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TITLE:

Novel spinal pathways identified by neuronal c-Fos expression after urethro-genital reflex activation in female guinea-pigs.

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ABSTRACT

Pudendal nerve-spinal pathways are involved in urethro-genital sensation, pain and sexual activity. However, details of these pathways and their modulation are unclear. We examined spinal pathways activated by the urethro-genital reflex (UGR) and visualised by c-Fos immunoreactivity in reflexly activated neurons within spinal cord. In anaesthetised female guinea pigs, a balloon was inserted into the urethra and inflated with short-repeat or long-continuous distension to activate the UGR. A second balloon recorded reflex contractions of the vagina and uterus. Two control groups had either no balloon or a vaginal balloon only. Ninety minutes after UGR activation, c-Fos immunoreactivity in L3 and S2 spinal segments was examined. Reflex activated c-Fos immunoreactivity also was investigated in some animals with acute spinal transections at either L4 or T12 levels. There was no significant difference in spinal c-Fos expression between the control groups. Short-repeat distension reliably induced a UGR and a 2-3 fold increase in c-Fos-expressing neurons throughout dorsal, intermediate and lateral spinal grey matter at S2 and about two fold increase in superficial dorsal horn at L3. T12 transection had little effect on c-Fos expression at either spinal level. However, after L4 transection, UGR generation was associated with a 4-6 fold increase in c-Fos-expressing neurons in lateral horn and central canal areas at S2, and but only 20-30% increase at L3. Thus, UGR activates preganglionic neurons projecting to pelvic viscera in both sacral and lumbar spinal cord. The reflex also must activate ascending and descending spinal inhibitory circuits that suppress c-Fos-expression in neurons at both sacral and lumbar spinal levels.

Keywords:

Pudendal nerve, Spinal reflex, Pre-ganglionic nerves, Female sexual function, c-Fos

INTRODUCTION

Female sexual dysfunction (FSD) affects a high proportion of women at some stage in their lives (Verit et al., 2006; Jha and Thakar, 2010; Bergeron, 2011). Some elements of FSD involve peripheral neural pathways. For example, vulvar vestibulitis syndrome (VVS) or dyspareunia can involve hypersensitivity of genital sensory nerves, whilst reduced vaginal lubrication ultimately is a failure of autonomic secretomotor and vasodilator pathways (Pukall et al., 2005). The spinal cord represents the first stage of processing genital sensory input and the final stage in generating autonomic motor output to the genital tract (Giraldi, 2004). Furthermore, the lumbo-sacral spinal cord has a well known ability to support sexual responsiveness at some level of the reflex in the absence of descending central control, as seen after spinal lesions (Chapelle, 1980; Sipski et al., 2004). Nevertheless, the spinal pathways activated during stimulation of the genital tract, especially in females, are poorly known.

In general, it is not feasible to identify spinal circuits in humans. However, spinal circuitry is well conserved across mammalian species, so that pathways identified in laboratory mammals are highly likely to have close homologues in humans. A spinal reflex response to mechanical stimulation of the urethro-genital region, known as urethro-genital reflex (UGR), has been reported in both male and female rats (McKenna et al., 1991; Vathy and Marson, 1998; Marson et al., 2003; Marson and Gravitt, 2004). The responses to the UGR activation in female animals resemble those seen during sexual activity, including significant increases in pelvic blood flow and rhythmic contractions of the vagina and uterus (Bohlen et al., 1982a, 1982b; Sipski, 2001). Afferent input arises from the pudendal nerves and the visceral responses themselves are produced by activation of autonomic pathways comprising

spinal preganglionic neurons, the hypogastric and pelvic nerves, with final motor neurons in the paracervical (anterior pelvic) ganglia (Morris and Gibbins, 1987; Keast, 1999; Jobling et al., 2003; Morris et al., 2005a; Wiedey, 2008). In guinea pigs, pudendal nerves project from sacral spinal cord mainly at S2 segment, hypogastric from rostral lumbar (eg. L3), pelvic nerves from caudal lumbar and sacral segments (Yuan et al., 2011). Most of them contain both afferents and efferents in the nerve bundles. In rats, hypogastric nerves mainly come from T13-L2 segments, pelvic nerves from L6 and S1, pudendal nerves from sacral segments (de Groat and Booth, 1993a). In human, it may be possible that there are some variations comparing with animals, but human most likely conserves a similar anatomical arrangement for these pathways (Wesselmann et al., 1997). Although the UGR has been regarded as a surrogate for sexual stimulation, it seems more likely that reflexes activated by mechanical stimulation of the urethra, especially its distension, are components of pain detection and response pathways.

In males and females, sacral parasympathetic pathways play a dominant role in producing vasodilation to increase blood flow to the reproductive organs (Dail et al., 1985; de Groat and Booth, 1993b; Papka and Traurig, 1993; Traurig and Papka, 1993 Sato et al., 1996; Cai et al., 2008). However, in several species, including humans, lumbar sympathetic pathways also can contribute to the change of pelvic blood flow and sexual arousal (Chapelle et al., 1980; Fahrenkrug and Ottesen, 1982; de Groat and Booth, 1993b; Sato et al., 1996; Sipski et al., 2004 Cai et al., 2008;). Indeed, many vasodilator neurons in guinea-pig paracervical ganglia receive their dominant preganglionic input from mid-lumbar (L3) spinal cord, often with convergent sacral spinal inputs (Jobling et al., 2003; Morris et al., 2005a).

Expression levels of an immediate-early proto-oncogene protein, c-Fos, in

spinal cord neurons are increased by activation of nociceptive sensory neurons, providing a valuable tool to identify neurons within spinal nociceptive pathways (Morgan et al., 1987; Marson et al., 2003; Marson and Gravitt, 2004; Coggeshall, 2005; Wiedey et al., 2008; Gao and Ji, 2009; Wang et al., 2010). Indeed, immunohistochemical mapping of c-Fos expression has allowed the identification of several populations of spinal neurons activated by stimulation of pelvic and pudendal nerves as well as the UGR (Marson et al., 2003). This work showed that the UGR activates neurons across most of the lumbar and sacral spinal cord, including autonomic preganglionic neurons and a wide range of neurons that must process different levels of afferent input. Such activation of c-Fos expression by the UGR further supports the interpretation that this reflex involves stimulation of nociceptive sensory pathways from the urinogenital tract.

Our recent electrophysiological studies in female guinea pigs demonstrated that activation of pudendal sensory nerves stimulates pelvic autonomic neurons controlling blood flow to the female genital tract via ascending spinal circuits projecting to both sacral and lumbar levels (Yuan et al., 2011). Many of these pelvic neurons in guinea pigs also receive descending central inputs from lumbar levels (L3) in addition to inputs from the sacral spinal cord (Jobling et al., 2003; Morris et al., 2005a; Yuan et al., 2011). However, outputs of the lumbar pathways to pelvic vasodilator and uterine motor neurons appear to be inhibited by spinal neurons acting via GABA_A receptors (Yuan et al., 2011). To date, the contributions of these neurons to pathways activated by the UGR reflex are not known. Building on our previous studies in guinea pigs (which confirm and extend observations made in other species, such as rats) (Jobling et al., 2003; Morris et al., 2005a; Yuan et al., 2011; Vilimas et al., 2011), we have adapted the UGR model for female guinea-pigs. We used c-Fos

immunohistochemistry to identify neurons that are activated by UGR in animals with intact spinal cord, and in animals following lesions of the spinal cord at different levels.

EXPERIMENTAL PROCEDURES

Young adult female guinea-pigs (pre-estrous 6-8 weeks old; 250-280 g body weight; Hartley-IMVS, Adelaide, Australia) were anaesthetised with 50% urethane (up to 1.8g/kg i.p.) and placed in a prone position on a heating pad to maintain body temperature at 37 °C. Oxygen was supplied continuously with a facemask during the experiment. Urethane provided stable anaesthesia to allow for repeated applications of stimuli to an animal *in vivo* and subsequent perfusion of the spinal cord for c-Fos immunohistochemistry. Animals were handled gently to avoid any overt activation of nociceptors that could increase background c-Fos expression in the spinal cord. Control animals were anaesthetised but otherwise had no other procedures in order to obtain baseline c-Fos expression with these anaesthesia and handling conditions. All experimental procedures employed in this study were approved by the Flinders University Animal Welfare Committee in accordance with national guidelines.

In vivo urethro-genital reflex (UGR) activation to induce spinal c-Fos expression

Two experimental groups were setup with urethral balloon distension to examine reflex-induced c-Fos expression in the spinal cord of anaesthetised animals. A small rubber balloon (urethral balloon: size 3F Fogarty arterial embolectomy catheter, Edwards Lifesciences, USA) was inserted into the urethra 1-2 mm away from the

external orifice and without stretching the wall of urethra. The balloon was inflated with 120 μ l distilled water for 30s every 10 minutes over 90 minutes (**short-repeat distension**) to activate the urethro-genital reflex. The degree of balloon distension was controlled to produce only limited increase in the diameter of the urethra. Such relatively mild distension was sufficient to induce UGR, which was used as threshold volume for urethral distension. In one group of animals with thoracic cord transection (T12, see below), the urethral balloon was inflated continuously for 30 minutes (**continuous distension**) to test for maintained activation of the UGR. A second balloon (vaginal balloon, VB: size 4F Fogarty arterial embolectomy catheter, Edwards Lifesciences, USA) was inserted into the area of vagina/lower uterus, but did not cross the cervix, and then inflated with 200 μ l distilled water to detect reflex contractions in response to urethral distension. Different sizes of the balloons used in urethra (3F) and vagina (4F) were determined by the sizes of luminal diameters of the urethra and vagina. Unlike the balloon in the urethra, vaginal balloon was distended to the level just filling up the lumen without significant stretching the wall of the organ, while sufficient to detect any vaginal spontaneous or reflex induced contractions. Both balloons were connected to Statham P23XL pressure transducers (Statham Medical Instruments, INC, USA) and recorded at 200 Hz sampling with a PowerLab/4s hardware (AD Instruments, Sydney, Australia) connected to a Power Macintosh computer using Chart version 4.0. Animals were left for a further 90 minutes under anaesthetic after completion of the stimulation protocol and then processed for detection of c-Fos immunoreactivity in the spinal cord.

At the beginning of each experiment, the threshold pressure in the urethral balloon required to elicit the UGR was determined. Reflex vaginal responses to this level of distension were allowed to stabilize before short-repeat or continuous

distension protocols were begun. These are the experimental groups (*Stimulated*) we examined for urethral balloon distension to induce reflex responses, which were detected by the balloon in vagina. Two control groups were used in these studies: the first control group (*Control-no VB*) had no balloon at all in both urethra and vagina in order to see the baseline c-Fos expression without any stimulation which may occur when the balloons were present in the lumen of urethra and vagina, while the second control group (*Control-with VB*) had only a vaginal recording balloon to see if there was any stimulation effect induced by presence of this balloon in vagina.

Spinal transection

To examine the involvement of ascending and descending spinal pathways in mediating reflex-induced c-Fos expression in spinal cord neurons, a total 35 anaesthetised animals were subjected to acute spinal cord transection prior to activation of the UGR. The region surrounding the thoracic (T12) or lumbar (L4) vertebrae of anaesthetised animals was exposed with a dorsal midline incision according to the anatomical landmark of the last ribs at T13. The spinal cord was transected with fine scissors in the area of T12 (n = 15) or L4 (n = 20). Because the mismatched level of the arrangement of spinal cord and vertebrae in the guinea pig, especially the lower level of the spinal cord, the accuracy of the spinal cord transection level was re-examined after removal of vertebral bone to expose the spinal cords after the experiments. All experiments with inaccurately transected spinal cords were discarded. The exposed areas were then covered with surgical gel foam and the incision was closed. After surgery the animals were allowed to stabilise for 30 minutes under maintained anaesthesia before stimulating the UGR. The purpose for using only 30 minutes post spinal cord transection was to see the acute effect of

blocking spinal pathways in these experiments. After the completion of the experiment, anaesthetised animals were perfused with fixative (see below) and the transected areas of the spinal cord were examined to ensure that complete transection of the spinal cord had been achieved at the correct level without any other significant damage, such as crushing the cord, spinal root avulsion or haemorrhage. A summary of the experimental setup is illustrated in **Figure 1**.

c-Fos, CGRP, SP, ChAT immunohistochemistry in spinal cord

Animals remained anaesthetised and were perfused (80 mmHg) via the left ventricle with 200 ml Dulbecco's minimal essential medium (DMEM, pH 7.4, Sigma) at 37 °C, followed by 500 ml Zamboni's fixative (0.5 % picric acid and 2% formaldehyde in phosphate buffer 0.1 M; pH 7.0). Spinal cords from lumbar (L2) to sacral (S3) segments were isolated and cut into approximately 5 mm long segments. Segments L3 and S2 were chosen for analysis, since they correspond to the peak autonomic outputs and sensory inputs to and from the pelvic viscera activated in the UGR. All spinal samples were stored in the same fixative for at least another 48 hours at 4°C. The tissue was cleared through 80%, 90%, 100% ethanol (twice), xylene (twice), 100%, 100%, 80%, 50% ethanol, water (0.5 hour each) and then stored in phosphate buffered saline with 0.01% sodium azide (PBS: 0.15 M NaCl in 0.01 M sodium phosphate, pH 7.1). L3 and S2 spinal segments were embedded in polyethylene glycol (PEG; MW = 1450) and cut transversely in 20 µm serial sections. Every 8th section was collected for immunohistochemistry.

After washing in PBS, free-floating sections were pre-incubated with 10% normal donkey serum for 30 minutes and then incubated over 2 nights at room

temperature in a humid chamber with antisera against the amino terminal of c-Fos protein (SC-54, raised in a rabbit, dilution: 1:600, Santa Cruz Biotechnology). In two experiments, sections were used for multiple labeling immunohistochemistry with a mixture of specific antisera against calcitonin gene-related peptide (CGRP) raised in a goat (1:1000; Arnel Products 1780, NY, USA), substance P (SP) raised in a rat (1:200; Chemicon International MAB356, Temecula, CA, USA) and choline acetyltransferase (ChAT) raised in sheep (1:4000; Chemicon). All these antisera are well characterised and do not show any significant cross-reactivity with inappropriate antigens in guinea-pig tissue (Jobling et al., 2003; Morris and Gibbins, 1987; Morris et al., 2005b; Vilimas et al., 2011). The sections were then washed in PBS (3 x 10 minutes) and incubated for 2 hours with species-specific secondary antibodies raised in donkey to rabbit, rat, sheep or goat immunoglobins and coupled with different fluorophores (dichlorotriazinylamino fluorescein, DTAF, or fluorescein isothiocyanate, FITC; indocarbocyanine 3, Cy3; indodicarbocyanine 5, Cy5; Jackson ImmunoResearch Laboratories, West Grove, PA) for double or triple-labeling fluorescence. All antisera were prepared with hypertonic PBS diluent (0.3M NaCl, pH 7.1) to minimise non-specific binding to tissue proteins.

Following further washing in PBS (3 x 10 minutes), tissues were mounted in buffered glycerol (2 parts glycerol in 1 part 0.5 M sodium carbonate buffer, pH 8.6) and viewed under an Olympus AX70 epifluorescence microscope with highly-discriminating filter blocks (Chroma Technology, Bellows Falls, VT). Digital images of labeled neurons were captured by a Hamamatsu ORCA cooled CCD camera (C4742-95, Japan) mounted on an AX70 microscope running AnalySIS acquisition software (version 5.0, Olympus Soft Imaging Systems).

Data analysis

To quantitatively measure changes in the number of c-Fos-immunoreactive (-IR) neurons in the spinal cord in response to UGR activation, the spinal cord was divided into 5 areas: (1) superficial (laminae I/II) and (2) deeper (laminae III-VI) layers of the dorsal horn, (3) lateral horn (LH, including intermediolateral (IML) cell columns and more medial gray matter), (4) central autonomic nuclei (CC) and (5) ventral horn (VH) (**Figure 2A**). The number of labeled neurons with strongly c-Fos-IR nuclei was counted in each area through the entire 20 μm thickness of each section. On average, 20 sections were counted at L3 level and 8-10 sections at S2 level for each animal. The sum of labeled neurons in all sections counted at each spinal level represents the final quantitative data for each animal. Labeled neurons were counted by an observer who was blinded to the experiment from which the sections came. The reliability of the counts was checked by recounting a sample of section.

Effects of different stimulation protocols and spinal transections on numbers of c-Fos-IR neurons detected were compared with multivariate and univariate analyses of variance (MANOVA/ANOVA) using the General Linear Model (GLM) procedure of SPSS 16.0 for Macintosh (SPSS, Chicago, IL, USA). Following detection of significant main effects or interaction terms in the complete data set ($P < 0.05$), either from the overall analysis of variance or from preplanned single-degree of freedom contrasts, pair-wise comparisons of estimated marginal means were made using Bonferroni adjustments for multiple comparisons with 95% confidence intervals. The total data set consisted of 93 separate neuronal counts for each of the 5 spinal regions taken across 18 combinations of experimental variables (ie. a total of 465 sets of neuronal counts). Summary data are expressed as mean \pm standard error from at least

four animals in each variable combination.

RESULTS

Pressure recordings from an intra-luminal vaginal balloon (VB) without any urethral balloon (UB) distension showed spontaneous vaginal contractions in 5 out of 10 experiments with a mean frequency of 18/hour, ranging from 6 to 60/hour ($n = 5$). Short (30 s) distension of the urethral balloon induced neuronal reflex responses recorded as pressure changes in the vaginal balloon (**Figure 2Ba**). Vaginal pressure increased from 0 to 9.6 ± 0.4 hPa ($n = 4$). This reflex response was repeatable at 10 minute intervals. The response includes an initial mechanical artifact followed by a rapid rising phase, peak and slow long-lasting recovery phase. The rising, peak and recovery phases were abolished by intra-luminal infusion of topical anesthetic (50 μ l of 4% lidocaine HCl) to the urethra, but not vagina, via an injection cannula in the urethra for 50 s indicating their underlying neural origin (**Figure 2Ba**). The component remaining after lidocaine infusion was the mechanical artifact induced by inflating the urethral balloon. This artifact was embedded in the initial rising phase and peak of the reflex response. Spontaneous contractions of the vagina were unaffected by lidocaine, indicating that they were not reflex induced responses and probably myogenic in origin (Granina et al., 2014) or perhaps due to local neural oscillator (Shafik et al., 2004) or pelvic floor muscle contraction. Continuous distension of the urethral wall for 30 minutes induced reflex responses similar to those induced by short-duration distension, but with a much longer recovery phase of the neuronal component (**Figure 2Bb**).

Examination of spinal cord from control animals that had neither a stimulating

urethral balloon nor a recording vaginal balloon revealed that only a relatively small number of neurons expressed detectable c-Fos-IR at both L3 and S2 spinal levels. Occasional labeled cells occurred in all regions of the spinal cord with comparable expression levels at L3 and S2 (**Figure 3**). There was no difference in the overall number of c-Fos-IR cells or in their distribution within the spinal cord of animals that had only a vaginal recording balloon ($P > 0.05$ for this protocol effect for each region of the spinal cord at each spinal level; ANOVA with preplanned contrasts). Thus, the insertion and inflation of the vaginal recording balloon generated no more c-Fos-IR in neurons than did the routine preparation of the animals for the experimental procedures.

Short-repeat urethral distension

c-Fos immunoreactivity in intact spinal cord

In contrast to simply inserting and inflating the vaginal balloon, short-repeat balloon distension of the urethra significantly increased the overall number of c-Fos-IR neurons in the spinal cord ($F_{(10,144)} = 8.6$, $P < 0.001$). However, there were significant differences in the changes of numbers of neurons with c-Fos-IR between sacral and lumbar levels ($F_{(5,71)} = 31.1$, $P < 0.001$). At S2 spinal level, urethral distension produced a 2- to 3-fold increase in the number of c-Fos-IR neurons throughout the dorsal horn (for example, in superficial dorsal horn layers I/II: control-no VB, 8 ± 2 ; control-with VB, 12 ± 1 ; stimulated, 27 ± 5 . Lateral horn: control-no VB, 5 ± 1 ; control-with VB, 6 ± 1 ; stimulated, 15 ± 2 . Central canal region: control-no VB, 5 ± 1 ; control-with VB, 5 ± 1 ; stimulated, 13 ± 3 . $P < 0.05$ for each comparison of stimulated condition with controls, $n = 5$). In contrast, at the L3 level, the same

stimulus generated a significant increase in the number of c-Fos-IR cells only in the superficial dorsal horn (I/II), where their number was doubled (control-no VB: 5 ± 1 ; control-with VB, 5 ± 1 ; stimulated, 10 ± 4 ; $P < 0.05$ for each comparison). There was no significant change in the number of c-Fos-IR neurons in the ventral horn at S2 and the remaining areas (III-VI, LH, CC, VH) at L3 ($n = 5$) (**Figure 3**).

c-Fos immunoreactivity in T12 transected spinal cord

Compared with animals with an intact spinal cord, acute spinal cord transection at T12 had no consistent effect on the number of spinal neurons expressing c-Fos-IR in response to short repeat distension of the urethra. Thus, the pattern of increase in the number of c-Fos-IR neurons at both the L3 and S2 segments was similar in T12 transected animals and the intact animals (ANOVA with preplanned contrasts and pairwise multiple comparisons using Bonferroni correction, $P > 0.05$ for comparisons for each area of the spinal cord, $n = 5$ transected animals, **Figure 4**). For example, we found that the pattern of increase of spinal neurons expressing c-Fos-IR in superficial dorsal horn laminae I/II (control-no VB, 12 ± 2 ; control-with VB, 17 ± 4 ; stimulated, 36 ± 6) and lateral horn (control-no VB, 7 ± 1 ; control-with VB, 7 ± 3 ; stimulated, 19 ± 3) at S2 with T12 transection were similar to those found in animals without spinal transection (**Figure 3**).

c-Fos immunoreactivity in L4 transected spinal cord

In contrast to spinal transection at T12 level, animals with acute spinal transection at L4 level, showed a dramatic increase in the number of c-Fos-IR neurons following

urethral distension (ANOVA: significant interaction between stimulation protocol and transection level, $F_{(20,296)} = 2.0$, $P = 0.008$). In particular, there was a 4- to 6-fold increase in the number of c-Fos-IR neurons in lateral horn and central canal areas of S2 spinal cord (for example, LH: control-no VB, 9 ± 1 ; control-with VB, 7 ± 2 ; stimulated, 28 ± 3 ; and CC: control-no VB, 4 ± 1 ; control-with VB, 4 ± 1 ; stimulated, 24 ± 3) and 20-30% increase in the same areas of L3 spinal cord (LH: control-no VB, 6 ± 1 ; control-with VB, 6 ± 1 ; stimulated, 8 ± 1 ; and CC: control-no VB, 5 ± 1 ; control-with VB, 4 ± 1 ; stimulated, 7 ± 1). These increases were significantly larger than those seen in intact or T12 transected animals (see below for details) (ANOVA with preplanned contrasts and pairwise multiple comparisons using Bonferroni correction, $P < 0.05$ for the appropriate comparisons). Compared with intact or T12 transected animals, spinal cord transection at L4 did not reveal any significant increase in the number of c-Fos-IR neurons in superficial laminae (I/II) of lumbar cord following urethral distension, indicating that the spinal cord transection at lower level abolished the activation of an ascending pathway from sacral to lumbar spinal cord (**Figure 5**).

Comparing the degree of increase in the number of c-Fos-IR neurons after urethral distension, particularly in the CC and LH areas at S2 level, L4 transection caused significantly larger increase in the number of c-Fos-IR neurons than intact or T12 transection group. For example, when stimulated animals were compared with control-with VB, at S2 level of the CC area: there was $611 \pm 140\%$ increase in L4 transected animals; $230 \pm 90\%$ increase in T12 transected animals; and $150 \pm 40\%$ increase in intact animals; at S2 level of the LH area: $410 \pm 160\%$ increase in L4 transected; $171 \pm 168\%$ increase in T12 transected; and $204 \pm 66\%$ increase in intact) (ANOVA with preplanned contrasts and pairwise multiple comparisons using

Bonferroni correction, $P < 0.05$). At L3 level, in both CC and LH areas the degree of increase in the number of c-Fos-IR neurons after stimulation is not significantly different among the intact, L4 and T12 transected groups (**Figure 6**).

Continuous urethral distension

c-Fos immunoreactivity in T12 transected spinal cord

Long lasting urethral distension significantly increased the number of c-Fos-IR neurons in the spinal cord (ANOVA, $F_{(5,28)} = 4.3$, $p = 0.005$). However, the pattern of increased c-Fos-IR in different areas of the spinal cord was similar to the change induced by the short-repeat stimulation in T12 transected animals (ANOVA, $F_{(5,28)} = 0.8$, $p = 0.6$), which in turn was not different from that seen in intact animals. This result indicated that variation in urethral distension time did not play a crucial role in determining the pattern of c-Fos expression (**Figure 7**).

Multiple labeling immunohistochemistry in spinal cord

Double or triple labeling immunohistochemistry with antibodies against c-Fos, CGRP, SP, ChAT revealed that c-Fos-IR spinal neurons activated by urethral distension within lamina I of the superficial dorsal horn were surrounded by a dense plexus of varicose fibres with strong immunoreactivity to both SP and CGRP, indicating that they were likely to be terminals of peptidergic nociceptors. Neurons with c-Fos-IR also were prominent in deeper dorsal horn lamina where they were distant from fibres containing SP-IR or CGRP-IR (**Figure 8**). Many c-Fos-IR neurons in the sacral parasympathetic nucleus and lumbar lateral horn and area around the

central canal were immunoreactive for ChAT, indicating that they were cholinergic autonomic preganglionic neurons (**Figure 8**).

DISCUSSION

This study has combined a range of experimental methods, including detection of neuronal c-Fos expression with immunohistochemistry, UGR activation by urethra distension and selective spinal transection, to take a novel approach to investigate UGR pathways in the female guinea pigs. We have found that the urethro-genital reflex induced by urethral distension activates spinal preganglionic neurons projecting to pelvic viscera through not only sacral levels but also lumbar levels of the spinal cord. We also showed the existence of ascending and descending spinal inhibitory circuits that suppress c-Fos-expressing neurons at both sacral and lumbar spinal levels during UGR activation.

c-Fos expression at sacral and lumbar spinal cord

Our data have revealed that the UGR induced by urethral distension increased c-Fos expression in several areas of the spinal cord at both lumbar and sacral levels in response to both short-repeat and continuous urethral distension. The low background levels of c-Fos expression observed in control preparations indicate that in untreated animals or in animals simply prepared for the experiment, any activation of sensory inputs from the urinogenital tract (or any other region) is insufficient to generate c-Fos expression in the great majority of dorsal horn neurons. Although the numbers of c-Fos labeled neurons in any section were small, differences due to treatment effects were consistent and reliable. Indeed, it is likely that relatively few neurons are

involved in spinal reflexes at each spinal level.

The sensory inputs responsible for the enhanced c-Fos expression most likely reach the spinal cord via the pudendal nerve which provides most of the sensory innervation to the urethra (Vilimas et al., 2011; Wiedey et al., 2008). The increased c-Fos expression at both sacral and lumbar levels was consistent with our previous electrophysiological observations, in which electrical stimulation of pudendal nerves activates not only a local pudendal nerve-sacral spinal cord-pelvic nerve pathway but also a more cranial pudendal nerve-lumbar spinal cord-hypogastric nerve pathway via ascending inter-segmental connections between sacral and lumbar spinal segments (**Figure 9A**) (Yuan et al., 2011). Our data also are consistent with studies in rats demonstrating that urethral distension significantly increased c-Fos expression in lumbar and thoracic spinal cord, especially at the level of L2-L4 (Marson et al., 2003).

We identified several groups of c-Fos expressing neurons in the spinal cord following the UGR. One group of activated spinal neurons occurred in superficial laminae (I/II) of both lumbar (L3) and sacral (S2) spinal cord. Many of them were surrounded by CGRP-immunoreactive fibres, suggesting that at least some of these neurons receive nociceptive inputs from the pudendal nerves and that neuropeptides could be involved in the spinal modulation of sexual function (Wilson et al., 2009). Neurons expressing c-Fos in deep dorsal horn (laminae III-VI) may receive inputs directly from peripheral mechanoreceptors. However, they are more likely to be interneurons in nociceptive circuits projecting from the superficial dorsal horn (Coggeshall, 2005). ChAT-immunoreactive neurons expressing c-Fos in the lateral horn and the region around central canal most likely were cholinergic preganglionic

neurons (Papka et al., 1998).

Pathways revealed by spinal lesions

Compared with animals with intact spinal cord or T12 transection, animals with acute spinal transection at L4 showed significantly increased c-Fos expression in the CC and LH areas of both lumbar and sacral spinal cord in response to the UGR. These changes are most probably the result of acute loss of inputs from adjacent spinal levels rather than trophic or epigenetic changes in the properties of the neurons, which are very unlikely to have developed in the short time frame of our experiments. Many of the labeled neurons expressed ChAT-IR, a marker for cholinergic neurons, and thus they are most likely to be autonomic preganglionic neurons (Papka et al., 1998).

The enhanced expression of c-Fos in L3 preganglionic neurons in L4 transected animals implies two features of the circuitry (**Figure 9B**). First, there must be direct sensory inputs to these levels activated by the UGR. The most likely pathway is via the hypogastric and lumbar splanchnic nerves, as concluded by previous studies using different experimental approaches (Papka and Traurig, 1993; Traurig and Papka, 1993). Second, under normal circumstances, there must be some ascending spinal inhibition of these neurons in response to the UGR, presumably generated by sensory inputs travelling via the pudendal nerve to spinal interneurons at sacral spinal levels, which project to lumbar spinal cord as seen in other animal species (Hubscher et al., 2010; McMahon and Morrison, 1982). Spinal interneurons labelled by different neurochemical markers (calretinin or parvalbumin) in deep dorsal horn area have been found to play a role in spinal sensory inhibitory pathway (Hughes et al., 2012) and contribute to local spinal neuronal circuitry (Liu et al.,

2010). Preganglionic neurons at L3 project to paracervical ganglion neurons that have significant vasodilator activity in the pelvic vasculature. These neurons also receive convergent preganglionic input from sacral spinal levels, suggesting that the lumbar and sacral pathways regulating their activity may mutually inhibit each other. Indeed, spinal transection at L4 also enhanced c-Fos expression in sacral preganglionic neurons in response to the UGR, implying there must be local descending inhibitory circuits as well. These observations are consistent with our recent electrophysiological study showing that spinally applied GABA_A antagonists enhanced activity in splanchnic or hypogastric nerves in response to stimulation of the pudendal nerve, as a result of blocking ascending inhibitory pathways from pudendal nerve sensory input to preganglionic neurons in lumbar spinal cord (Yuan et al., 2011).

The exact physiological function of these ascending and descending inhibitory spinal pathways is not clear. One possibility is that they modulate the excitability of spinal efferent pathways controlling other pelvic organs for different physiological functions, such as urination and defecation, during sexual arousal. For example, it has been reported that the myo-electrical activity recorded from rat external urethral sphincter was significantly increased when genital stimulation was applied (Pastelín et al., 2012). They also suggested that the dorsal nerve, originating from the pudendal nerve, innervates both distal urethra and female genitalia. There are neural pathways connecting between these two regions, most likely via spinal cord. This finding also indicates that sexual stimulation can activate a pathway to control the urination. Because the UGR induced in our study mimics the activities induced by genital stimulation the ascending inhibitory pathway found in our study may play a role in control of urination through genital-spinal-urethral pathway. Indeed, sexual

dysfunction commonly seen in women was reported also to have urinary incontinence (Sutherst, 1979; Laumann et al., 1999; El-Azab et al., 2011). Anatomically, there is a considerable degree of overlap and interaction between neural pathways to different pelvic organs (eg. rectum and urogenital system) with considerable potential for integration or modulation of their activity within the spinal cord (Berkley, 2006; Pezzone et al., 2005; Rudick et al., 2007; Ustinova et al., 2006; Ustinova et al., 2010).

In contrast to the effects of L4 spinal transection, the lack of significant effect of acute spinal transection at T12 on c-Fos expression is somewhat surprising (**Figure 9A**). Previous studies, mostly in males, have indicated that lumbar preganglionic outflow receives both excitatory and inhibitory descending spinal input (de Groat and Booth, 1993b; de Groat et al., 1993). In our previous study of female guinea-pigs, electrical stimulation of descending spinal tracts above T12 level provides predominantly excitatory input to lumbar preganglionic neurons projecting out the hypogastric nerve (Yuan et al., 2011). The simplest interpretation of our data is that the descending inputs are not tonically active under our experimental conditions. Instead, tonic inhibition of preganglionic neurons seems to be arising either from lower spinal levels (for lumbar preganglionic neurons) or via local inhibitory circuits (for sacral preganglionic neurons). Presumably, descending central inputs play only an intermittent role in the control of lower spinal autonomic pathways to the pelvic viscera, and may be active only during centrally-generated sexual behaviour. However, we cannot rule out the possibility that the c-Fos immunohistochemistry we used in this study may not be sufficiently sensitive to detect the effects of blocking tonic activity of pathways descending from above T12 level.

This work provides further evidence for complex sensory pathways from the urethra and distal genital tract to lumbar and sacral spinal cord. These pathways are most

likely responsible for the sensations of urethro-genital pain induced by mechanical stimulation of the areas innervated by the pudendal nerve, which could occur in the presence or absence of sexual activity. Two aspects of our data predict that these spinal pathways are related to pain perception: (1) c-Fos expression in superficial dorsal horn neurons is up-regulated almost exclusively by nociceptive inputs (Coggeshall, 2005); and (2) many of these c-Fos expressing neurons were found to be surrounded by CGRP-immunoreactive fibres, which are known to be primarily nociceptive (Morris et al., 2005b). The complexity of the pathways is further demonstrated by the presence of three sensory-autonomic pathways regulating pelvic organs activity: (1) pudendal nerve inputs to sacral spinal cord with outputs via the pelvic nerve; (2) pudendal nerve inputs to sacral spinal cord with ascending pathways to lumbar outputs via lumbar splanchnic and hypogastric nerves; and (3) hypogastric nerve inputs to lumbar spinal cord with outputs via lumbar splanchnic and hypogastric nerves (Dail et al., 1985; Jobling et al., 2003; Morris and Gibbins, 1987; Morris et al., 2005a; Papka and Traurig, 1993; Sato et al., 1996; Traurig and Papka, 1993; Wiedey et al., 2008; Yuan et al., 2011). In addition, there is a significant degree of intra-spinal modulation within both ascending and descending pathways (Yuan et al., 2011).

The most common problem affecting the female urethro-genital area is infection-induced inflammation with on-going pain (Farage and Galask, 2005). Independent of any effect of the pain itself on sexual behaviour, our data suggest there are likely to be spinal reflex mechanisms that alter autonomic motor control in these regions, which in turn are likely to further modulate the experience of sexual activity. It is usually assumed that the UGR reflects the activation of reflex activity in normal sexual activity (Marson and Gravitt, 2004). However, c-Fos activation of neurons in the spinal cord is more likely under conditions of noxious sensory stimulation

(Coggeshall, 2005). If so, most of the c-Fos activation we and others have observed after the UGR probably is not the consequence of normal sexual activity but is more likely related to noxious stimulation of the urethro-genital region, presumably mediated by polymodal mechano-nociceptors. Indeed, our recent immunohistochemical analyses revealed complex anatomical relations between the endings of presumed low-threshold mechanoreceptors and polymodal nociceptors in the distal urinogenital tract of females (Vilimas et al., 2011). Dissociating these elements experimentally remains a major challenge.

It is clear that both lumbar and sacral spinal cord act as centres for processing sensory information from the pelvic and urethro-genital regions. As details of these spinal pathways emerge, new strategies may develop to understand and potentially treat patients with lower cord spinal injury. Although the damaged spinal cord is unlikely to regain its normal physiological function in the detection and regulation of urethro-genital activity, compensatory nerve pathways may well be candidates for targeted activation after injury. In particular, focusing attention on the lumbar sympathetic pathways which play an important role in control of pelvic blood flow and sexual arousal could be most beneficial (Cai et al., 2008; Chapelle et al., 1980; Dail et al., 1985; de Groat and Booth, 1993b; Fahrenkrug and Ottesen, 1982; Sipski et al., 2004).

CONCLUSIONS

This work provides new evidence that urethro-genital reflex induced by urethral distension activates preganglionic neurons projecting to pelvic viscera in not only sacral but also lumbar spinal cord. The reflex also must activate intra-spinal ascending

and descending inhibitory circuits that suppress c-Fos-expression in neurons at both sacral and lumbar spinal levels.

AUTHOR CONTRIBUTIONS:

SY Y designed and interpreted experiments, prepared the manuscript; PIV carried out experiments and c-Fos analysis; VPZ contributed to experimental design and interpretation; ILG oversaw experimental design, analysed the data, and prepared the manuscript.

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FIGURE CAPTIONS

Figure 1: Diagram of proposed spinal pathways to be examined during the activation of the urethro-genital reflex (UGR) and the locations of spinal cord transection in anaesthetised female guinea-pigs. The spinal reflex pathways projecting to the paracervical ganglia (PG, filled circles) controlling the pelvic organs were examined by detection of reflex contraction in vagina/uterine during urethral distension. Descending spinal and ascending pudendal-lumbar spinal pathways activate sympathetic preganglionic neurons (filled circle) projecting via L3 lumbar splanchnic nerves (LSN), the inferior mesenteric ganglion (IMG) and hypogastric nerves (HN) to the paracervical ganglia. Sacral spinal output to the paracervical ganglia is via parasympathetic preganglionic neurons projecting out the pelvic nerve (PN). Most of HN, PN and pudendal nerves project to the peripheral from more than one spinal segment. For example in guinea pigs, pudendal nerves project from sacral spinal cord mainly at S2, S3 segment, hypogastric from rostral lumbar (eg. L3), pelvic nerves from caudal lumbar and sacral segments. Spinal transection at T12 was used to block descending central inputs (solid lines with arrows) to lumbar and sacral cord and also ascending sensory inputs to supra-spinal levels and brain. In addition to the effect of T12 spinal transection, transection at L4 was also used to interrupt spinal connections (the solid line with arrow for descending and the broken lines with arrow for ascending) between lumbar and sacral cord and to reveal the underlying pathways connecting two spinal levels.

Figure 2. A. Cross section of L3 spinal cord of female guinea pig illustrating the areas in which c-Fos-IR neurons were identified and counted. Left side shows the laminae of the spinal cord; right side shows the areas used for analysis. SDH,

superficial dorsal horn; DDH, deep dorsal horn; LH, lateral horn including intermediolateral (IML) cell columns and more medial gray matter; CC, central canal; VH, ventral horn. **B.** Example of urethro-genital reflex responses recorded from a vaginal balloon induced *in vivo* by short repeat (a) and continuous (b) urethral distensions in anaesthetized young female guinea pig. (a) Reflex contraction of the vagina (upper trace) was induced by urethral wall distension 30 s in duration (lower trace). The reflex response comprised with initial rapid rising-phase (see arrow), peak and long-lasting recovery phase, All of which were abolished by intra-luminal infusion of topical anesthetic (4% lidocaine HCl) for 50 s. The remaining component after anaesthetic infusion is the mechanical artifact from inflating the urethral balloon, which was embedded in the initial rising phase and peak of the reflex response. The spontaneous contraction is unaffected by lidocaine indicating its non-reflex induced response. (b) Reflex response of the vagina (upper trace) induced by continuous distension of the urethral wall for 30 minutes (lower trace). The reflex response is similar to the response induced by short duration distension, but with a much longer neuronal component.

Figure 3. Example of c-Fos immunohistochemistry in spinal cord sections (A) and the distribution of c-Fos-IR neurons (B) in animal with short-repeat urethral distension but no spinal cord transection. A: c-Fos-IR neurons in superficial dorsal horn (laminae I/II) of S2 spinal cord (arrows). Compared with control (top), UGR stimulation (bottom) significantly increased the number of c-Fos-IR neurons in these areas. Solid lines mark the edge of the sections, broken lines mark the margins of grey matter. B: Group data (mean \pm S.E, n = 5 animals) show that a significant increase was found in most areas at S2, but only in laminae I/II at L3 (ANOVA, $p < 0.05$). *

Significant increase due to stimulation compared with controls with or without presence of balloon in vagina (VB). There is no difference between the two control groups. These labeled neurons include spinal cord sensory and preganglionic neurons. n = number of animals examined. Calibration bar in A: 100 μ m.

Figure 4. Example of c-Fos immunohistochemistry in spinal cord sections (A) and the distribution of c-Fos-labeled neurons (B) in a **T12 transected** animal with short-repeat urethral distension. A: c-Fos-IR neurons in superficial dorsal horn (laminae I/II) of S2 spinal cord (arrows). Compared with control (top), UGR stimulation (bottom) significantly increased the number of c-Fos-IR neurons in these areas in T12 transected animal. Solid lines mark the edge of the sections, broken lines mark the margins of grey matter. B: Group data (mean \pm S.E) from T12 transected animals show that the increase in c-Fos-IR neurons induced by UGR at S2 and L3 was similar to that in spinal cord intact animals showing in Figure 3. * Significant increase due to stimulation compared with controls with or without presence of balloon in vagina (VB; ANOVA, $p < 0.05$). These labeled neurons include spinal cord sensory and preganglionic neurons. n = number of animals examined. Calibration bar in A: 100 μ m.

Figure 5. Example of c-Fos immunohistochemistry in spinal cord sections (A) and the distribution of c-Fos-IR neurons (B) in an **L4 transected** animal with short-repeat urethral distension. A: c-Fos-IR neurons in the grey matter of LH (arrows) of S2 spinal cord. Compared with control (top), UGR stimulation (bottom) significantly increased the number of c-Fos-IR neurons in LH at S2 level. B: Group data (mean \pm

S.E) from L4 transected animals show that the pattern of the increase induced by UGR at S2 was similar to that in animals without spinal cord transection. With the exception in the regions of CC and LH the increase was statistically much greater than that in animals with or without spinal cord transected at T12 (see Figure 3B & 4B). * Significant increase (ANOVA, $p < 0.05$). n = number of animals examined. Calibration bar in A: 100 μm .

Figure 6. Comparison among three groups of L4, T12 spinal transection and intact animals for the increase in the number of c-Fos-IR neurons in the CC and LH areas after urethral stimulation. Top panel: at S2 level L4 transection caused significantly larger increase (between stimulated and control-with VB) in the number of c-Fos-IR neurons in both CC and LH areas than those seen in T12 transection or intact (no transection) groups. Bottom panel: at L3 level the increase in the number of c-Fos-IR neurons in both CC and LH areas after L4 spinal transection was small to T12 transection or intact groups and the difference among the three groups was not statistically significant. * Significant increase (ANOVA, $p < 0.05$). n = number of animals examined.

Figure 7. Distribution of c-Fos-IR neurons in spinal cord with continuous urethral distension for 30 minutes in a **T12 transected** animal. Group data (mean \pm S.E) show that long lasting distension also induced a significant increase (ANOVA, $p < 0.05$) in the number of c-Fos-IR neurons in most areas examined at S2 and in laminae I/II at L3 in T12 transected spinal cord. The increase in different spinal areas was similar to that with the short-repeat distension in T12 transected animals (see Figure 4). n =

number of animals examined.

Figure 8. Example of multiple labeling immunohistochemistry in spinal cord sections after urethro-genital reflex activation. **A** and **B**: Triple labeling immunohistochemistry with antibodies against c-Fos, CGRP and SP revealed that many reflex activated sacral spinal neurons with c-Fos immunoreactivity in nuclei (red, indicated with arrow heads) were found in superficial dorsal horn (see **B**, photo taken in the area was marked with ^x in **A**) and surrounded by fibres with strong immunoreactivity to SP (green) and CGRP (blue). These spinal neurons most likely processing UGR sensory inputs. **C** and **D**: c-Fos labeled spinal neurons (red) in lumbar lateral horn (area marked with * in **A**) were immunoreactive to ChAT (green with arrows). These spinal neurons are autonomic cholinergic preganglionic neurons activated within the UGR pathways. Examples of photographs (**A**, **B**, **C**, **D**) were taken from different sections of the preparation. Calibration bar: 200 μm for **A**; 100 μm for **B**, **C** and **D**.

Figure 9. Summary of proposed spinal reflex pathways projecting to the paracervical ganglia (PG, filled circles) controlling the pelvic organs. Sensory pathways are indicated by solid double lines; intra-spinal pathways by dashed lines; descending central pathways by dashed double lines. Excitatory connections indicated by arrows; inhibitory connections by dotted lines capped by bars. **A**: Sensory fibres in the pudendal nerve (PudN) activated by urethral distension stimulate preganglionic neurons projecting to pelvic viscera, not only at sacral spinal levels but also at lumbar spinal levels, mainly by an ascending intra-spinal pathway (single dashed lines), with

potential additional sensory input directly via the hypogastric nerve (HN). The lumbar pathways activate sympathetic preganglionic neurons, projecting via L3 lumbar splanchnic nerves (LSN), inferior mesenteric ganglion (IMG) and hypogastric nerves to paracervical ganglia (PG). Sacral pathways send spinal output to PG via spinal parasympathetic preganglionic neurons projecting out the pelvic nerve (PN). Descending spinal pathways from brain or other supra-spinal level (dashed double lines) did not have a strong influence on UGR as shown by the lack of significant effect of T12 spinal transection. **B:** UGR also activates local spinal inhibitory circuits (dotted lines) that suppress c-Fos expression in spinal preganglionic neurons projecting from both lumbar and sacral levels to the pelvic viscera. These local spinal inhibitory circuits include ascending (from sacral to lumbar) and descending (from lumbar to sacral) pathways.

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Fig. 1

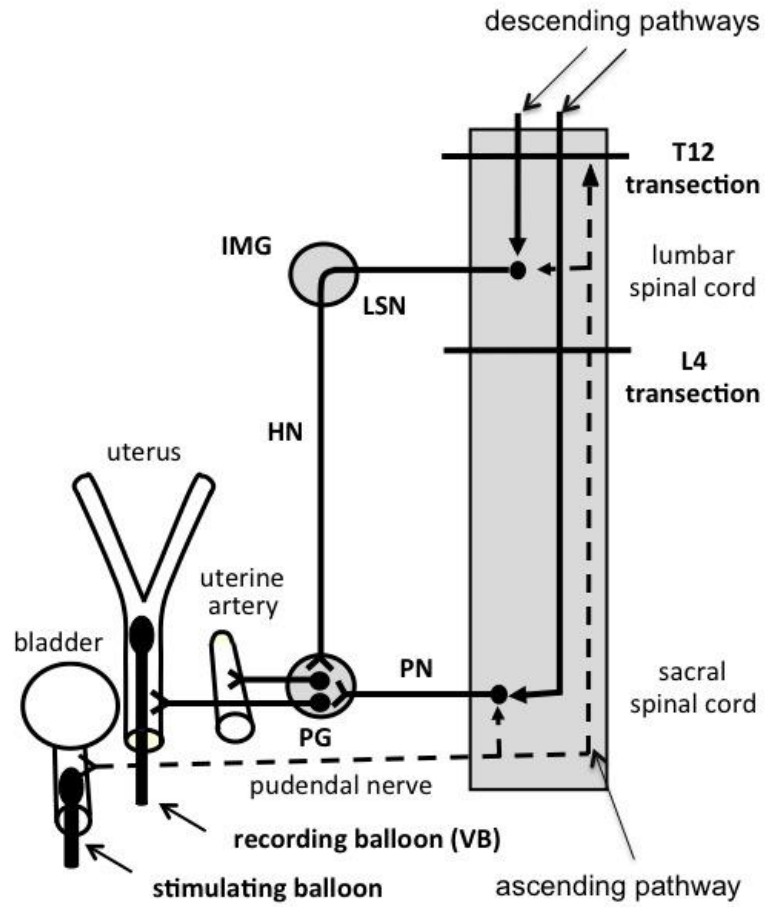
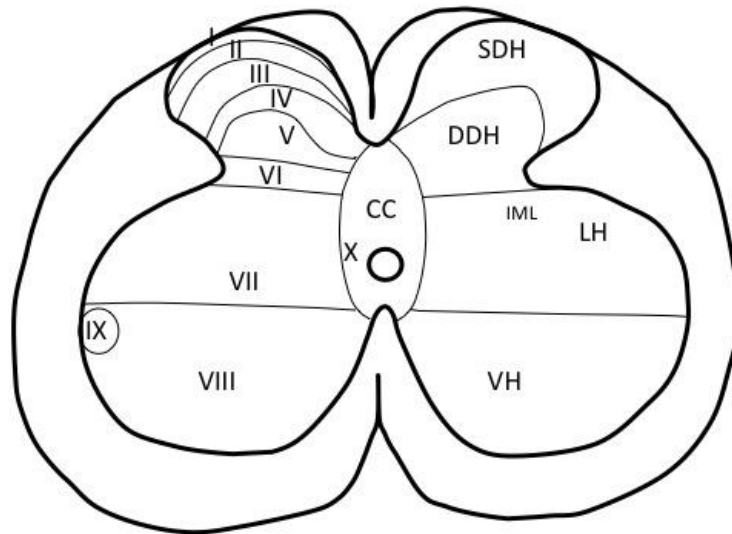


Fig 2

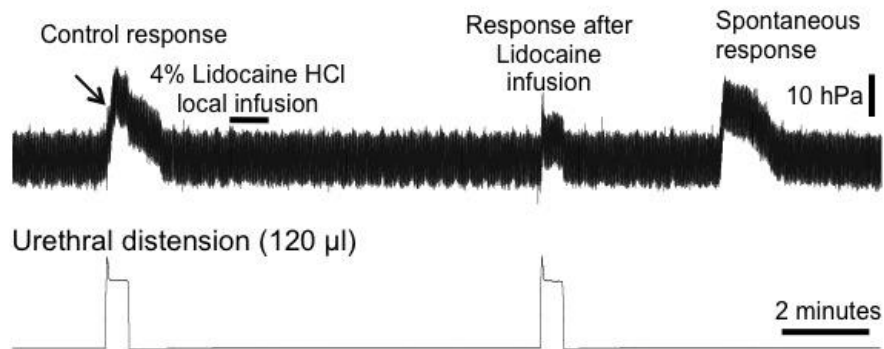
A



B

a. Reflex responses to short-repeat urethral distension

Vaginal pressure



b. Reflex responses to continuous urethral distension

Vaginal pressure

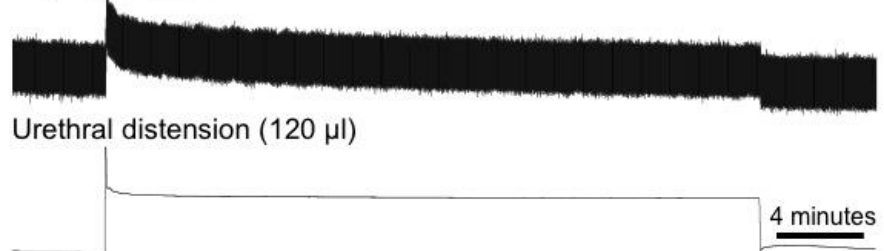


Fig 3

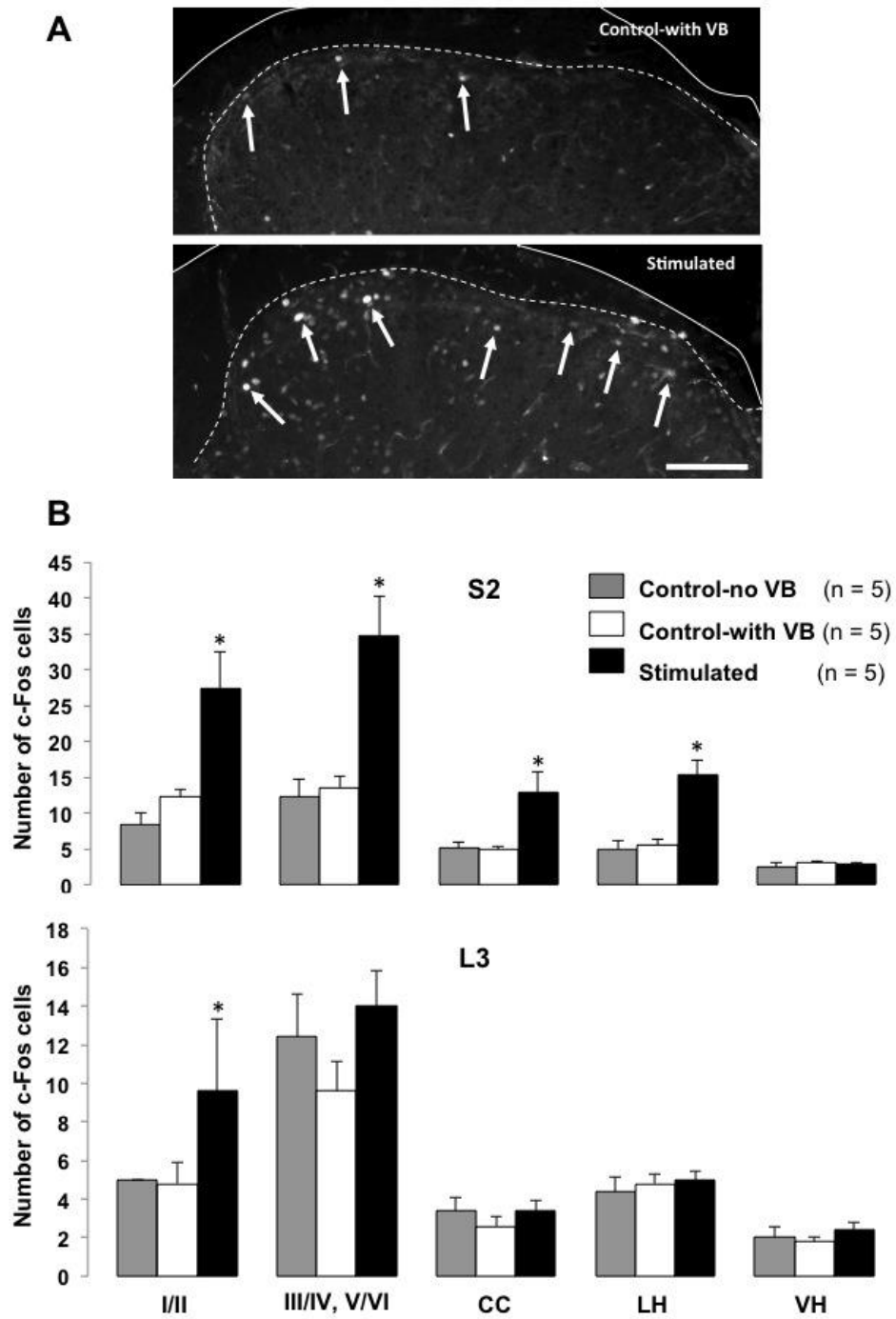


Fig 4

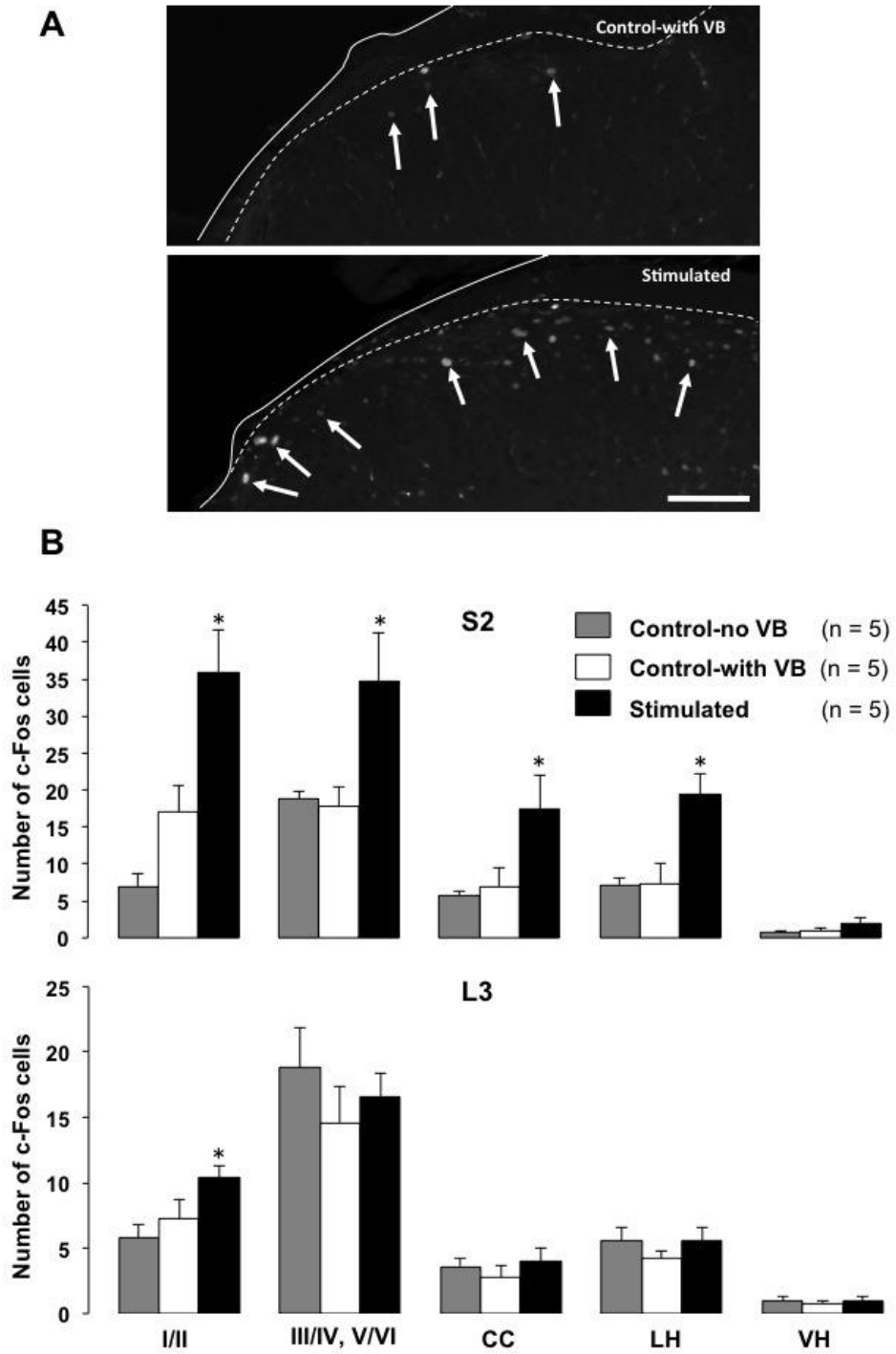


Fig 5

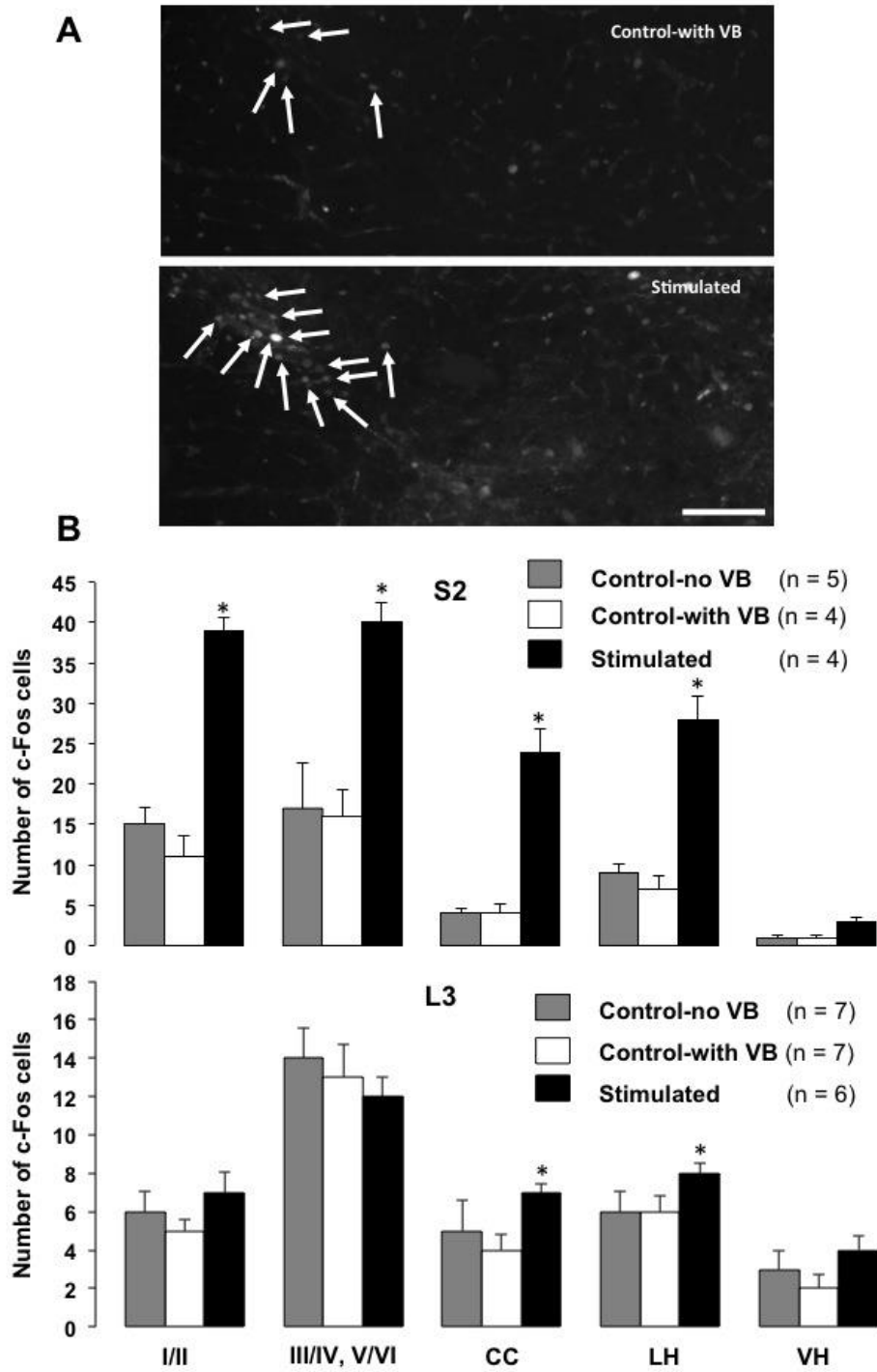


Fig 6

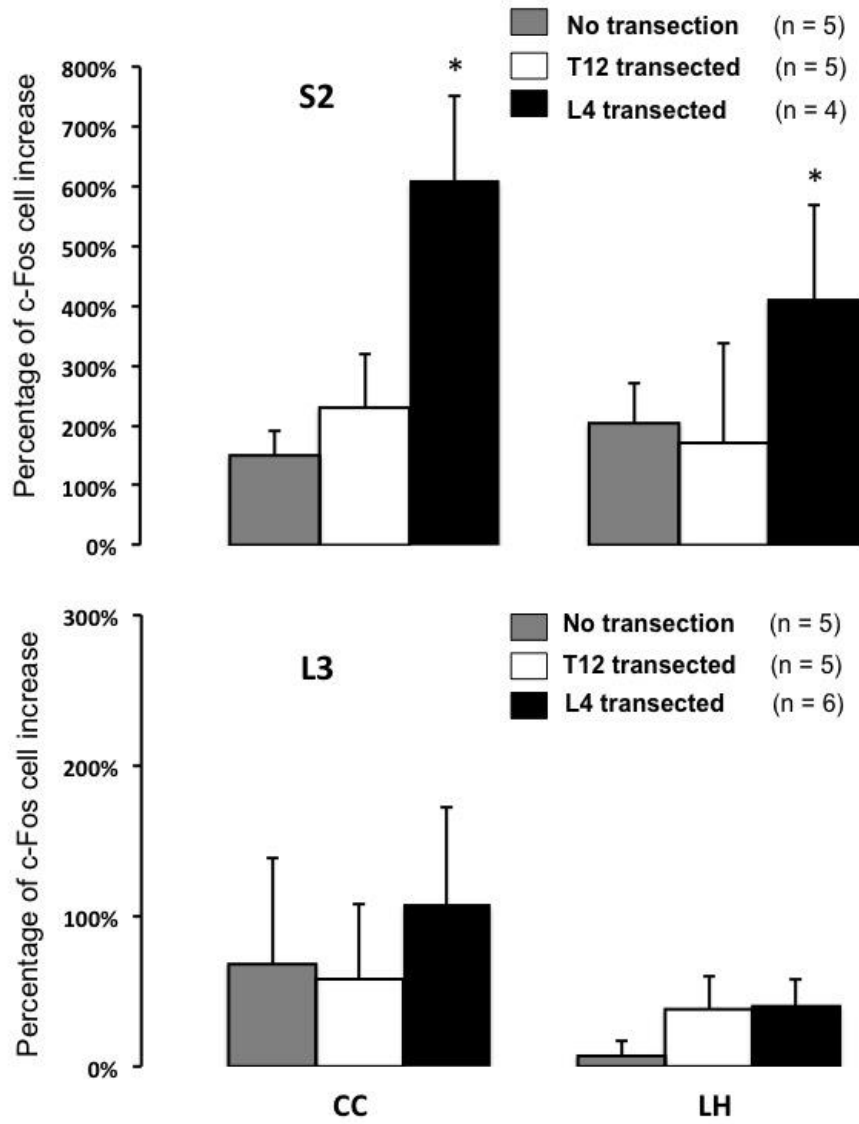


Fig 7

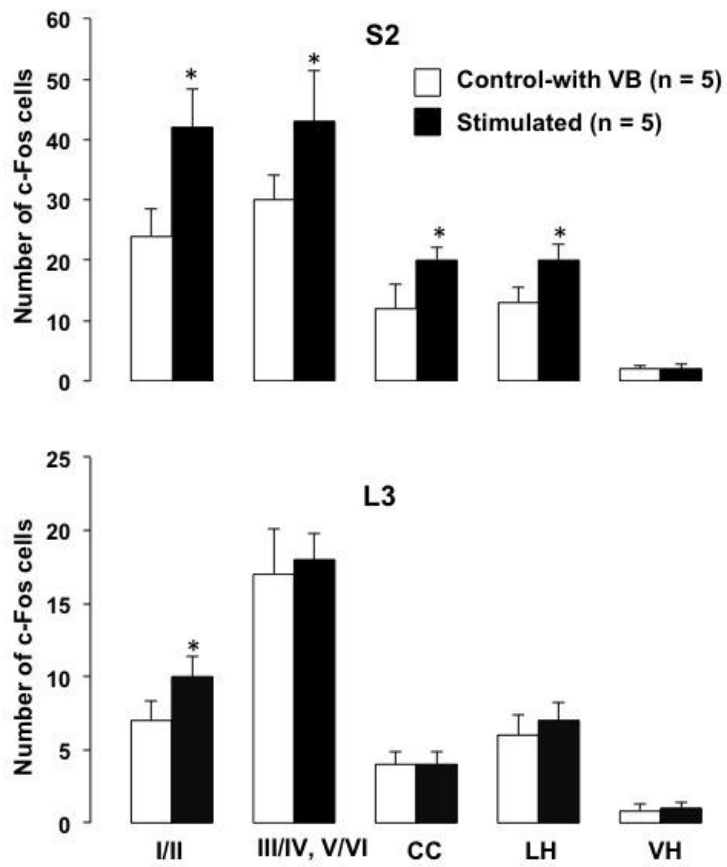


Fig 8

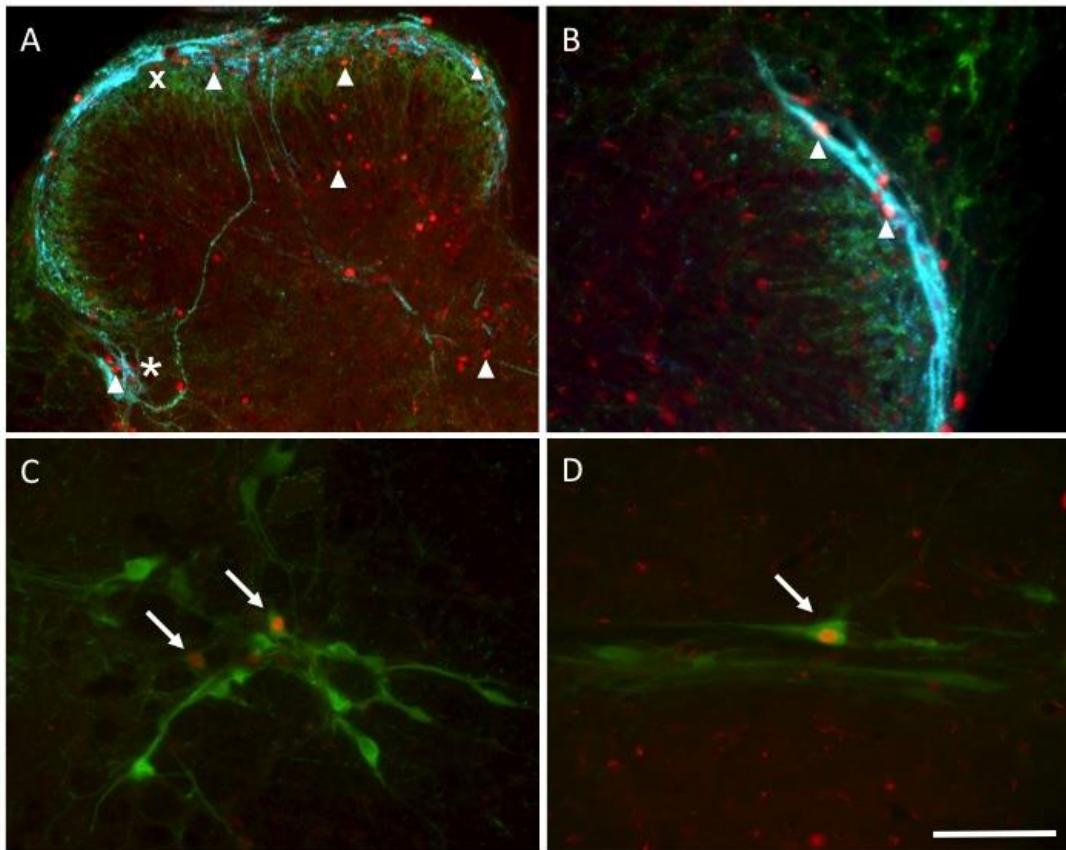


Fig 9

