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Retrograde tracing of spinal cord connections to the cervix with pregnancy in mice

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Abstract

In contrast to the uterus, the cervix is well innervated during pregnancy and the density of nerve fibers increases before birth. To assess neural connections between the cervix and the spinal cord, the cervix of pregnant mice was injected with the trans-synaptic retrograde neural tract tracer pseudorabies virus (PRV). After 5 days, the virus was present in nerve cells and fibers in specific areas of the sensory, autonomic, and motor subdivisions of the thoracolumbar spinal cord. In nonpregnant controls, the virus was predominantly distributed in laminae I–III in the dorsal gray sensory areas with the heaviest label in the substantia gelatinosa compared with the autonomic or motor areas. Labeled cells and processes were sparse in other regions, except for a prominent cluster in the intermediolateral column (lamina VII). Photomicrographs of spinal cord sections were digitized, and the total area with the virus was estimated. Compared with nonpregnant controls, the area with PRV was significantly decreased in all the spinal cord subdivisions in pregnant mice except in the intermediolateral column. However, areas with the virus were equivalent in mice injected with PRV at 4 days or 1 day before birth. These findings suggest that the predominant innervation of the murine cervix is from the sensory regions of the thoracolumbar spinal cord, and that these connections diminish with pregnancy. The results raise the possibility that the remaining connections from sensory and autonomic subdivisions, particularly the intermediolateral column, of the thoracolumbar spinal cord may be important for increased density of nerve fibers in the cervix as pregnancy nears term.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Introduction

The cervix is a well-innervated part of the reproductive tract and nerve fibers are abundant in women who are not pregnant, as well as during pregnancy and near term (Tingaker *et al*. 2006). In rodents, more nerve fibers are present in the cervix by the day before birth than earlier in pregnancy or in nonpregnant controls (Kirby *et al*. 2005, Yellon *et al*. 2008, Boyd *et al*. 2009). The preterm increase in nerve fibers in the cervix temporally correlates with several pivotal events in the process of cervical remodeling, including immigration of immune cells, degradation of extracellular collagen matrix, and changes in biophysical capabilities to allow sufficient stretch for birth (Leppert 1995, Mackler *et al*. 1999, Buhimschi *et al*. 2004). Recent evidence indicates that of the two major spinal cord pathways that innervate the cervix, transection of the pelvic nerve, but not of the hypogastric nerve, affects remodeling of the cervix and forestalls birth (Higuchi *et al*. 1987, Boyd *et al*. 2009, Mackay *et al*. 2009). Thus, understanding the CN connections with the cervix during pregnancy has important implications for sensory perception and potential neural effector functions in the process of parturition.

Innervation of the uterine cervix has been extensively studied in nonpregnant females. Projections from the lower thoracic and upper lumbar spinal cord to the lower uterus and cervix include sensory, motor, and autonomic innervation (Baljet & Drukker 1980, Owman 1981, Steinman *et al*. 1992, Lee & Erskine 2000). These spinal connections have sympathetic and motor projections primarily through the hypogastric nerve, as well as inputs from parasympathetic fibers through the pelvic nerve (Papka *et al*. 1996, Houdeau *et al*. 1997). Sensory neuropeptidergic fibers are reported in both spinal cord projections (Berkley *et al*. 1993, Houdeau *et al*. 1998). The hypogastric and pelvic nerves originate within multiple thoracolumbar spinal cord segments and make connections with interneurons and other fibers that decussate across the midline through the dorsal spinal gray (from laminae I to V) and the ventral anterior white commissure (Collins *et al*. 1999, Coleman & Sengelaub 2002). The topographic innervation of the lower uterus and cervix, distinct from that for the uterine horns, led Houdeau *et al*. (1998) to suggest that region-specific innervation may be important for the control of the uterine cervix. This contention is supported by findings that activity of sensory nerves from the lower genital tract varies with respect to the estrous cycle or pregnancy (Robbins *et al*. 1992, Liu *et al*. 2008). The possibility that innervation of the cervix may change during the dramatic remodeling of the reproductive tract that occurs with pregnancy and in preparation for parturition comes from the evidence that steroids have organizational effects on synaptic connections within the CN (Matsumoto 1991, Beyer & Gonzalez-Mariscal 1994). Conceivably, an increased presence of nerve fibers by the day before birth in rodents may reflect more connections with the CN or a local extension of existing nerve fibers as pregnancy nears term. Thus, the objective of this study was to test the null hypothesis that central innervation of the cervix from the spinal cord remains unchanged during pregnancy. A multisynaptic retrograde neural tract tracer was used to test this hypothesis because traditional neural tract tracers would have only identified primary neurons whose cell bodies reside in paracervical, inferior mesenteric, and dorsal root ganglia. Rather, the approach of the present investigation was to use pseudorabies virus (PRV) to study the distribution of neurons in the thoracolumbar spinal cord that synapse on

primary neurons with terminal fibers in the cervix from pregnant and nonpregnant mice. The findings suggest that plasticity in cervical connections to specific nociceptive and autonomic regions of the spinal cord occurs with pregnancy.

Results

The cervix from mice injected with PRV contained numerous dark brown labeled particles that were sequestered exclusively within cells. These PRV-labeled cells were distributed throughout the stroma and sub-epithelium near the lumen of the cervix (data not shown). Based upon size and morphology, cells that had sequestered PRV resembled monocytic phagocytes. No PRV labeling was found in extracellular spaces, or in the uterine body, or in adherent fat or fasciae that were external to the cervix. Based upon histology of the cervix, endothelial morphology and thickness indicate that the nonpregnant mice were in the diestrus or early proestrus phase of the ovarian cycle, a time of increased estradiol (E_2) and relatively low progesterone concentrations in circulation compared to that during estrus phase or the latter part of pregnancy (Grota & Eik-Nes 1967, Butcher *et al.* 1974).

In the thoracolumbar spinal cord, the anatomical distribution of PRV label was consistent among individuals. In general and irrespective of group, PRV-stained cells and fibers were prominent in sensory regions of the dorsal horns of the spinal cord (Fig. 1, top panels). PRV-labeled neurons and fibers were most densely distributed in superficial lamina (Fig. 1, top left inset). In the autonomic subdivision, PRV-stained neurons and fibers were fewer and sparsely distributed. Virus-labeled structures were found throughout the intermediate zone of lamina VII (midline tissue cross section that includes the central canal) and in small diameter fibers that extended laterally from the intermediolateral nucleus through the intermediolateral gray matter into the dorsolateral funiculus toward the dorsal spinal root, as well as in the area surrounding the central canal (lamina X) and dorsal gray commissure (Fig. 1, top middle and right panels). In the motor subdivision of the ventral horn, PRV-labeled cells and fibers were relatively sparse. Isolated cells, most often small to intermediate in size, and fibers were limited to more lateral edges of laminae VIII and IX.

With pregnancy, the overall density and distribution of PRV labeling was diminished in the spinal cords of mice injected with PRV into the cervix. Although the extent of label varied among individuals within each group, fewer PRV-infected cells and fibers were present in each subdivision of the thoracolumbar spinal cord in pregnant mice following injection of PRV into the cervix compared to that in nonpregnant controls (Fig. 1, bottom panels). The density and distribution of PRV label in each spinal cord subdivision were similar whether mice were injected with PRV on day 15 or 18 of pregnancy (Fig. 1, bottom insets). However, in the intermediolateral column, a prominent cluster of PRV-labeled cell bodies and fibers was present in all nonpregnant and pregnant mice.

Image analysis of area of PRV label in subdivisions of the thoracolumbar spinal cord

Photomicrographs of spinal cord sections were digitized to determine the area of tissue with PRV label. Digitized images approximate the dense PRV stain in photomicrographs. In nonpregnant mice, most of the PRV label was present in the sensory subdivision of the thoracolumbar spinal cord (Fig. 2). PRV-stained cells and fibers were widely distributed in

laminae I–III of the superficial dorsal horns in all mice, more than tenfold greater than in autonomic or motor subdivisions of these segments of the spinal cord. Compared with nonpregnant controls, the sensory area with PRV label in mice injected with PRV into the cervix on day 15 or 18 of pregnancy was reduced more than 25-fold ($P<0.05$ ANOVA, $F>3.7$; Fig. 3). Similar findings were evident in autonomic and motor subdivisions, i.e. the area with PRV label was reduced nearly sevenfold compared to that in nonpregnant controls ($P<0.008$ ANOVA; $F>6.2$). However, in the intermediolateral column of the autonomic subdivision, a prominent proportion of PRV-labeled cells and fiber remained evident in all mice. The area of PRV in the intermediolateral column of the thoracolumbar spinal cord segments averaged $0.2804\times10^6/\mu m^2$ with an overlapping S.E.M. average of $0.0675\times10^6/\mu m^2$ ($P=0.84$, $F=0.179$). Finally, there were no significant differences in the area with PRV label among any spinal cord subdivisions in mice injected with PRV on day 15 or 18 of pregnancy ($P>0.1$ ANOVA).

Discussion

PRV has proven useful to investigate a variety of neural pathways including those from the spinal cord to the cervix. The strengths of this approach have been discussed at length along with concerns about individual variability in infectivity which may diminish the reliability of quantitative assessment of stain intensity and number of virus-labeled cells (Lee & Erskine 2000). The present study addressed these considerations in several ways. First, standardized methods directly delivered the same dose and volume of PRV into the approximately same location within the cervix of each mouse. Secondly, standardized analyses identified PRV stain for each pixel in the digitized photomicrographs of the spinal cord sections based upon dark brown-colored settings, not on stain intensity or subjective cell/fiber counts, to distinguish specific label from the background (see Cummings & Cotman 1995). The present results, though, underestimate the total area of stain because small isolated thin fibers and sparse punctate fibers in cross section were below the limit of detection. More precise estimates would magnify significant differences in the presence of PRV in the spinal cord sections between nonpregnant and pregnant mice. These differences exceeded variability in data between individuals in each treatment group. Variations in PRV label in the spinal cord of mice within each group, decreased in sensory dorsal columns and no change in the autonomic intermediolateral column with pregnancy, could reflect nerve terminals in contact with the virus, or an unknown factor that might affect retrograde transport or replication of the virus (Card *et al*. 1993, Card & Enquist 1999, Lee & Erskine 2000). However, area-specific differences in PRV label do not support the contention that pregnancy globally affects the infection rate or transport of the virus. The paucity of information about the effects of the endocrine milieu of pregnancy or gonadal steroids on PRV as a transneural circuit tracer is counterbalanced by findings that estrogen treatment of ovariectomized rats does not affect the infectivity or transport of PRV to a variety of brain areas following injection of the virus into the cervix (Papka *et al*. 1998, McCarthy 2008). Moreover, PRV has proven useful to demonstrate that E_2 promotes novel connections between nuclei in the brain (Horvath *et al*. 2002). Furthermore, neither estrus cycle nor pregnancy affected infectivity by PRV (rodents: Papka *et al*. 1998, Lee & Erskine 2000, Tanaka & Mannen 2008), or number of virus-infected cells in various ganglia after injection

of PRV into the cervix in the spinal cord of nonpregnant rats (Weiss *et al*. 2001), or transport, shedding, and replication of other viruses (cat: Boggess *et al*. 1997, human: Mungall *et al*. 2007). In our own preliminary study, PRV infected specific regions in the forebrain and, as in the spinal cord, brain areas were differentially labeled after the virus was injected into the cervix of pregnant mice versus nonpregnant controls (Williams *et al*. 2005). These findings suggest that the virus is comparably transported to the structures upstream from the spinal cord irrespective of reproductive or endocrine status. In conjunction with the differential region-specific presence, the normal distribution of data, and semi-quantitative analysis that reinforces qualitative observations, evidence collectively suggest that virus transport cannot account for findings in the present report.

Collectively, evidence in this study indicates that the majority of PRV-stained cells and fibers were present in the sensory regions of the dorsal horns in the lower thoracic through the upper lumbar spinal cord of mice. These findings replicate results in nonpregnant rats in which PRV was used to trace spinal cord pathways to reproductive structures (Papka *et al*. 1998, Lee & Erskine 2000, McKenna 2002). Similar to these reports, virus-labeled neurons and fibers were found in sensory regions of the dorsal horns, the midline dorsal gray commissural nucleus, and the intermediolateral cell column of the thoracolumbar spinal cord. Moreover, the present findings indicate significantly fewer connections between the sensory subdivision in these spinal cord segments and the cervix of pregnant mice compared to those in nonpregnant controls. The area of PRV-labeled cells in the sensory dorsal horn laminae closely corresponds to cells in the spinothalamic tract which conveys signals for nociception and temperature from the lower pelvis in nonpregnant rats (Kobayashi 1998, Johnson 2006). The reduced area of PRV stain in this subdivision suggests fewer synapses with ascending neural connections via the spinothalamic tract during pregnancy. This finding raises the possibility that diminished nociceptive information from the cervix could mitigate perception of pain as pregnancy progresses and during birth with passage of the fetus.

Counterbalancing this possibility is the evidence that nociceptive and other nerve fibers are abundant in the cervix and increase prior to term in pregnant rodents (Collins *et al*. 2002, Kirby *et al*. 2005, Boyd *et al*. 2009). Similar findings can be surmised in the cervix of peripartum women taking into consideration the tissue hypertrophy with pregnancy (Bryman *et al*. 1987, Stjernholm *et al*. 1999, Tingaker *et al*. 2005). Enhanced cervical fiber density may account for the evidence in a recent neurophysiologic study that suggests enhanced responsiveness to uterine cervical distension by nociceptive afferents in the hypogastric nerve as pregnancy nears term (Liu *et al*. 2008). Such responses are unlikely to be mediated by uterine nerves, a structure that is relatively denervated during pregnancy (Zupko *et al*. 2005, Liu *et al*. 2008). The greater density of fibers in the cervix by the day before birth could, by summation of generator potentials, decrease the threshold for afferent firing in the hypogastric nerve. This increase in neural activity may not necessarily reflect enhanced transmission of sensory information to the brain, given evidence for reduced connections between the cervix and the spinal cord in the present study. Rather, a greater local density and activity of nerve fibers in the cervix may be associated with fewer synaptic connections in the thoracolumbar spinal cord by day 15 post breeding, i.e. 4 days before birth. Estrous

cycle and pregnancy-related declines in neurogenesis, axonal sprouting, and dendritic plasticity have been reported in several specific brain regions that project to the thoracolumbar spinal cord in rodents (Gerrits *et al*. 2008, Pawluski *et al*. 2009). Thus, the present findings raise the possibility that an enhanced area of nerve fiber distribution in the cervix and activity by those nerves before birth may serve other functions in the prepartum cervix.

In nonreproductive tissue, sensory nerves have a variety of effector functions that are relevant for remodeling of the prepartum cervix. Increased sensory nerve activities, such as those mediated by neurotransmitters, including neuropeptides in late pregnancy, have been considered as a possible mechanism to promote vascular permeability, as well as recruitment and activation of immune cells (Collins *et al*. 2002, Richardson & Vasko 2002, Klede *et al*. 2003, Mowa *et al*. 2003). Immigration and activation of leukocytes may account for local increases in cytokines, prostaglandins, and nitric oxide in the prepartum cervix (Yellon *et al*. 2003, Maul *et al*. 2006). These components characterize the inflammatory process that remodels the cervix before birth (Word *et al*. 2007). Whether sensory afferents are essential for timing of birth is not known, but spinal cord innervation is critical for the normal timing of birth since transection of the pelvic nerve, but not of the hypogastric nerve, forestalls parturition (Burden *et al*. 1984, Boyd *et al*. 2009). These findings raise the possibility that spinal cord innervation of the cervix plays an important role in the process of cervical remodeling and the normal timing of birth.

In addition to finding a decline in sensory connections to the cervix with pregnancy, PRV was less prevalent in autonomic and motor subdivisions of the spinal cord in pregnant versus nonpregnant mice. Autonomic innervation of the cervix may be important for the regulation of vascular tone and blood flow as in other tissues (Markiewicz *et al*. 2003). PRV in decussating fibers that extend from the lateral edge gray matter near the intermediolateral cell column, an area of preganglionic parasympathetic neurons, may reflect the second-order infections from pelvic nerve fibers that terminate in this region (Morgan *et al*. 1981). The ascending pathway in the intermediolateral cell column projects to specific regions of the medulla related to the vagus nerve and the paraventricular nucleus of the hypothalamus in rats (Sawchenko & Swanson 1982, Ortega-Villalobos *et al*. 1990, Lee & Erskine 2000). Plasticity in these regions of the medulla during the estrous cycle suggests hormone-dependent modulation of visceral signals from the spinal cord (Gerrits *et al*. 2008). Pathways in the intermediolateral cell column are oxytocinergic and vasopressinergic (Steinman *et al*. 1992). The prominent persistence of this ascending projection with pregnancy and near term may be related to neuroendocrine reflexes that are associated with the parturition process or postpartum lactation.

In summary, evidence indicates a reduction in thoracolumbar spinal cord innervation of the cervix with pregnancy. The decline in infection of cells in the sensory subdivisions of the dorsal horns and autonomic region suggests that thoracolumbar processing and transmission of nociceptive information are reduced with pregnancy. The findings also support the hypothesis that limited remaining CN connections with the cervix may account for the increased density of neural fibers in the cervix by the day before birth. In combination with a previous study (Collins *et al*. 2002), these results raise the possibility that local neurogenic

inflammatory processes promote leukocyte migration and collagenolytic activity as part of the mechanism by which the cervix remodels in preparation for birth.

Materials and Methods

Animals

Adult C3H/HeN mice were purchased from Charles River Laboratories (Wilmington, DE, USA). Mice were housed in individual cages with access to food and water *ad libitum*. All experimental procedures were approved by the Loma Linda University Committees for Animal Care and Use, as well as for Biosafety and Hazardous Materials, in conformation with NIH guidelines for research using laboratory animals.

Tracing neural connections between the cervix and the spinal cord

Neural connections between the cervix and the spinal cord were studied using procedures similar to that previously reported (Owman *et al*. 1980, Papka *et al*. 1998, Lee & Erskine 2000, Weiss *et al*. 2001). PRV was selected as a retrograde transneuronal tract tracer because it is rapidly and preferentially taken up by small afferent nerve terminals or axons and transported back to the cell nucleus for replication (Card *et al*. 1993, Papka *et al*. 1998, Daniels *et al*. 2001, Enquist 2002). Nonpregnant or pregnant mice were anesthetized with Nembutal (2 mg/kg), secured on a foam platform with limb restraints, and a specially designed speculum with three 25-gauge guide cannulas inserted into the vaginal canal. Prior to insertion, the speculum tip was dipped in Newskin (Medtec, Jackson, WY, USA) to insulate the tip from surrounding vaginal tissue as done previously (Papka *et al*. 1998). The speculum was secured to the hindlimbs to maintain alignment with the cervix. Three Hamilton syringes (10 μl with a fixed 30-gauge needle) were preloaded with fresh PRV (1×10^6 pfu Bartha K strain, initial supply was a generous gift from Dr Lynn Enquist, Princeton University) and inserted into each of the cannula guides; a spacer ensured that the tip of each needle precisely extended 5 mm beyond the cannula tip into the cervix. The contents of each syringe containing a total of 4–5 μl, i.e. average of 1.5 μl per syringe, were simultaneously injected into the cervix. A 15-min extended time for delivery of the small volume facilitated extracellular diffusion of the injectate into tissue and minimized the backflow of solution from the injection site. Mice recovered from the anesthesia and, thereafter, showed no signs of illness.

Tissue processing

Similar to previous reports in other tissues, including the prostate (Orr & Marson 1998) and the cervix of nonpregnant rat (Strack & Loewy 1990, Collins *et al*. 1999, Aston-Jones & Card 2000), a 5-day survival period was empirically determined to label CN neurons following PRV injection into the cervix without evidence of necrosis in the cervix or disruption of the spinal cord cytoarchitecture. Mice were euthanized with CO_2, and then PBS (0.1 M PBS, pH 7.4, 37 °C) was perfused through the heart, followed by 4% paraformaldehyde in PBS. The cervix and whole vertebral column were dissected, postfixed overnight, and then transferred to 30% sucrose for cryoprotection. Tissues were embedded in OCT (Sakura Finetek, Torrance, CA, USA) and sectioned on a cryostat at 20 μm. Coronal cross sections of the cervix and every fourth section of the spinal cord from the mid-thoracic

through the upper lumbar segments were collected onto subbed glass slides and air-dried on a slide warmer and were then stored at 4 °C until processed. At the injection site, PRV that is not taken up by nerve terminals is rapidly sequestered by phagocytic cells and is not available for further uptake after about 24 h as determined by the microscopic examination of PRV label in sections of cervix following immunohistochemistry (Card *et al*. 1993).

For immunocytochemistry, slides were pretreated with 10% goat serum containing 0.3% Triton X-100 in 0.1 M PBS for 1 h (PBS, to reduce nonspecific labeling) and then overnight at 20 °C in a 1:10 000 dilution of a rabbit polyclonal anti-PRV primary antibody (a generous gift from Dr Lynn Enquist, Princeton University). The next day, slides were washed in PBS and incubated for 2 h at room temperature with biotinylated donkey anti-rabbit secondary antibody (1:600; Fitzgerald Industries International, formerly Research Diagnostics Inc., Concord, MA, USA) and treated with an avidin–biotin HRP complex (0.32% avidin–biotin HRP complex, Vectastain Elite ABC Kit, Vector Labs, Burlingame, CA, USA). Slides were then washed to visualize PRV, reacted for 10 min with a 0.01% hydrogen peroxide–diaminobenzidine (2% DAB; Dako Industries, Carpinteria, CA, USA). Sections not incubated with primary antisera served as a negative control for each run. Sections were counterstained with a 0.1% solution of cresyl violet acetate to identify cell nuclei and spinal cord laminae, and distinguish white matter from gray matter. Sections were washed with buffer, dehydrated with a graded series of ethanol, cleared with absolute xylene, and coverslipped with Permount.

Image analysis of PRV stain

Spinal cord sections were analyzed with a Zeiss Axio Imager A1 brightfield microscope. Photomicrographs were taken with an Apogee camera using a 4× objective to include as much as possible of the spinal cord cross section in one frame. PRV-infected cells and fibers were dark brown labeled against a relatively clear background and were similar to those described by Kobayashi (1998). Of all mice that received PRV, one nonpregnant mouse and one pregnant mouse had no PRV label in the cervix or in the segments of the spinal cord. Thus, the success rate for PRV infection of nerve terminals in the cervix was 86%.

Photomicrographs of the spinal cord sections from the lower thoracic to the upper lumbar segments (origins of hypogastric and pelvic nerve innervation of the cervix) were analyzed for area of tissue with PRV. Each spinal cord section was subdivided into three regions: sensory – laminae I–III in dorsal horns, autonomic – intermediate lateral horn zone, and motor – laminae below a horizontal line drawn just below the spinal canal. An atlas by Sidman *et al*. (1983), as well as descriptions and images in Molander *et al*. (1984) helped identify spinal cord segments, subdivisions, laminae, and cytoarchitectonic landmarks within each level. To estimate the area of tissue with PRV label, i.e. sum of pixels in a digitized image of stained cells and fibers, we adapted the approach and methods described in a study of area of immunocytochemistry-identified amyloid-β deposits in the cortex of patients with Alzheimer's disease (Cummings & Cotman 1995).

For the present study, Image ProPlus software (V3.0, Media Cybernetics, Silver Springs, MD, USA) was used to outline a spinal cord subdivision of interest based upon a photomicrograph taken using a 10×lens. The photomicrograph was converted into a

digitized image to determine the total area with PRV label in cells and fibers in the sensory, autonomic, and motor regions of interest. For this conversion, the RGB true color menu was used to adjust pseudo-color ranges to closely match the size and shape of regions with stain in the pixelated image with that in the photomicrograph and to label actually observed in the slides. To differentiate specific stain from background, color was converted into a gray scale threshold and the same settings were used for all other sections from the same animal. In the 'Measure', 'Count/Size Menu', and 'Filter Ranges' options of Image Pro plus, the minimum area filter range was three pixels. The total number of pixels times 1.61 μm^3/pixel was based upon the calibration of the field in view in the microscope. On average, the area of PRV stain was quantified in 26 sections from the thoracolumbar segments of each spinal cord. This approach provides an accurate estimate of the area of viral label in the subdivisions of interest in the spinal cord. The use of the same RGB threshold setting for different sections from each mouse provided replicable data for the area with PRV stain across sections, as well as facilitated comparisons among animals in different groups. Less than 5% variability was found in replicate measures of the same region of interest when analyzed by the same or another independent individual who was blind to the study design. Although the pixelated representation in each digitized image accurately reflected the density and distribution of PRV label in photomicrographs of stained sections, the analysis probably underestimated the total PRV area. Thin fibers and small punctate structures (e.g. dendrites and small diameter axons) that were visualized with the higher power objective (40×) were often below the limits of sensitivity of detection.

Statistical analyses

Data were analyzed by one-way ANOVA (SPSS, Chicago, IL, USA) after Levene's test for homogeneity of variability indicated a normal distribution. $P<0.05$ was considered statistically significant.

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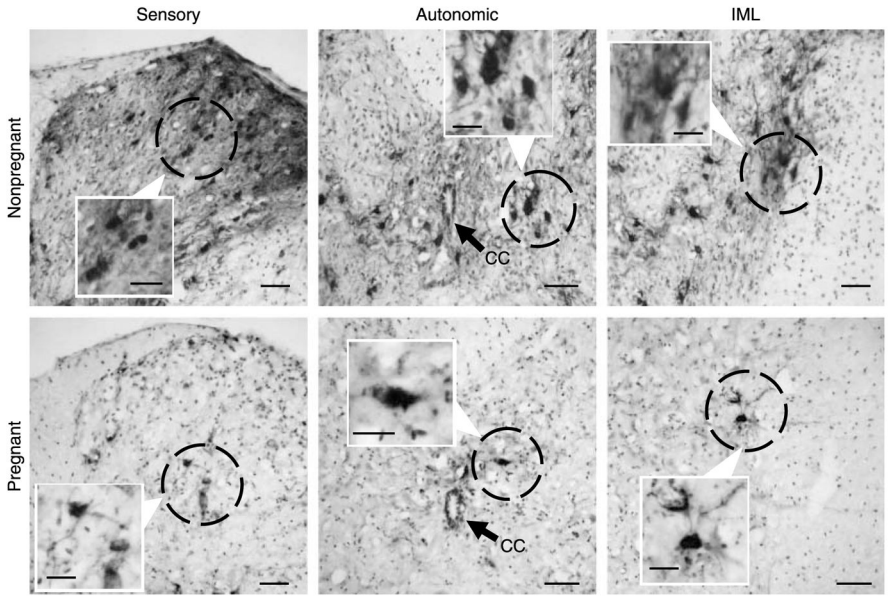

Figure 1.

Photomicrographs of pseudorabies virus-labeled cells and fibers in sections in the lower thoracic spinal cord (T11) from a mouse that was injected with pseudorabies virus into the cervix when nonpregnant (top) or on day 15 of pregnancy (bottom). Pseudorabies virus label was most prevalent in sensory (dorsal horn) and autonomic (region surrounding and lateral to the central canal) subdivisions, as well as in the intermediolateral cell column (IML). Insets highlight details of virus-labeled neuronal cell bodies and fibers. Scale bar=100 μm and, in insets, 50 μm.

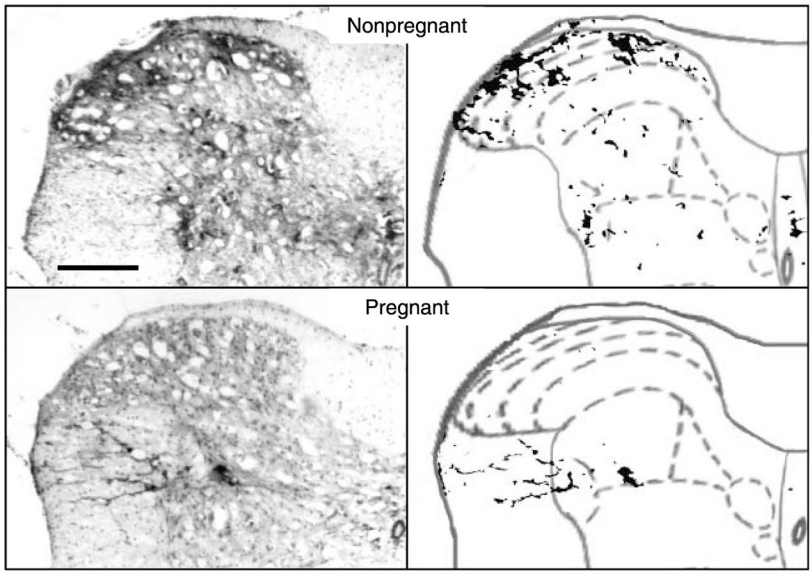

Figure 2.

(Left) Photomicrographs of thoracic segment 11 spinal cord sections from a mouse that was injected with pseudorabies virus into the cervix when nonpregnant (top) or on day 15 of pregnancy (bottom). Dark stain indicates pseudorabies virus-labeled cells and fibers. (Right) Digitized pixelated images of pseudorabies virus label generated with Image Pro plus software were superimposed upon a drawing of the spinal cord segment cytoarchitecture from Molander *et al*. (1984). Isolated thin fibers or small punctate structures that reflect fibers in cross section were below the limit of imaging threshold detection. Scale bar=100 μm.

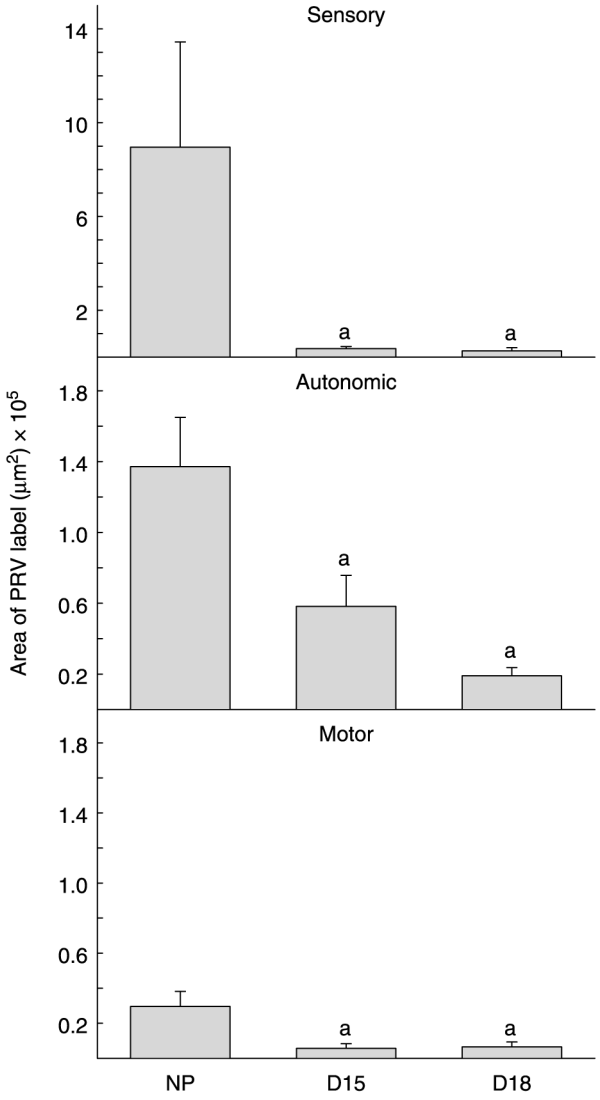

Figure 3.

Estimated area of cells and fibers that contained pseudorabies virus in sensory, autonomic, and motor subdivisions of the lower thoracic and upper lumbar segments of spinal cord in mice that were injected with pseudorabies virus into the cervix when nonpregnant (NP), or on day 15 of pregnancy (D15), or on day 18 of pregnancy (D18). Data for area of cervix with pseudorabies virus (PRV) label were the sum of the number of pixels times the area/pixel in the thoracolumbar spinal cord (mean±S.E.M., n=4/group; see details in Materials and Methods). ‘a’ indicates P<0.05 versus NP group.