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Expression of vesicular glutamate transporters in sensory and autonomic neurons innervating the mouse urinary bladder

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Runninghead: VGLUTs in **neurons innervating** the urinary bladder

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Key Words: botulinum toxin, DRG, glutamate, neuropeptides, pain, urinary bladder.

Abstract

Purpose: Vesicular glutamate transporters (VGLUTs), essential for loading glutamate into synaptic vesicles, are present in various neuronal systems. However, the expression of VGLUTs in neurons innervating the urinary bladder has not yet been analyzed. Here, we study the presence of VGLUTs type-1, -2 and -3 ($VGLUT_1$, $VGLUT_2$ and $VGLUT_3$, respectively) in mouse urinary bladder neurons. **Materials and Methods:** Expression of $VGLUT_1$, $VGLUT_2$ and calcitonin gene-related peptide (CGRP) was analyzed by immunohistochemistry in retrogradely labeled primary afferent and autonomic neurons of BALB/C mice after injecting Fast Blue in the urinary bladder wall. To study $VGLUT_3$, retrograde tracing of the urinary bladder was performed in transgenic mice where $VGLUT_3$ is identified by detection of enhanced green fluorescent protein (EGFP). **Results:** Most urinary bladder DRG neurons expressed $VGLUT_2$. A smaller percentage of neurons also expressed $VGLUT_1$ or $VGLUT_3$. Coexpression with CGRP was only observed for $VGLUT_2$. Occasional $VGLUT_2$-immunoreactive (IR) neurons were seen in the major pelvic ganglion (MPG). Abundant $VGLUT_2$-IR nerves were detected in the urinary bladder dome, trigone and also the urethra; $VGLUT_1$-IR nerves were discretely present. **Conclusions:** We present novel data on the expression of VGLUTs in sensory and autonomic neurons innervating the mouse urinary bladder. The frequent association of $VGLUT_2$ and CGRP in sensory neurons suggests interactions between glutamatergic and peptidergic neurotransmissions, potentially influencing commonly perceived sensations in the urinary bladder, such as discomfort and pain.

Abbreviations and Acronyms:

CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; eGFP, enhanced green fluorescent protein; FB, fast blue; IR, immunoreactive; Li, like-immunoreactivity; LS, lumbosacral; LSC, lumbar sympathetic chain; MPG, major pelvic ganglion; NPs, neuron profiles; TL, thoracolumbar; TRPV1, transient receptor potential cation channel, subfamily V, member 1; VGLUTs, vesicular glutamate transporters

The urinary bladder is profusely innervated by sympathetic, parasympathetic and also primary afferent neurons [1,2]. The main neurotransmitters involved in autonomic functions are noradrenaline and acetylcholine, produced by sympathetic and parasympathetic neurons, respectively. Presence of these neurotransmitters in neurons and nerve fibers is usually shown by identification of associated molecules such as tyrosine hydroxylase (TH) or the norepinephrine transporter-1 (noradrenergic) and choline acetyltransferase or the vesicular acetylcholine transporter (acetylcholinergic). Visceral primary afferent neurons, like non-visceral ones [3], are classically described as being glutamatergic and often capable of coexpressing a variety of molecules, including neuropeptides, various kinds of receptors (see [4]) and even markers of autonomic neurons (TH [5]).

The earliest morphological demonstration of the glutamatergic nature of urinary bladder dorsal root ganglion (DRG) neurons was published over 12 years ago, when Keast and Stephenson [6] reported immunohistochemically detectable glutamate in approximately 50% of rat urinary bladder DRG neurons. For its use in neurotransmission, glutamate is taken up into synaptic vesicles by means of vesicular glutamate transporters (VGLUTs). Different types of VGLUTs have been recently identified, resulting in the reliable immunohistochemical characterization of various subpopulations of glutamatergic neurons, both in the central and the peripheral nervous systems [7]. In the latter, VGLUTs have been characterized in **rodent** non-visceral (see [8] and references therein) and colorectal primary afferent neurons [9], as well as in some lumbar sympathetic chain (LSC) neurons after peripheral nerve injury [10].

To date, analysis of the presence of VGLUTs in DRG and autonomic

neurons projecting to the urinary bladder is not available. Here, we present a comprehensive immunohistochemical study of the expression of the three known VGLUTs in DRG, LSC and major pelvic ganglion (MPG) neurons innervating the mouse urinary bladder.

MATERIALS AND METHODS

Male BALB/c (Taconic, Germantown, NJ, USA; 7-8 weeks old) and 129S6/SvEvTac-C57Bl/6 mice ([11]; 6 weeks old) were used in **all** experiments. All research protocols adhered to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care Use Committee of the Universities of Pittsburgh and California in San Francisco.

Of all commercial antibodies against $VGLUT_3$ currently available, none efficiently label DRG neurons. Therefore, we also utilized a transgenic mouse expressing EGFP under the control of $VGLUT_3$ regulatory sequences that reliably identify $VGLUT_3$ expressing neurons in these mice (see [11]).

The urinary bladders of five BALB/c male mice, and three $VGLUT_3$-EGFP male mice, 6 weeks old, were injected with the fluorescent retrograde neuronal tracer Fast Blue (FB, 2% in saline; EMS-Chemie, Gross Umstadt, Germany) according to previously reported surgical procedures (see [9]).

Twelve days after injection of FB, mice were deeply anaesthetized using sodium pentobarbital (60 mg/kg, i.p.; Ovation Pharmaceuticals, Deerfield, IL, USA) and perfused via the ascending aorta with previously described fixative mixtures (see [9]). Three naïve BALB/C mice were similarly anesthetized and perfused. Thoracolumbar (T8-L1; TL) and lumbosacral (L6-S2; LS) DRGs, MPG, LSC and urinary bladder were dissected out and processed following previously described immunohistochemistry protocols (see [8,9]). Table 1 lists all antibodies used. Further details on these antibodies can be found in previous publications (see [9] and references therein).

Single- or double-staining experiments, utilizing tyramide signal amplification (TSA plus, NEN Life Science Products, Inc., Boston, MA, USA)

and/or indirect immunofluorescence techniques, were performed (see [9]). Briefly, sections were incubated with one or two antibodies, for single or double staining, respectively, and processed first following the TSA technique. Additional TSA or indirect immunofluorescence protocols were employed for double staining experiments. Nonspecific staining by the secondary antibody was tested in a few sections by omission of the primary antibody. CGRP, VGLUTs and EGFP antisera have been thoroughly characterized in previous publications (see [8,9] and references therein).

Immunofluorescence sections were examined on a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) provided with a Retiga 2000 R Fast CCD camera (Q-Imaging, Surrey, British Columbia, Canada) using IPLab software (Scanalytics Inc., Vancouver, Canada). Colocalization analysis was done using a Fluoview FV 1000 confocal microscope (Olympus, Tokyo, Japan). Resolution, brightness and contrast of the images were optimized using Adobe Photoshop CS3 software (Adobe Systems Inc., San Jose, CA). Because confocal imaging of FB was not possible due to lack of appropriate filters, some images (MPG) were composed by merging separate optical (FB) and confocal (other markers of interest) photomicrographs.

Retrogradely traced neuron profiles (NPs) were quantified in every fifth section of TL and LS DRGs (5-8 sections/ganglion). Percentages of VGLUT-expressing urinary bladder NPs were obtained by comparing the total number of FB-positive urinary bladder vs. those expressing each marker. Cell body diameters of a representative sample of each type of quantified NP (more than 7 and up to 48 neurons per type) were measured using the public domain NIH program, Image J (http://rsb.info.nih.gov/ij/download.html), and these data used to correct the raw

counts, based on the Abercrombie correction factor [12]. Only NPs exhibiting an intensity of immunostaining higher than 2 orders of magnitude were counted.

Data are presented as mean ± SEM and were statistically analyzed using the Student's t-test for independent samples; $P<0.05$ was considered significant.

RESULTS

Retrograde tracing from the urinary bladder revealed a discrete number of FB-positive NPs of different sizes per section in TL and LS DRGs that were easily differentiated from FB-negative NPs (Figs. 1, 2).

All VGLUTs were expressed in mouse DRG neurons projecting to the urinary bladder, although in different proportions (Table 2; Fig. 1). On average, 32.4 ± 3.6%, 94.1 ± 3.0% and 18.1 ± 1.3% of urinary bladder DRG NPs expressed $VGLUT_1$, $VGLUT_2$ or eGFP-$VGLUT_3$, respectively. Further analysis showed that the expression of VGLUTs differed between TL and LS DRGs. $VGLUT_1$ was present in a greater proportion of TL (38.9 ± 4.2%) than LS (25.9 ± 3.6%) urinary bladder DRG NPs (P<0.05; Table 2; Fig. 1A-C); the majority of $VGLUT_1$-IR NPs appeared to be medium- to large-sized (Fig. 1A-C). $VGLUT_2$ was detected in similar, high proportions in TL (93.8 ± 2.0%) and LS (94.3 ± 4.1%) urinary bladder DRG NPs of all sizes (Table 2; Fig. 1D-I); occasional urinary bladder DRG neurons lacking $VGLUT_2$ were also found (Fig. 1G-I). eGFP-$VGLUT_3$ was expressed in a greater proportion of TL (27.9 ± 2.0%) than LS (8.3 ± 0.6%) urinary bladder DRG NPs of small size, respectively (P<0.01; Table 2; Fig. 1J-L). Finally, in addition to urinary bladder neurons, abundant $VGLUT_2$- or eGFP-$VGLUT_3$-IR, and several $VGLUT_1$-IR, FB-negative neurons were detected in all DRGs (Figs. 1, 2).

Colocalization analysis showed that only $VGLUT_2$ coexpressed with CGRP in urinary bladder DRG NPs (Fig. 2). Thus, while no $VGLUT_1$ urinary bladder DRG NPs coexpressed CGRP (Fig. 2A-D), 54.7 ± 3.4% of TL and 52.2 ± 9.9% of LS urinary bladder DRG NPs coexpressed $VGLUT_2$ and CGRP (Fig. 2E-H). FB-negative $VGLUT_2$-IR NPs coexpressing CGRP (Fig. 2F-H) or CGRP-only urinary bladder DRG neurons were also detected (Fig. 2A-D; I-L). As observed for

$VGLUT_1$, virtually no eGFP-$VGLUT_3$ urinary bladder DRG NPs coexpressed CGRP (Fig. 2I-L). Such lack of coexpression with CGRP was also noted for FB-negative $VGLUT_1$- (Fig. 2A-D) or eGFP-$VGLUT_3$-IR NPs (Fig. 2I-L).

Retrograde labeling of LSC (Fig. 3A, D) and MPG neurons (Fig. 3G, J. M. P) from the urinary bladder was also noted. In the LSC, no FB-positive neurons expressed $VGLUT_1$ or $VGLUT_2$ (Fig. 3A-C and Fig. 3D-F, respectively). Nevertheless, some $VGLUT_2$-IR fibers, and what appeared to be $VGLUT_2$-IR synaptic varicosities, were detected in the LSC (Fig. 3D-F). Similarly, FB-positive neurons in the MPG also lacked $VGLUT_1$-like immunoreactivity (Li) (Fig. 3G-I), which was only associated with a few varicose profiles (Fig. 3H, I). In contrast, occasional $VGLUT_2$-IR urinary bladder MPG neurons were found (Fig. 3J-L), along with many $VGLUT_2$-IR fibers and varicosities (Fig. 3K, L, N, O, Q, R). Some of these varicosities exhibited a basket-like appearance, surrounding non-urinary bladder MPG (Fig. 3P-R) neurons.

In the urinary bladder, $VGLUT_1$-Li was commonly observed in the muscular (Fig. 4A) and, to a lesser extent, in the submucosal layers (Fig. 4B) of the organ. In comparison, $VGLUT_2$-IR nerves formed a profuse fiber network spanning all layers of the urinary bladder wall throughout the dome (Fig. 4C, D), trigone (Fig. 4E) and the urethra (Fig. 4F, G). Colocalization analysis showed that $VGLUT_1$ and CGRP coexpression in urinary bladder nerve fibers was virtually nonexistent (Fig. 5A-C). In contrast, $VGLUT_2$ and CGRP were often colocalized in nerve fibers in the urinary bladder (Fig. 5D-L).

DISCUSSION

Although it is well established that sensory neurons are often glutamatergic [6], information on the types of VGLUTs involved in the synaptic vesicle uptake of glutamate in such neurons is only recently receiving attention (see [8]). Evidence for the expression of VGLUTs in visceral sensory neurons was first reported by Tong et al. [13], in ganglia innervating the rat stomach. Since then, expression of VGLUTs has been confirmed in the peripheral projections of vagal and DRG neurons innervating the gut of guinea pig, rat and mouse (see [9] and references therein). More recently, we showed that over 95% of mouse colorectal DRG neurons express $VGLUT_2$, and in a much lower proportion, $VGLUT_1$ [9]. Similarly, we demonstrate in the present study that mouse urinary bladder DRG neurons also predominantly express $VGLUT_2$, and to a lesser extent $VGLUT_1$ or $VGLUT_3$.

The urinary bladder, like the colorectum, is innervated by lumbar splanchnic and pelvic nerves, with cell bodies in TL and LS DRGs, respectively. We found that urinary bladder sensory neurons expressing $VGLUT_1$ or $VGLUT_3$, unlike those synthesizing $VGLUT_2$, are more abundant in TL than in LS DRGs. An increasing number of reports reveal significant differences in both the functions and neurochemical expression of various molecules in afferent neurons comprising the two pathways of innervation (see [5] and references therein). For example, 66% of mouse TL bladder neurons express the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) mRNA, whereas TRPA1 expression in LS bladder neurons is scarce (~2.5%) [14]. In contrast, TH is expressed in threefold (colorectum) and fivefold (urinary bladder) greater

proportions in LS DRGs [5]. The differences in expression of $VGLUT_1$ and $VGLUT_3$ between TL and LS bladder neurons reported here could imply differences in neuronal physiology. This has already been shown in the mouse colorectum, where a higher expression of transient receptor potential cation channel, subfamily V, member 1 (TRPV1) in TL than in LS DRGs corresponds with a stronger response to applied capsaicin in colorectal TL nerve terminals [15].

We found that of the 94% of retrogradely traced urinary bladder DRG neurons expressing $VGLUT_2$, about half coexpressed the peptidergic marker CGRP. These figures correlate with previous studies in rat showing that up to 69% of DRG neurons innervating the urinary bladder express CGRP (see [4]). In the mouse colorectum, more than 80% of all retrogradely traced DRG neurons coexpressed $VGLUT_2$ and CGRP [9], in accordance with the expression of CGRP in the vast majority of DRG neurons innervating the rodent colorectum (see [4]). Accordingly, it can be concluded that a large proportion of visceral glutamatergic sensory neurons also synthesize, and potentially co-release, CGRP. In contrast, in DRGs innervating non-visceral tissues only ~31% of the ~65% $VGLUT_2$-IR neurons colocalize CGRP [8], suggesting potentially variable levels of co-release of glutamate and CGRP between DRG neurons innervating different tissues.

$VGLUT_1$ and $VGLUT_3$-IR urinary bladder neurons did not coexpress CGRP. However, since virtually all neurons innervating this organ synthesized $VGLUT_2$, it is highly likely that $VGLUT_1$ and/or $VGLUT_3$ are coexpressed with $VGLUT_2$ in its non-peptidergic subpopulation (see above). Coexpression of more than one type of VGLUT was first shown for some neurons in the central nervous system [16], and later confirmed for $VGLUT_1$ and $VGLUT_2$ in non-visceral primary afferent neurons and their projections (see [8] and references therein). Our studies

in mouse colorectum [9] and urinary bladder (current study) further support the supplementary contribution of different VGLUTs in glutamatergic physiology in sensory neurons.

$TRPV_1$ is a non-selective cation channel activated by pH, heat and capsaicin and strongly implicated in urinary bladder nociception (see [17]). $TRPV_1$ is expressed in many peptidergic urinary bladder DRG neurons in both rat [18] and mouse [14,15,18]. Considering the prominent expression of $VGLUT_2$ in peptidergic urinary bladder DRG neurons, $VGLUT_2$ undoubtedly coexists with $TRPV_1$. In support, coexpression of $VGLUT_2$ with $TRPV_1$ has been previously demonstrated in nerve fibers terminating in the mouse rectum [19]. Interestingly, $TRPV_1$ and glutamate receptors (GluRs) also colocalize in non-visceral small diameter primary afferent neurons, and it has been shown that the induction of c-Fos expression in the spinal dorsal horn induced by subcutaneous injection of capsaicin is prevented by concomitant subcutaneous administration of ionotropic or metabotropic glutamatergic receptor antagonists [20]. Peripheral release of glutamate, likely from primary afferent nerve terminals, has been demonstrated in the hindpaw skin of rats exposed to intraplantar injection of the irritant formalin (see [21] and references therein). Moreover, the local injection of glutamate induces nociceptive behaviors both in rats [22] and humans [23]. Thus, it could be speculated that a $TRPV_1$-glutamate receptor interaction, both at central as well as peripheral nerve terminals, and potentially driven by glutamate released from primary afferent neurons, could have a role in the physiopathology of sensation and pain of the urinary bladder.

As suggested above, urinary bladder nociception could be influenced by the peripheral release and action of glutamate. In recent years, botulinum toxin A has been used in the treatment of urinary bladder pain associated with interstitial

cystitis. Although originally described as a potent inhibitor of acetylcholine release in the neuromuscular junction (see [24]), it is increasingly accepted that botulinum toxin A also blocks transmitter release at non-acetylcholinergic synapses. Thus, it has been hypothesized that botulinum toxin A desensitizes peripheral afferent nerves by inhibition of the vesicular release of ATP, as well as the release of peptides such as CGRP and SP. Reduction of the axonal expression of TRPV1 from urothelial and suburothelial nerve endings has also been proposed (see [25] and references therein). In humans, the synaptic vesicle protein 2, a high-affinity receptor for botulinum toxin A, and the synaptosomal-associated protein 25 (SNAP-25), one of the SNAP (Soluble NSF-attachement Proteins) receptors (SNAREs) involved in vesicle fusion prior to neurotransmitter release and essential for the blocking action of botulinum toxin A, have been found in at least half of the peptidergic nerve fibers present in the urinary bladder [26]. In rodents, most of the non-visceral [8], colorectal [9] and urinary bladder (present study) DRG neurons/nerves expressing $VGLUT_2$, and often synthesizing peptides, are most likely provided with the SNARE machinery for synaptic neurotransmitter release. Therefore, it can be speculated that in these neurons/nerves, botulinum toxin A could also affect the docking of glutamatergic synaptic vesicles, limiting the release of glutamate, and thus contributing to the analgesic effects of the toxin. In fact, the benefits of using botulinum toxin have already been successfully explored in animals with non-visceral neuropathy [24].

Finally, we also detected a few MPG neurons projecting to the urinary bladder that also expressed $VGLUT_2$, whereas none of the retrogradely traced neurons in the LSC showed positive immunostaining for any VGLUT. Thus, in normal conditions, not only DRGs, but also the MPG may be a source of $VGLUT_2$

immunoreactive fibers in the urinary bladder. Interestingly, we have recently shown that upon injury of the pelvic nerve, a subpopulation of LSC neurons upregulates $VGLUT_2$ [10]. In that study, we did not trace the neurons, and thus it remains to be established if some of the LSC neurons upregulating $VGLUT_2$ projected either to the urinary bladder or the colorectum. Likewise, it could be speculated that MPG neurons, either projecting to visceral organs or not, may upregulate $VGLUT_2$ after injury of their postganglionic axons.

In conclusion, we present an immunohistochemical account of the expression of VGLUTs in sensory and autonomic neurons innervating the mouse urinary bladder. The finding of a preponderant expression of $VGLUT_2$, as recently also demonstrated in the mouse colorectum [9], suggests a relevant role for this transporter in glutamatergic neurotransmission in neurons innervating pelvic visceral organs, from which the principal conscious sensations that arise are discomfort and pain. Glutamate, which is cointained in the central terminals of primary afferents, spinal interneurons and terminals of fibers that descend from the medulla oblongata [27], is well established as important to nociceptive transmission from pelvic organs [1,28]. Thus, a complex interaction between spinal ionotropic (NMDA and AMPA) receptors has been shown in the spinal processing of nociceptive input from the irritated lower urinary tract (LUT) [29]. Likewise, a facilitatory role in urinary bladder primary afferent processing has been ascribed to the metabotropic receptor mGluR5, both in normal conditions as well as during inflammation of the LUT [28]. However, it should be noted that the role of peripheral glutamate release in the urinary bladder is unclear. Thus, the intravesical application of metabotropic glutamate

receptor antagonists has no effect on urinary bladder contractility and pelvic nerve afferent firing in normal rats [30]. Studies using animal disease models will be necessary to establish the potential involvement of peripheral glutamate/glutamatergic receptors in the urinary bladder.

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Figure legends

Table 1. Technical specifications of the primary and secondary antibodies, and fluorophores used in this study.

Table 2. Percentages of thoracolumbar **(TL)** or lumbosacral **(LS)** DRG urinary bladder NPs expressing $VGLUT_1$, $VGLUT_2$ or $VGLUT_3$. Combined TL and LS data are also shown. *P<0.05; ***P<0.001.

Figure 1. Expression of VGLUTs in DRGs innervating the urinary bladder. Optical immunofluorescence photomicrographs of sections of S1 (A-C), L6 (D-F), T10 (G-I) or T11 (J-L) DRGs incubated with $VGLUT_1$ (B), $VGLUT_2$ (E, H) or eGFP-$VGLUT_3$ (K) antisera. In the merged panels (C, F, I, L), retrogradely labeled urinary bladder DRG neurons containing FB (A, D, G, J) are shown in purple; $VGLUT_1$-, $VGLUT_2$- or eGFP-$VGLUT_3$-IR neurons are shown in green; colocalizations are shown in white (VGLUT+FB). Double arrowheads in panels A-C, D-I or J-L show $VGLUT_1$-, $VGLUT_2$ or eGFP-$VGLUT_3$-IR urinary bladder NPs, respectively. Arrows in panels A, G or J show urinary bladder NPs lacking $VGLUT_1$, $VGLUT_2$ or eGFP-$VGLUT_3$-Li, respectively. Arrowheads in panels B, E or K show non-traced NPs expressing $VGLUT_1$, $VGLUT_2$ or eGFP-$VGLUT_3$. Scale bars: 50 µm (I=G, H; L=A-F, J-K).

Figure 2. Patterns of co-expression of VGLUTs with CGRP in DRGs innervating the urinary bladder. Optical immunofluorescence photomicrographs of sections of T10 (A-D), L6 (E-H) or S2 (I-L) DRGs incubated with $VGLUT_1$ (B), $VGLUT_2$ (F) or eGFP-$VGLUT_3$ (J) antisera. In the

merged panels (D, H, L), retrogradely labeled urinary bladder DRG neurons containing FB (A, E, I) are shown in red; $VGLUT_1$-, $VGLUT_2$- or eGFP-$VGLUT_3$-IR neurons are shown in green; CGRP-IR neurons are shown in blue; colocalizations are shown in white (VGLUT+FB+CGRP), orange (VGLUT-FB) or purple (CGRP+FB). White double arrowheads in panels A-D, E-H or I-L show $VGLUT_1$- $VGLUT_2$- or eGFP-$VGLUT_3$-IR urinary bladder NPs lacking CGRP-Li, respectively. Black double arrowheads in panels A-D or I-L show urinary bladder NPs expressing CGRP and lacking VGLUTs-Li. Double arrows in panels E-H show $VGLUT_2$-IR urinary bladder NPs coexpressing CGRP. White arrowheads in panels B, F or J show non-traced $VGLUT_1$-$VGLUT_2$ or eGFP-$VGLUT_3$-IR DRG NPs lacking CGRP-Li, respectively. Black arrowheads in panels E-H show non-traced $VGLUT_2$-IR NPs coexpressing CGRP. Scale bar: 50 μm (L=A-K).

Figure 3. Expression of VGLUTs in LSCs and MPGs innervating the urinary bladder. Optical (A, D, G, J, M, P) and confocal (B, C, E, F, H, I, K, L, N, O, Q, R) immunofluorescence photomicrographs of sections of the LSC (A-F) and the MPG (G-R) after incubation with $VGLUT_1$ (B, H) or $VGLUT_2$ (E, K, N, Q) antisera. In the merged panels (C, F, I, L, O, R), retrogradely labeled urinary bladder DRG neurons containing FB (A, D, G, J, M, P) are shown in purple; $VGLUT_1$- or $VGLUT_2$-IR neurons are shown in green; colocalizations are shown in white (VGLUT+FB). Arrows in panels A and G, or in D, J and P show urinary bladder NPs in the LSC (A, D) or the MPG (G, J, P) lacking $VGLUT_1$- or $VGLUT_2$-Li, respectively. White arrowheads in panels H or E, K and Q show $VGLUT_1$- or $VGLUT_2$-IR varicosities in the LSC (E) or the MPG (H,

K, Q), respectively (white arrowhead in panel H is shown at higher magnification in I). Black arrowheads in E, K and Q show $VGLUT_2$-IR fibers in the LSC (E) or the MPG (K, Q). $VGLUT_2$-IR varicosities surrounding urinary bladder NPs lacking the transporter are detected (combined arrow and white arrowhead in F, also shown in inset in F). Double arrows in panels J-L show $VGLUT_2$-IR urinary bladder NPs (shown at higher magnification in M-O). White double arrowhead in panel Q shows a $VGLUT_2$-IR basket surrounding non-traced NPs lacking the transporter (also shown in inset in R). Scale bars: 50 µm (L=A-K; O=M, N; R=P, Q); 10 µm (all insets).

Figure 4. Confocal immunofluorescence photomicrographs of transverse (A-D) and sagittal (E-G) sections of the urinary bladder dome (A-D), trigone (E) and urethra (F, G), after incubation with $VGLUT_1$ (A, B) or $VGLUT_2$ (C-G) antiserum. White double arrowheads in panels A and B, or C and D show $VGLUT_1$- or $VGLUT_2$-IR nerve fibers in the muscular layer of the urinary bladder, respectively. White arrowheads in panels B, or C and D show $VGLUT_1$- or $VGLUT_2$-IR nerve fibers in the submucosal layer of the urinary bladder, respectively. Arrows in panel E show $VGLUT_2$-IR nerve fibers in the urinary bladder trigone. Black arrowheads in panels F and G show $VGLUT_2$-IR fibers penetrating the urethra. White arrowheads in panels F and G show VGLUT2-IR nerve fibers innervating the urethra. Scale Bars: 100 µm (D=A-C; E; G=F).

Figure 5. Confocal immunofluorescence photomicrographs of transverse sections of the urinary bladder after co-incubation with $VGLUT_1$ (A) or

$VGLUT_2$ (D, G, J) and CGRP (B, E, H, K) antisera. In the merged panels (C, F, I, L), $VGLUT_1$- or $VGLUT_2$-IR fibers are shown in green; CGRP-IR fibers are shown in purple; colocalizations are shown in white (VGLUT+CGRP). Arrowheads in panels A, or D and J show $VGLUT_1$- or $VGLUT_2$-IR nerve fibers lacking CGRP-Li in the urinary bladder dome, respectively. Arrows in B and H show CGRP-IR nerve fibers lacking VGLUTs-Li in the muscular layer of the urinary bladder dome. Double arrows in panels G-I or J-L show $VGLUT_2$- and CGRP-IR nerve fibers in the mucosal (inset 1 in F, shown at higher magnification in G-I) and muscular layers (inset 2 in F, shown at higher magnification in J-L) of the urinary bladder, respectively. Scale bars: 50 µm (F=D, E), 25 µm (C=A, B; I=G, H; L=J, K).

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PRIMARY ANTIBODIES		
Target antigen and host species	**Dilution**	**Reference or source**
Rabbit anti-VGLUT$_1$	1:4,000 $_{(T)}$	Kawamura et al., 2006
Guinea Pig anti-VGLUT$_2$	1:8,000 $_{(T)}$	Miyazaki et al., 2003; Brumovsky et al., 2007; 2011a, b
Rabbit anti-EGFP	1:4,000	A11122; Molecular Probes
Rabbit anti-CGRP	1:8,000 $_{(I)}$ / 1:40,000 $_{(C;T)}$	C8198; Sigma
SECONDARY ANTIBODIES/FLUOROPHORES		
HRP-donkey anti-guinea pig	1:200	713-035-003; Jackson Immunoresearch
HRP-donkey anti-rabbit	1:200	711-035-152; Jackson Immunoresearch
TRITC-donkey anti-rabbit	1:400	711-025-152; Jackson Immunoresearch
Fluorescein tyramide	1:700	NEL741; Perkin Elmer

	$VGLUT_1$	$VGLUT_2$	$VGLUT_3$
Thoracolumbar	**38.9** ± 4.2 %	**93.8** ± 2.0 %	**27.9** ± 2.0 %
Lumbosacral	**25.9** ± 3.6 %	**94.3** ± 4.1 %	**8.3** ± 0.6 %
Combined	**32.4** ± 3.6 %	**94.1** ± 3.0 %	**18.1** ± 1.3 %

* ***

Figure 1

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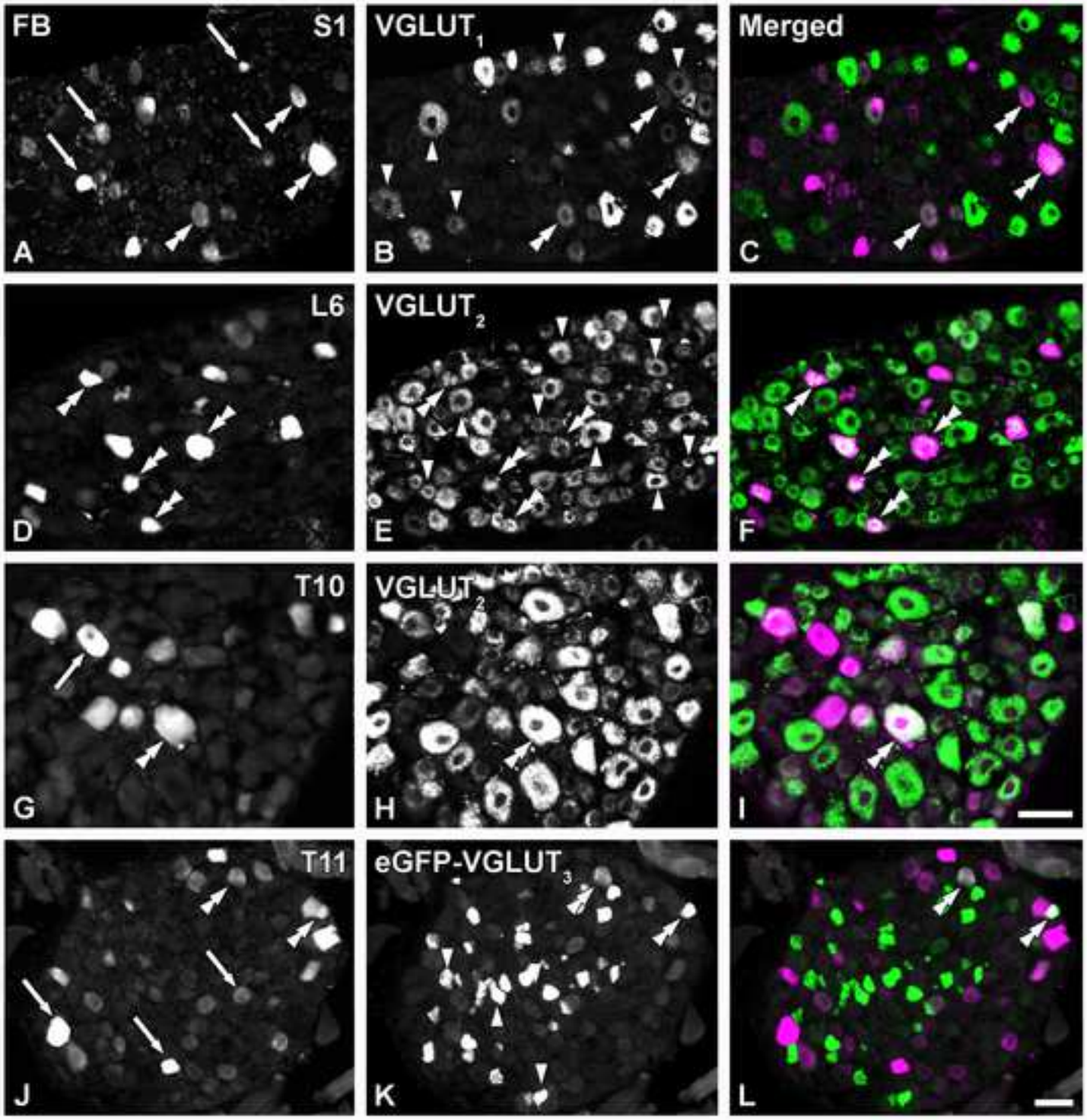

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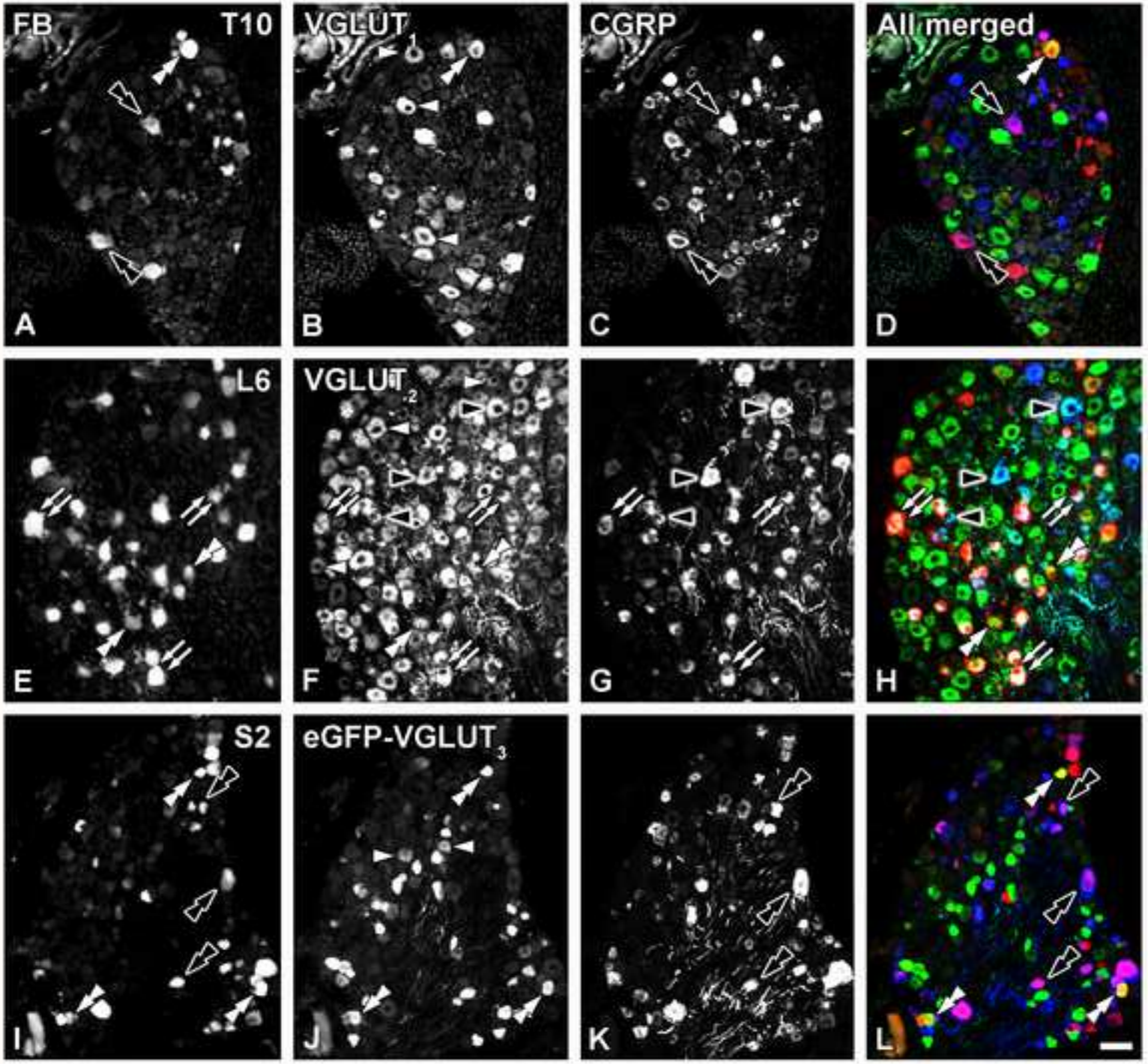

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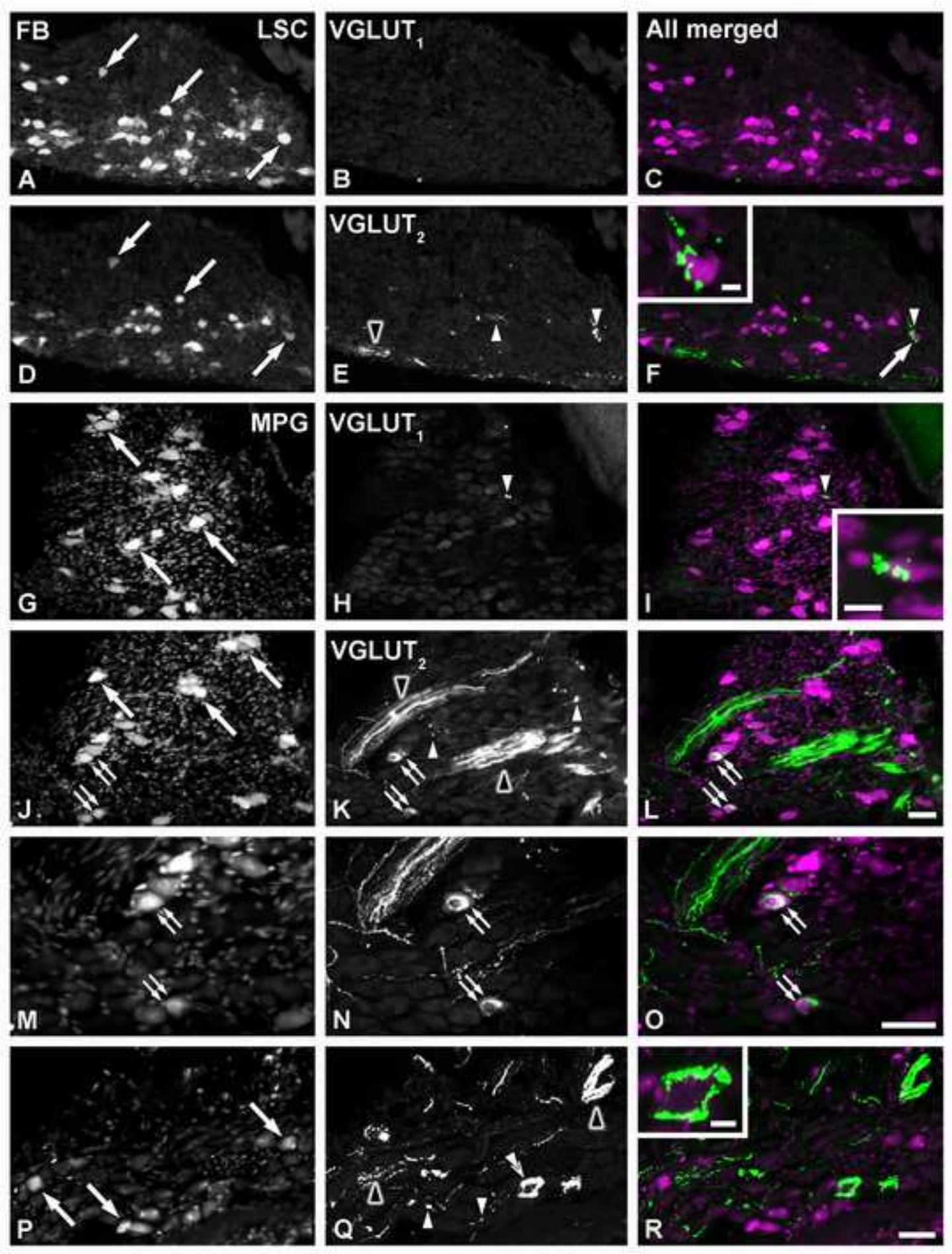

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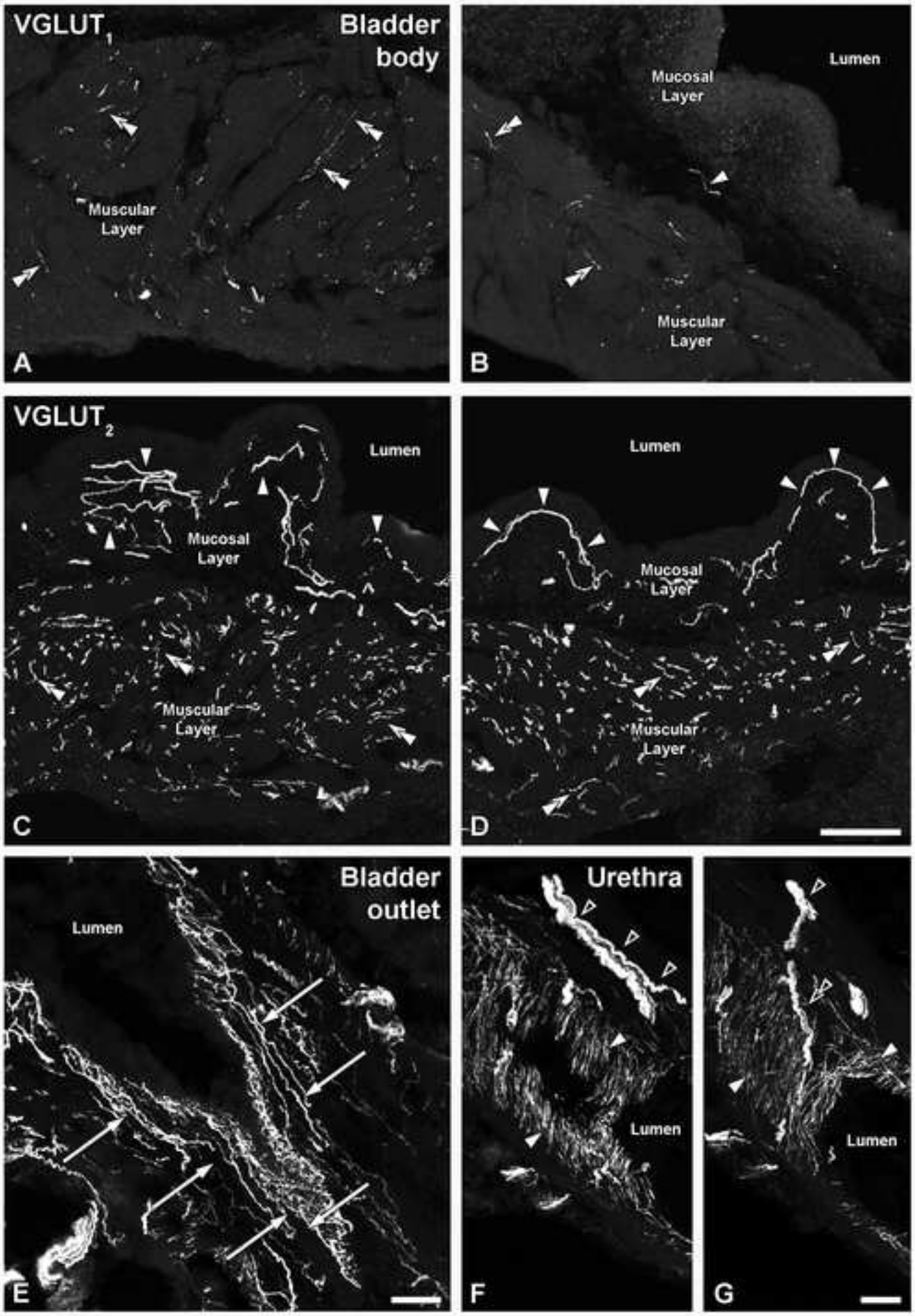

Figure 5

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