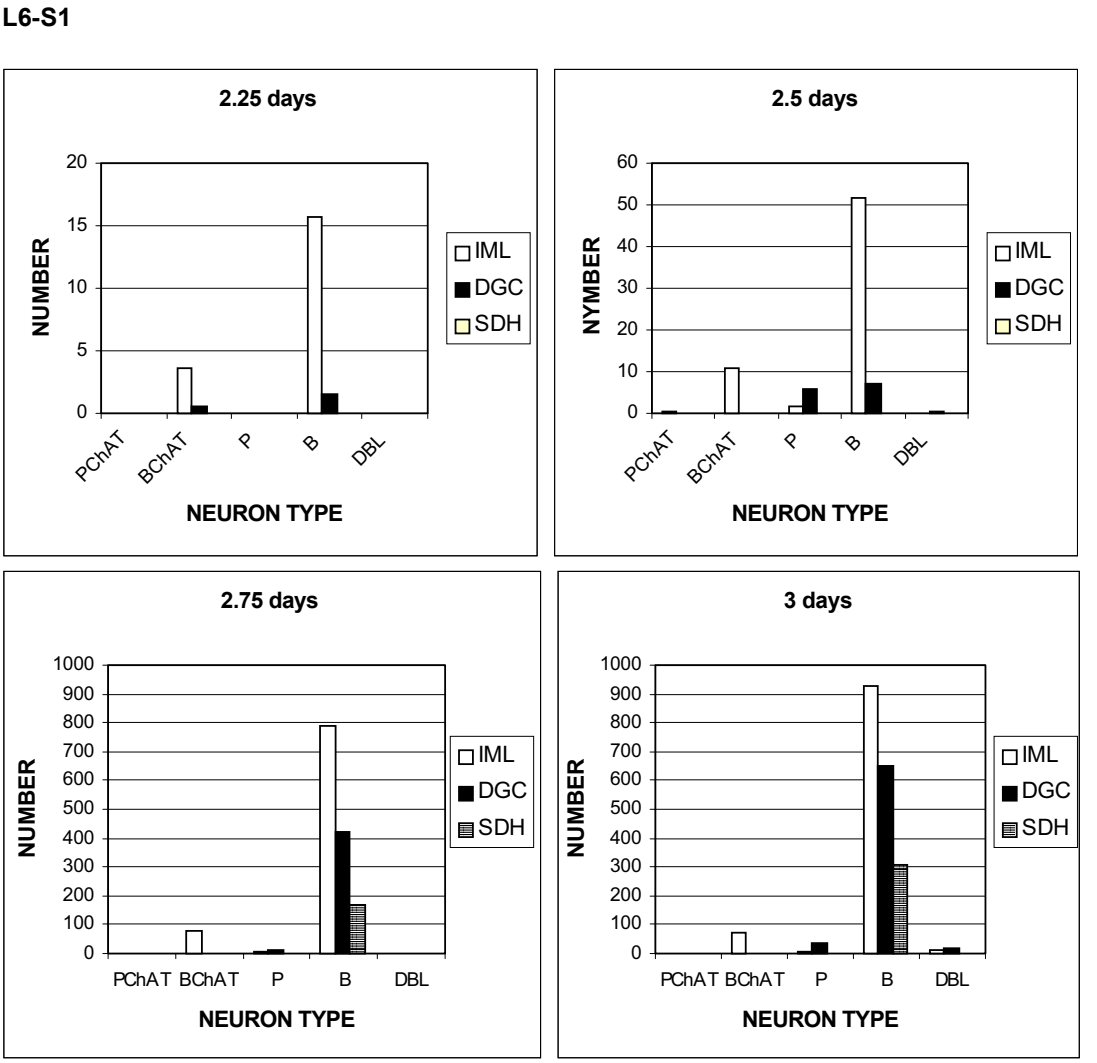

Figure 4
Types of neurons in the L6-S1 spinal cord segments as a function of incubation time The number of neurons within each category and location increases with incubation time. The majority of labeled neurons are bladder (B) neurons; there are very few neurons labeled by virus from the prostate (P). Cholinergic neurons (BChAT) are restricted to the intermediolateral area (IML) and their number remains approximately constant. The number of non-cholinergic bladder neurons (B) increases dramatically at longer incubation times. These are presumably interneurons and are found in the intermediolateral area and the dorsal gray commissure (DGC) and, after three days, also in the superficial dorsal horn (SDH).

Table 1: Data means and standard errors

	L1-L2		L6-S1		
2.25d	**IML**	**DGC**	**IML**	**DGC**	**SDH**
PChAT	4.33 (±2.33)	9.33 (±4.33)	0	0.00	0.00
BChAT	0.67 (±0.66)	0.00	3.67 (±3.18)	0.50 (±0.41)	0.00
P	0.00	2.67 (±0.88)	0	0.00	0.00
B	0.00	0.00	15.67 (±14.19)	1.50 (±1.22)	0.00
DBL	0.00	0.00	0	0.00	0.00
2.5d					
PChAT	9.60 (±4.30)	20.20 (±6.63)	0.33 (±0.33)	0.00	0.00
BChAT	1.00 (±0.58)	0.00	11.00 (±4.36)	0.00	0.00
P	0.20 (±0.26)	12.40 (±4.66)	1.67 (±0.88)	6.00 (±3.6)	0.00
B	0.60 (±0.77)	0.00	51.67 (±30.35)	7.00 (±4.73)	0.00
DBL	0.00	0.20 (±0.26)	0.00	0.33 (±0.33)	0.00
2.75d					
PChAT	9.50 (±4.04)	22.40 (±6.86)	1.33 (±0.88)	0.00	0.00
BChAT	2.75 (±3.68)	1.00 (±0.58)	77.67 (±17.75)	0.00	0.00
P	5.75 (±5.94)	50.60 (±21.64)	5.33 (±3.93)	13.0 (±5.69)	0.00
B	4.00 (±3.71)	14.00 (±4.26)	792 (±125)	421 (±123)	167 (±73)
DBL	0.00	5.20 (±2.69)	2.33 (±2.33)	2.67 (±2.19)	0.00
3.0d					
PChAT	17.00 (±5.82)	26.50 (±9.32)	0.25 (±0.25)	0.00	0.00
BChAT	28.50 (±7.55)	2.00 (±0.41)	71.00 (±7.95)	0.50 (±0.50	0.00
P	32.50 (±26.87)	71.50 (±49.44)	9.00 (±5.20)	36.50 (±26.43)	0.00
B	42.50 (±16.98)	25.75 (±4.72)	929 (±174)	648 (±226)	308 (±101)
DBL	2.25 (±1.93)	14.75 (±3.84)	10.50 (±8.23)	19.25 (±12.15)	0.00

Numbers of virus-labeled neurons in the lumbosacral spinal cord Means and standard errors of the different types and locations of neurons in spinal cord segments L1-L2 and L6-S1 as a function of incubation time. There were at least three experiments at each incubation time.

at 3 days, an increase by a factor of only 5. The mean total number of interneurons was 263 compared to the mean number of all labeled neurons of 439.

The corresponding numbers in L6-S1 were 17.17 at 2.25 days, 1623 at 3 days for the interneurons and 4.17 at 2.25 days and 71.75 at 3 days for the preganglionic cholinergic neurons. The mean total number of interneurons was 2939 compared to a total number of labeled neurons of 3574.

Double-labeled neurons were found in the cord in the dorsal gray commissure and intermediolateral regions (DGC>IML). Preganglionic neurons labeled by virus from the prostate were only found in the L1-L2 cord and were divided between the DGC and the IML with the larger fraction in the DGC. No preganglionic neurons innervating the prostate were found in L6-S1. Preganglionic neurons labeled by virus from the bladder were mainly found in the L6-S1 cord and mainly IML. Some were located in L1-L2 in the DGC and IML areas (IML>DGC).

Figure 5 displays maps showing the locations of labeled neurons in representative sections from the L6-S1 (5A) and L1-L2 (5B) spinal cord segments three days post infection. Virus-labeled bladder neurons (red) were mainly located in the L6-S1 whereas virus-labeled prostate neurons (green) were mainly located in L1-L2. Also shown are non virus-labeled cholinergic neurons (blue) located mainly in the ventral horn and the intermediolateral areas.

Figure 6 displays photomicrographs of virus-labeled and cholinergic neurons from the L6-S1 spinal cord in IML (A: virus-labeled, B: ChAT-labeled). Several neurons (a, b, c) were both virus-labeled and cholinergic.

Brain

In the brain labeled virus neurons first appeared four days post-infection. Labeled neurons were found in the raphe nuclei, gigantocellular reticular nucleus, A5 adrenergic nucleus, locus coeruleus, sub coeruleus, Barrington's nucleus, periaquaductal gray, red nucleus, paraventricular nucleus, lateral thalamus, and medial preoptic area. Approximately equal numbers of bladder and prostate neu-

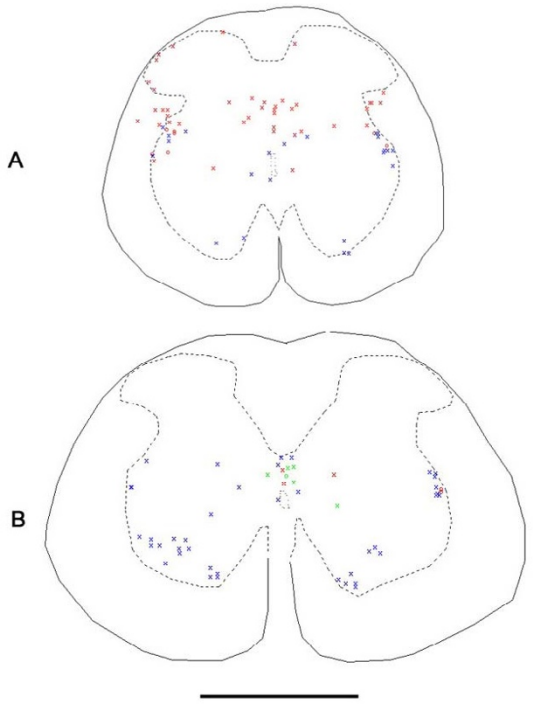

Figure 5
Neurons labeled from either the bladder or the prostate three days post infection Maps of sections from the lumbosacral spinal cord showing neurons labeled from either the bladder or the prostate three days post infection. Neurons labeled by virus that infected the bladder are red ("x" or "o") and those that are labeled by virus that infected the prostate are green ("x" or "o"). The "o" means that the neuron is also cholinergic (preganglionic). Blue neurons ("x") are cholinergic neurons that are not labeled by either virus. **A:** Section from the L6-S1 spinal cord. Cholinergic bladder neurons (parasympathetic preganglionic) are restricted to the intermediolateral area. Bladder neurons that are not cholinergic are found in the IML and DGC (interneurons) and in SDH. Cholinergic neurons that are not labeled by either virus (blue "x") are found in the ventral horn (somatic motor neurons), the intermediolateral area (preganglionic motor neurons that innervate other visceral organs) and around the central canal. Note that, in this particular section, there are no neurons labeled by virus that was injected into the prostate. **B:** Section from the L1-L2 spinal cord. Note that there are neurons labelled by virus that infected the bladder as well as the prostate. There are cholinergic (preganglionic, located in the intermediolateral and commissural areas) and non-cholinergic neurons labeled by virus from both organs. Bar: 1 mm.

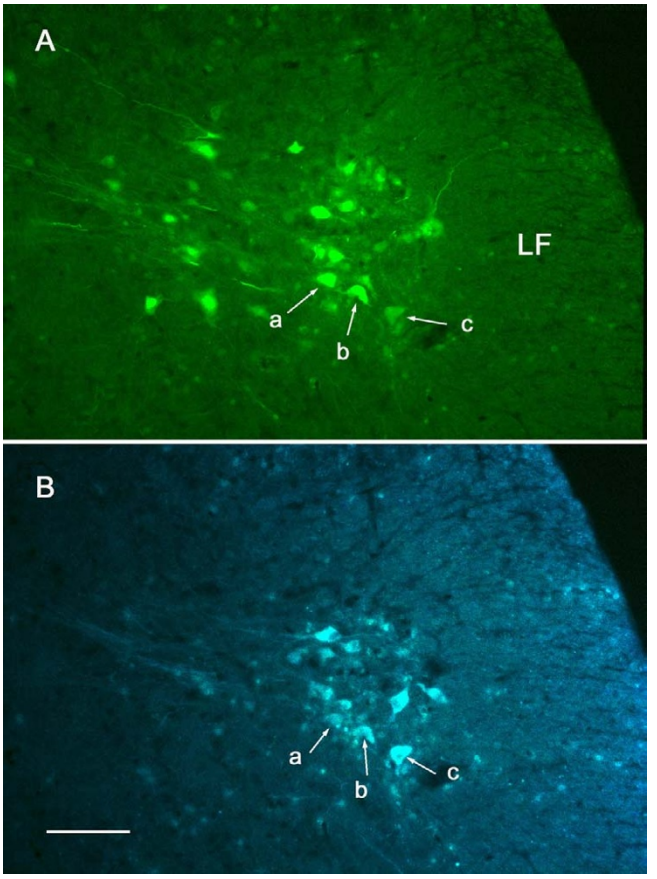

Figure 6
Virus-labeled bladder neurons and cholinergic neurons in the L6-S1 IML three days after infecting the bladder A: Virus labeled neurons in IML. **B:** Cholinergic neurons in IML. Three of the neurons in A (arrows) are also cholinergic (arrows in B) and are therefore preganglionic neurons that innervate bladder-designated postganglionic neurons in the major pelvic ganglion. Also in A are neurons that are labeled by bladder virus that are not cholinergic. These are interneurons. There are cholinergic neurons in B: that are not bladder neurons. These innervate other visceral organs (e.g. colon). Up is dorsal and left is medial. LF: lateral funiculus. Bar: 200 microns.

rons were found in all locations except for Barrington's nucleus and the medial preoptic area. The later two regions contained mainly neurons labeled by virus from the bladder and only a few labeled by virus from the prostate. Figure 7 displays examples of virus-labeled bladder and prostate neurons from five of these areas. Many neurons labeled from the bladder or prostate were found in the raphe nuclei, the A5 adrenergic nucleus, the locus coeruleus and the paraventricular nucleus. In each of these areas there were examples of doubly labeled neurons. In the red nucleus (not shown) mainly neurons labeled by virus

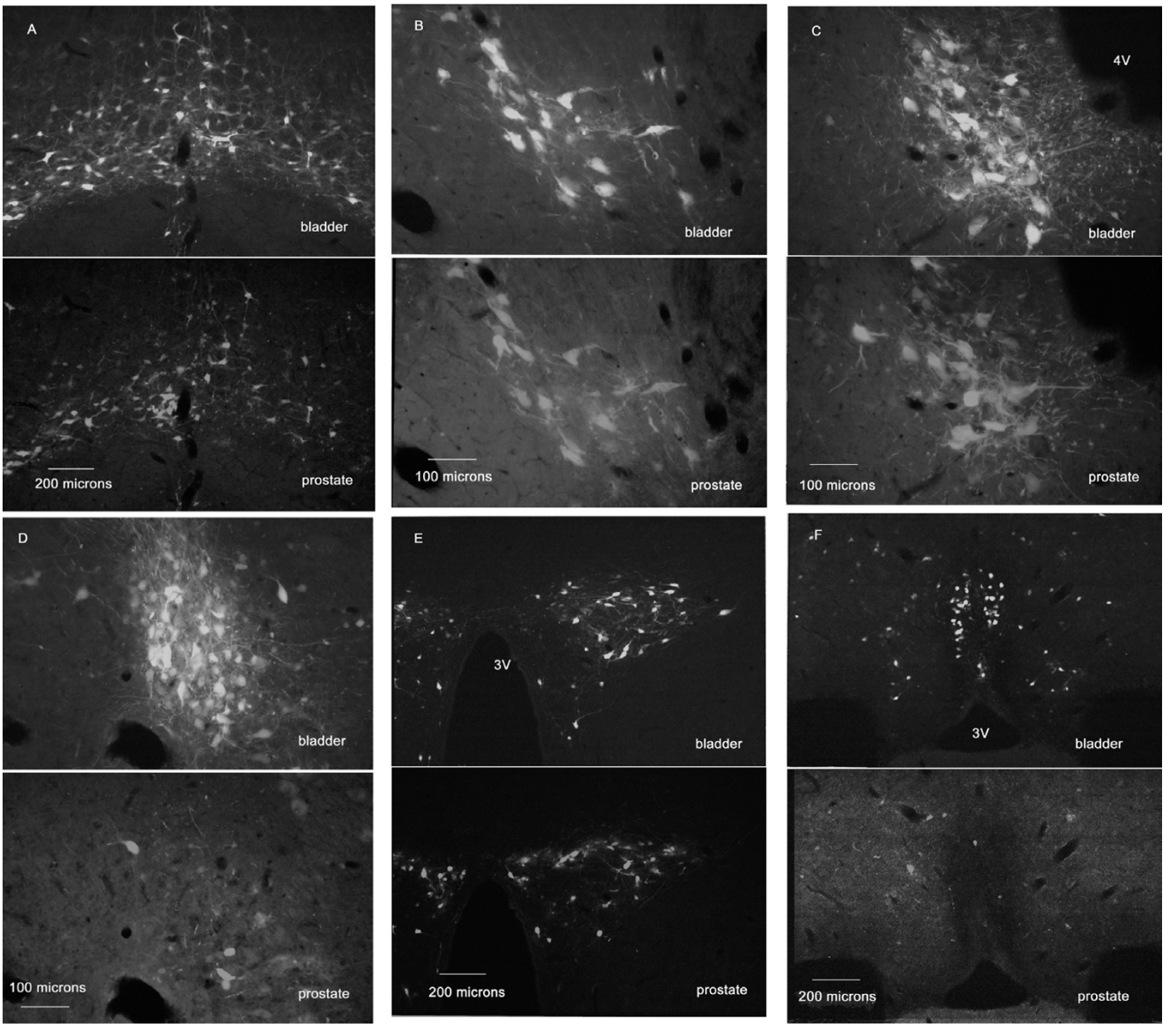

Figure 7
Prostate- and bladder-labeled neurons in the brain Neurons in selected brain nuclei, arranged in caudal to rostral order labeled by virus from the bladder and virus from the prostate. **A:** Raphe and gigantocellular reticular nuclei. Double-labeled neurons are common. **B:** The A5 adrenergic nucleus. Note that there are numerous double-labeled neurons. **C:** The locus coeruleus. Several neurons are double-labeled. **D:** Barrington's nucleus (the pontine micturition center). Bladder virus labels the overwhelming number of neurons. Only a few neurons contain prostate virus. **E:** The paraventricular nucleus. Approximately equal numbers of bladder and prostate neurons. Some are double-labeled. **F:** The medial preoptic nucleus. Only bladder neurons are found.

from the bladder were observed with only a few labeled by virus from the prostate.

Discussion

The use of two immunohistochemically distinguishable viruses allows one to detect central nervous system neurons, within a single tissue section, that were labeled by both viruses and therefore to examine the possibility of interactions between the two organs that have been injected.

One consideration that must be addressed concerns the rates of infection along the routes traveled by the two viruses used in these experiments. Although precise experiments designed to address this question have not been published, preliminary results in our laboratory demon-

strate that the two viruses infect the same neurons at about the same rate. Therefore, until more precise data is available, we shall assume that these rates are the same and that therefore numbers of neurons in a particular location infected by the viruses can be compared.

A second question concerns whether both viruses can simultaneously infect a single neuron. This point has not been directly addressed in specific experiments, but previously published works ([9,10]) have demonstrated examples of double labeled neurons in brain and spinal cord. We shall therefore assume that there is some proof that responds to this point.

In general neurons that are associated with the prostate or the bladder were found in those areas (intermediolateral and dorsal gray commissure) known to be associated with autonomic function.

The first neurons to be labeled from either the bladder or the prostate were located in the major pelvic ganglion. There were no pelvic ganglion neurons infected by both viruses. Seventy seven percent of those that were associated with the prostate were also adrenergic as would be expected since the prostate is under sympathetic control. This compares to 68% reported by Keast [3] in her study of fluorogold-labeled rat prostate neurons. The remaining 22% could also receive their innervation from the hypogastric nerve but, in addition, could be cholinergic thus representing a sympathetic-cholinergic pathway that might be related to the production of prostatic fluid [2]. However there is evidence for cholinergic fibers [11] and muscarinic receptors on the epithelial tissue surrounding the alveolar lumens [12] supporting this concept.

The main finding in this experiment is that the CNS distribution of bladder is different from the prostate. In the spinal cord the L1-L2 segments cells were mainly labeled by virus from the prostate. As shown in Table 1, there were few prostate preganglionic neurons located in the L6-S1 segments at any time points. This is understandable since the prostate is mainly under sympathetic control. The report by Keast [3] of prostate neurons in the major pelvic ganglion contacted by fibers running in the pelvic nerve suggests, though only indirectly, that these come from preganglionic neurons in the intermediolateral region of the L6-S1 spinal cord and that therefore more such neurons labeled by the virus injected into the prostate should have been found. We do not have an explanation of why we did not see more prostate preganglionic neurons in L6-S1. However, a recent report [13], demonstrated the existence of afferent fibers that contacted the postganglionic neurons in the major pelvic ganglion. Therefore there is evidence that fibers other than those from sacral preganglionic neurons contact these neurons and additional investigation is needed to completely resolve the question.

The bladder receives its major innervation from the L6-S1 segments and that is where most of the preganglionic neurons labeled by virus from the bladder were found. There were only a relatively small number of double-labeled interneurons. The majority of these were found in the L6-S1 segments. In the brain, all areas examined except for two contained cells labeled by the bladder virus or the prostate virus and there were always a few that were labeled by both viruses. There was no area that was specifically labeled by the virus that was injected into the prostate. Barrington's nucleus contained mainly cells infected by bladder virus. These neurons directly innervate the preganglionic neurons in the sacral parasympathetic nucleus that supply the excitatory impulses to the bladder and are among the first neurons in the brain to be labeled from the spinal cord. Our present findings do not agree with previous published results [14,15] which demonstrate labeling of Barrington's nucleus by virus from the prostate. In those studies the virus used was the Bartha strain of pseudorabies virus that is more virulent than the viruses used in the current work [16]. It is possible that the Bartha strain results in a much more extensive labeling of central structures and demonstrates that, if a long enough time elapses from the initial infection to the time of observation, many brain structures will be labeled. For example pseudorabies virus injected into the trachea [17] labeled neurons in Barrington's nucleus after an incubation time of six to seven days and pseudorabies virus injected into the pancreas [18] labeled neurons in Barrington's nucleus after an incubation time of six days. This demonstrates the importance of following the spread of virus as a function of time carefully to find those areas that are labeled first. The second area that did not receive any virus from the prostate is the medial preoptic area. This area is directly and reciprocally connected to Barrington's nucleus (i.e. the pontine micturition center) [19] and will therefore have neurons that are infected with the bladder virus but not with the prostate virus.

Conclusions

Unlike the distributions observed for bladder and external urethral sphincter [10] where there was considerable overlap as well as a large number of double-labeled neurons, the results for bladder and prostate in this work are quite different. There is very little overlap of the two distributions and very few double-labeled neurons. This result implies that there is little mutual interactive control of these two pelvic visceral organs and their functions are clearly separate and independent.

Materials and Methods

Surgery

All procedures involving rats were performed in compliance with the USDA Animal Welfare Act and amendments thereto and the revised Guide for the Care and Use of Laboratory Animals DHEW (NIH) and were approved by the Animal Studies Subcommittee of the Bay-Pine Veterans Administration Medical Center. Animals used in these experiments were male Sprague-Dawley rats (260–300 gm). The rat was anesthetized with Halothane initially at 4% in 95% O_2 + 5% CO_2 in a plastic box and, when the animal had succumbed, via a plastic nose cone at a maintenance level of 1.5%. An abdominal incision was made to expose both the urinary bladder and the prostate.

Pseudorabies double labeling procedure

The unique design of these experiments is the use of two immunohistochemically distinguishable pseudorabies viruses. This enables the investigator to examine the neuronal distribution of two possibly functionally related organs within a single spinal cord section and to suggest putative interactions between them. The two pseudorabies viruses used in these experiments were PRV-Bartha Blu and PRV-152 and were donated by Dr. L.W. Enquist. PRV-Bartha Blu (Ba-Blu) is a modified Bartha virus that contains a gene expressing β-galactosidase and can be detected with an appropriate antiserum. PRV-152 (PRV-GFP) is a Bartha virus modified to contain the green fluorescent protein (GFP) and can be visualized with the appropriate excitation wavelength or with a polyclonal antiserum against GFP. Each virus had a titer of about 10^8 plaque-forming units per ml. The PRV-GFP virus was injected into the ventral wall of the urinary bladder detrusor and the Ba-Blu virus was injected into the ventral lobes of the prostate. In some experiments the order of viruses was reversed with Ba-Blu injected into the urinary bladder and PRV-GFP injected into the prostate. Results were not dependent upon which virus was injected into the organ. Injections were made with a 0.3 ml insulin syringe with a 28-gauge tapered needle (Becton-Dickinson Co.). Two injections of 5 μl each were made into each organ under visual control with the operating microscope. In order to avoid the possibility of spread of virus between the two organs, the injection to the bladder was confined to the bladder body and was not near the prostate. The injections were observed to be confined to the tissue injected. To further reduce the possibility of spread to nearby tissues other than those injected, the areas were washed with saline and the liquid blotted after each injection. Five groups each comprising at least three animals were infected and allowed to survive for 2.25, 2.5, 2.75, 3 and 4 days post infection. One animal in each group had the viruses reversed. At the end of these periods animals were anesthetized with pentobarbital (80 mg/kg i.p.) and perfused transcardially with 200 ml of cold Krebs ringer followed with 200 ml of cold 4% paraformaldehyde in phosphate buffer (pH 7.4). Major pelvic ganglia, spinal cord and brain were removed and stored in cold fixative until ready for sectioning. Twenty-four hours prior to sectioning, the tissue was exposed to a solution of phosphate buffer containing 20% sucrose.

Immunohistochemical processing

Cryostat sections (14 microns) from the major pelvic ganglia were cut and thaw-mounted on gelatinized slides. Major pelvic ganglia sections from experiments where the prostate was infected with the Ba-Blu virus were processed with antiserum against β-galactosidase (see below) and also with an antiserum against tyrosine hydroxylase to identify those neurons that were adrenergic. Cryostat sections (20 microns) from spinal cord and brain were cut and stored in phosphate buffer. Spinal cord sections were collected sequentially in individual bins each containing six sections. The brain was cut in half and both portions mounted on a block. Brain sections were collected sequentially in individual bins each containing two cuts each consisting of one section from each brain portion. Slides were prepared containing a complete series of sections representing either the spinal cord segments or the brain. In the case of the spinal cord one section of the six in each bin was mounted. In the case of the brain, one section from each pair was mounted thus representing a complete profile of the brain. The slides were processed immunohistochemically with antisera against β-galactosidase (5 Prime → 3 Prime, Inc.) to visualize neurons labeled by Ba-Blu and choline acetyl transferase (ChAT, Chemicon, Inc.) to visualize cholinergic neurons. Free floating sections were incubated in a combination of rabbit anti β-galactosidase at a dilution of 1/1 k and goat anti ChAT at a dilution of 1/100, overnight at 4°C followed by a combination of donkey anti rabbit tagged with tetramethyl rhodamine isothiocyanate (TRITC) and donkey anti goat tagged with aminomethylcoumarin acetate (AMCA) each at a dilution of 1/50, at room temperature for 60 minutes. The sections were mounted on gelatinized slides and coverslipped. Neurons labeled with β-galactosidase appeared red under epifluorescent illumination. If they were also cholinergic they appeared blue under epifluorescent illumination. Neurons labeled by PRV-GFP were detectable because they were autofluorescent. Neurons were divided into three categories: Ba-Blu positive, PRV-GFP positive, or both Ba-Blu and PRV-GFP positive. Neurons that were cholinergic were also noted. All these types of neuron could be detected within a single section using epifluorescent illumination with appropriate filters. Counts of each type of neuron were made from each section of a complete spinal cord series. The counts were combined for all the animals in each group, averaged and a standard error computed. Maps showing the location and character of the different types of labeled neurons

were made with the MDPLOT software (Minnesota Datametrics). Photomicrographs were made of interesting portions of the sections to illustrate particular results and processed using Adobe Photoshop software (Adobe Systems Inc).

Authors' contributions

IN and PLV participated in the design of this work. IN conducted the experiments and the data analysis and drafted the manuscript. AMS participated in some of the experiments.

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