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ORIGINAL ARTICLE

Vanilloid receptor type 1-immunoreactive nerves in the rat urinary bladder and primary afferent neurones: the effects of age

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The vanilloid receptor (VR1) is a molecular integrator of various painful stimuli, including capsaicin, acid and high temperature. VR1 protein functions both as a receptor for capsaicin and a transducer of noxious thermal stimuli. In addition, VR1 is well characterised at the terminals of sensory nerves involved in the pain pathway. VR1 is also expressed in a capsaicin-sensitive and peptide-containing sub-population of primary sensory nerves.

Indirect immunohistochemistry was used to examine the distribution of nerves immunoreactive (ir) for VR1 in the base of the urinary bladder and in the neurones of the lumbosacral dorsal root ganglia (L1-L2 and L6-S1) of young adult (3 months) and aged (24 months) male rats. Semi-quantitative estimations of nerve densities were assessed and quantitative studies were also used to examine the effects of age on the percentage of VR1-ir dorsal root ganglion neurones.

The bladder base in young adults showed dense VR1-ir fibres within the urothelium and in the subepithelium and fibres ranging from sparse to moderate in number in the muscle coat. In comparison to the young animals, the aged rats showed sparse to moderate densities of VR1-ir nerves in the subepithelium and sparse fibres in the muscle layers. In the lumbosacral dorsal root ganglia the percentage of VR1-ir neuronal profiles showed a significant reduction from (mean ± SEM) 17.8 ± 2% in the young adult to 12 ± 1.6 in the aged rats.

The present findings suggest that the effects of VR1 on bladder function (nociception and reflex micturition) are influenced by age and the reduction with age of VR1-ir neurones in the dorsal root ganglia could also have important implications for the micturition reflex.

Key words: VR1, immunohistochemistry, sensory neurones, dorsal root ganglia

INTRODUCTION

The vanilloid receptor type 1 (VR1) protein functions both as a receptor for capsaicin and as a transducer of noxious stimuli [19]. VR1 is a non-selective cation channel expressed in about half the dorsal root ganglion neurones [9, 30]. The VR1-expressing sub-population of primary sensory neurones includes the majority of small and medium-sized cells that synthesise neuropeptides, such as substance P (SP) or calcitonin gene-related peptide (CGRP) and carbohydrates that bind isolectin B4 (IB4) [1, 19]. *In vitro*, VR1-expressing cells can be activated by protons (pH < 6) or heat (> 43°C) as well as by vanilloids, such as capsaicin or resiniferatoxin [9]. Recent

findings showing that mice lacking the VR1 gene do not respond to vanilloids and have reduced sensitivity to noxious heat and low pH confirmed that VR1 is responsible for vanilloid susceptibility and contributes to heat and proton sensitivity *in vivo* [14].

There has been experimental and clinical evidence to show the importance of VR1 in the lower urinary tract [5]. In humans this receptor has been detected in nerve endings of primary sensory neurones, smooth muscle and connective tissue cells [22]. Moreover, VR1 has also been detected in the urothelium of the rat urinary bladder [4]. A large proportion of sensory fibres innervating the urinary bladder are sensitive to capsaicin [7, 26, 31, 32].

Consistent with the nociceptive nature of capsaicin-responsive primary afferents, intravesical instillation of capsaicin has been shown to produce burning pain in humans [25] and pain-related behaviour [20] and induction of spinal c-*fos* expression in animals [11]. On the other hand, capsaicin-sensitive bladder afferents also appear to participate in micturition control. Intravesical capsaicin application induces detrusor contractions and reduces the bladder volume threshold for reflex micturition in humans [26] and rats [20]. In addition, capsaicin-sensitive bladder afferents contribute to a lowering of the volume threshold for reflex micturition in experimental animals subjected to bladder inflammation [24] or chronic spinal cord injury [15].

Previous studies have revealed that aged rats showed slight reductions in the densities of CGRP, SP and nitric oxide synthase innervation in all regions of the rat urinary bladder [28, 29]. In addition, in the lumbosacral dorsal root ganglia (DRG) the percentage of all of these peptides and NOS--immunoreactive (ir) neuronal profiles showed a significant reduction with age [29]. Moreover, attrition of both sympathetic neurones in the major pelvic ganglion of the rat and sympathetic innervation of the urinary tract [35] has been observed with increasing age. These changes in sympathetic innervation imply that a decrease in neuronal control of the urinary tract may take place with age and it is therefore important to investigate whether VR1 innervation is also affected by age. However, to date there have been no studies on the effects of ageing on VR1-ir neurones in the rat urinary bladder and the primary afferent neurones (L1-L2, L6-S1).

Therefore this study aimed to:
1. examine the distribution of VR1 in the male rat urinary bladder;
2. compare the distribution of VR1-ir axons between the urinary bladder of young adult (3 months) and aged (24 months) rats;
3. quantify the VR1-ir primary sensory neurones L1--L2 and L6-S1 DRG in both young and aged rats.

MATERIAL AND METHODS

Eight white male Wistar rats aged 3 months (young adult) and 24 months (aged) (n = 4 for each age group), maintained under conditions of constant temperature, humidity and lighting, were used in this study. The rats were deeply anaesthetised on a halothane anaesthetising unit (Fluorovac 240 V) by inhalation of 5% halothane in oxygen administered at 3 L/min. The animals were terminally anaesthetised by injecting 2 ml of Euthatal (20 mg/ml pentobarbitone sodium; Rhône Mérieux, Dublin) into the liver. A 2 ml syringe containing 1 ml Heparin (Multiheparin, 5000 U/ml; CP Pharmaceuticals) and 1 ml of 1% sodium nitrite (Fisons) was injected into the left ventricle. This was followed by perfusion of fixative solution of 4% paraformaldehyde in 100 mM sodium phosphate-buffered saline (PBS) intracardially at a higher pressure (116 ml/min). The DRG (L1, L2, L6 and S1) as well as the urinary bladder were dissected out and postfixed in 4% paraformaldehyde for 2 hours. After being rinsed in PBS, the tissues were stored overnight at 4°C in PBS containing 20% sucrose.

DRG were isolated by cutting the roots as close to the ganglion as possible. The base of the urinary bladder (which contains the trigone) was dissected out from the rest of the bladder tissues and the procedure for immunohistochemistry was followed. The base of the urinary bladder was selected for the study as it is a highly innervated region compared to the body and the dome (apex) regions [28, 35]. Transverse sections, including the whole bladder lumen, urothelium, muscular and adventitial layers, were collected from the lower end of the bladder base. Serial (10 μm) sections were collected onto slides coated with poly-L-lysine (Sigma, UK) and allowed to dry for 30 minutes. The sections were then incubated in PBS containing 5% normal swine serum (1 h), rinsed in PBS + 0.5% bovine serum albumin (BSA).

The sections were then incubated for 48 h at 4°C with polyclonal rabbit anti-VR1 (dilution: 1:2000) (Chemicon International, CA, USA). After being rinsed in PBS + 0.5% BSA (3 × 10 min), the sections were further incubated in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (Dakopatts Ltd) (dilution: 1:50) for 2 hours at room temperature. This was followed by rinsing in PBS + 0.5%

BSA and mounting in Vectashield antifade mountant. Control slides (sections were treated by omitting the primary antibody) showed no immunostaining. Finally, the slides were examined with a fluorescence microscope.

The density of the VR1-immunofluorescent neural elements was assessed semi-quantitatively by assigning values on the basis of a (–) to (+ + +) scale, where (–) represents lack of VR1-ir, (+) very sparse innervation, (+ +) sparse to moderate innervation and (+ + +) dense innervation. Regarding DRG neurones, only those with clearly visible nuclei and nucleoli were counted. Counts of all VR1-ir neurones and non-immunostained cells were made from three equally spaced (50 μm) sections for each young and aged rat DRG. This protocol was used to avoid counting any individual neurone twice. The average number of VR1-ir cells (Mean ± S.E.M) per ganglia was then calculated. The ratio of labelled/unlabelled neuronal profiles was presented as a percentage [6, 23]. In addition, the percentage was determined of VR1-ir cells in all young and all aged (L1, L2, L6 and S1) DRG. Statistical comparison between each level of young and aged DRG was performed using analysis of variance (ANOVA). Moreover, comparisons between total young and total aged VR1-ir cells were made using the unpaired t test. Values of p < 0.05 were considered statistically significant.

RESULTS

Age-related pigment was observed in all sections of the aged bladder. These pigments could be differentiated from the bright green FITC immunofluo-rescence by the highly intense granular brownish-yellow autofluorescence (Figs. 1B, 2C, D) which did not mask the immunofluorescence.

Nerve fibres were distinguishable from the background autofluorescence by the bead-like appearance of their varicosities and their bright fluorescence, which enabled the densities of immunoreactive nerves of different parts of the urinary bladder to be estimated semi-quantitatively in both age groups by assigning values on the basis of – to + + + (Table 1).

Urinary bladder base

Intraepithelial VR1-ir nerve fibres that ramified from subepithelial axons were often observed in the base of the urinary bladder in both age groups (Fig. 1A). These VR1-ir axons were slightly varicose. An increase in the number of subepithelial varicosities (running just under the epithelium) occurred more commonly among young animals. The lamina propria of the bladder base showed axons of similar shape with varicosities these were fewer in number. In the muscle layers most of the smooth muscle bundles were innervated by VR1-ir axons (Fig. 1A, B). There were many varicosities, which ran parallel to and inside the muscle bundles. These varicosities were also observed along most of the blood vessels (arteries and large veins) located in submucosal and muscular connective tissues. They had small varicosities and ran in a longitudinal and circular manner in the adventitial layer of the vessels. Serial sections of the bladder base showed no differences in the distribution of VR1 immunostaining between the trigone and other regions of the bladder base. While
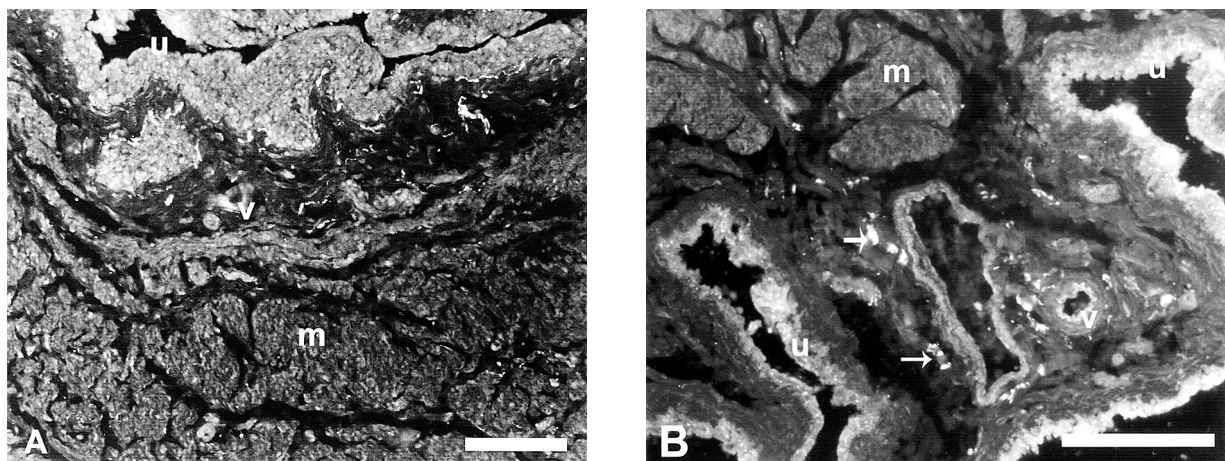
Figure 1. Young (**A**) and aged (**B**) rat urinary bladder base immunostained with VR1. Both age groups showed positive immunoreactivity to VR1. Age-pigment (arrows) was prominent mainly in the aged rat urothelium; m — muscle; u — urothelium; v: — vessel; → — age pigments. Scale bar: 400 μm.

Table 1. Semi-quantitative estimation of VR1-ir nerve densities in the urinary bladder base in young (3-month-old) and old (24-month-old) rats; +: sparse; + +: moderate; + + +: dense

	3 months			24 months		
Region	Subepith.	Muscles	Blood vessels	Subepith.	Muscles	Blood vessels
BASE	+ + +	+ +	+	+ +	+	+
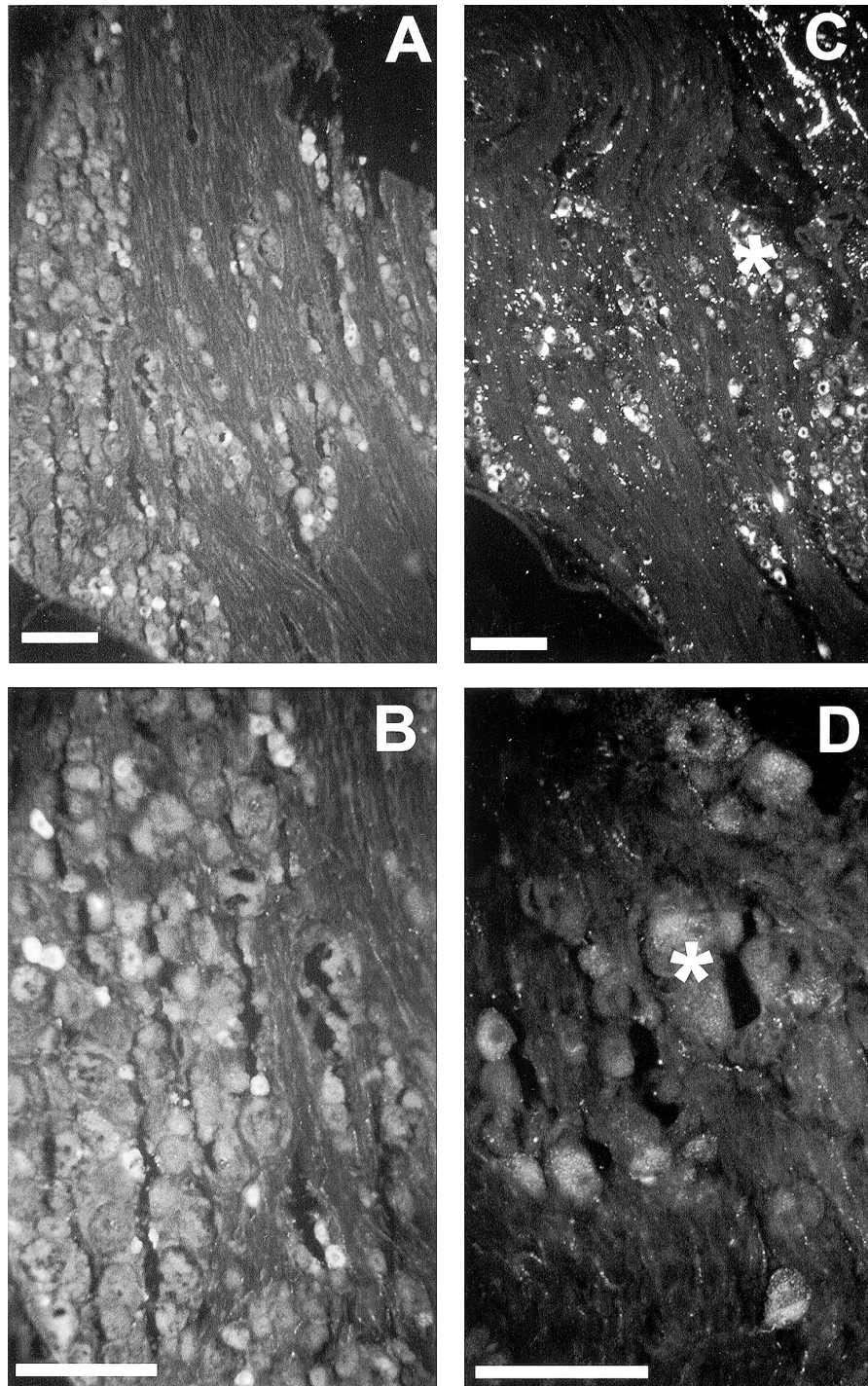
Figure 2. L1 (**A**) and L6 (**B**) DRG of the young rats showed more VR1-ir neuronal cell profiles (+) than those of the aged rat L1 (**C**) and L6 (**D**) DRG. VR1-ir was restricted to small and medium-sized neurones. Age pigments (*) were also noticed in all aged rat sections. Scale bar: 30 μm (A, C); 40 μm (B, D).

VR1-ir varicosities in the mucosa are free terminal endings close to the epithelial cells, in the muscular layer they encroach into a shallow groove in the surface of the smooth muscle fibres.

It is noteworthy that the overall pattern of innervation of the young rat urinary bladder base decreased with age (Table 1). The young rat subepithelial region of the urinary bladder showed dense VR1-ir nerve axons compared to only moderate innervations in the aged rat tissues. In addition, the muscular layers of the young rat urinary bladder base revealed moderate innervations of VR1. On the other hand, the aged rat bladder base showed only sparse VR1-ir nerve axons. However, blood vessels in both young and aged animals showed no remarkable changes in VR1 immunoreactivity. It was also observed that the aged rat bladder had long VR1-ir axons (varicosities) in the subepithelial region of the base, but these were short and less dense in the muscle layers (Figs. 1A, B).

Dorsal root ganglia

VR1-ir neuronal profiles were present in all sections of young and aged DRG (L1-L2, L6-S1). Within in the dorsal root ganglia VR1-ir was restricted to small (15–25 μm) and medium (25–40 μm) sized neurons. The uniformity of immunostaining within a perikaryon varied in young and aged animals. VR1-ir neurons occurred in a variety of patterns. Small cells were usually intensely stained, with the immunoreactivity homogeneously distributed within the cytoplasm (Figs. 2A, B, D). On the other hand, the immunostaining in the medium cells was usually weaker, whereby the immunoreactivity exhibited a patchy staining around the nucleus.

The quantitative data of the present study revealed that the mean percentage of young rat L1,

L2, L6 and S1 DRG neurones immunoreactive for VR1-ir showed a significant ($p < 0.05$) decrease with age (Table 2) in all DRG levels. L6 DRG showed the highest percentage of VR1-ir cells in both young (20%) and aged (13.4%) rats. Moreover, the mean percentage of VR1-ir cells in all aged DRG samples (12 ± 1.6) showed significant decreases ($p < 0.05$) compared to the mean (17.8 ± 2) in all young rat DRG (Table 2).

DISCUSSION

In this study we examined the distribution of VR1 in the male rat urinary bladder and lumbosacral (L1--L2 and L6-S1) DRG in both young and aged rats. These DRG were specifically chosen for study on the basis of previous observations which have shown that they are the main segmental levels of primary afferent neurones innervating the rat urinary bladder, the majority of which run in the pelvic nerve to L6-S1 [21]. In the present study, however, we found that the bladder base and its lumbosacral primary afferents were innervated by VR1-ir fibres.

The distribution pattern of nerve axons within the walls of the bladder base of the 3-months old rats observed in the present study is similar to that previously reported in young rats and young guinea pigs [2]. In addition, the distribution of the VR1-ir fibres underneath the epithelium and around the smooth muscle cells was similar to that of sensory neuropeptides such as SP CGRP in the rat bladder [4, 27, 29, 31, 32].

Changes in neuronal circuitry could lead to perturbation of the micturition reflex during ageing. The sub-epithelial localisation of VR1 fibres is consistent with the nociceptive function of VR1-expressing primary sensory neurones, whereas the intimate connection between VR1-expressing nerves and smooth muscle fibres suggests that the former play a role in mechanical sensitivity. As VR1 is not mechanosensitive, co-expression with a mechanotransducer could be another explanation.

Previous study has revealed the occurrence of VR1-ir nerve varicosities in shallow grooves on the surface of smooth muscle cells, suggesting a functional relationship between these profiles. In plain electron micrographs of the bladder such coupling has been interpreted as a possible neuromuscular junction between autonomic nerve fibres and smooth muscle cells [17]. Since at the periphery VR1 seems to be expressed exclusively in primary sensory neurones [33, 34], our results are suggestive that this coupling may have a mainly sensory function.

Table 2. Age-related changes in the percentage of L1, L2, L6 and S1 DRG neurones immunoreactive to VR1 in both young adult and aged rats; *$p < 0.05$

Percentage of VR1-ir neurons	3 months	24 months
L1	16%	10.2%
L2	18%	11.9%
L6	20%	13.4%
S1	17.4%	12.5%
(Mean±SD)	17.8 ± 2*	12 ± 1.6*

As previous data indicate that capsaicin-sensitive bladder afferents are activated by changes in the bladder volume [12, 13, 26, 34], it is reasonable to assume that the coupling between VR1-ir nerve endings and smooth muscle cells may act as a mechanoreceptor. However, the couplings may also serve as an area where sensory neuropeptides bind to neurokinin receptors which modulate detrusor smooth muscle cell contractility [3].

In the mucosa VR1-ir varicosities occurred as free terminal endings close to the base membrane of the epithelial cells or between the latter. Free terminal endings have previously been found in the human bladder mucosa [16], some of them showing immunoreactivity to SP [36]. As free nerve endings are usually associated with nociception [21], our morphological findings further strengthen the putative role of VR1-expressing nerve terminals in pain perception at the surface of the bladder.

The downregulation of VR1-ir cell profiles in the aged rat DRG raises the possibility that the direct nociceptive function of VR1 and/or the sensation or the transmission of sensory information from the urinary bladder may be compromised as age progresses. The present finding of a significant decrease with age in the percentage of VR1-ir neurons in (L1, L2, L6 and S1) DRG confirms previous reports on the effects of age on the percentage of SP-ir and CGRP-ir neurons in the rat DRG [6, 29]. This appears to be in line with the distribution of VR1-ir in unmyelinated C fibres [8], suggesting that the nociceptive function of VR1 may change with age.

Although the exact physiological role of VR1 in the nerves of the bladder is still uncertain, previous studies in humans [12, 13] and rats [4] have shown that intravesical administration of vanilloids decrease the mechanosensitivity of bladder primary afferents. In addition, intravesical vanilloids abolish reflex bladder contractions triggered by ice-cold water instillation [18]. This reflex is dependent on sensory input conveyed by C-fibres [10] and becomes overactive in certain pathological conditions of the human bladder [18]. However, VR1 is not sensitive to pressure [9] and under normal conditions only responds to temperatures above 43°C [10]. Therefore the loss of mechanical and cold sensitivity after intravesical vanilloid application can hardly be attributed to VR1 desensitisation. It is, however, possible that vanilloids interfere with the function of other receptors besides VR1. As intravesical vanilloids do not induce

terminal afferent degeneration [4], a P2X3 (ATP-activated receptors involved in pain sensation) functional impairment, to occur, should be attributed to post-translational changes induced by the increase of intracellular Ca^{2+} concentration that follows VR1 activation [4]. In addition, Ca^{2+} might also interfere with the function of other specific mechanosensitive ion channels already identified in capsaicin-sensitive fibres [7]. However, the contribution of these mechanoreceptors to bladder control is yet unknown. Alternatively, a general decrease in the bioelectrical activity of bladder primary afferent fibres after vanilloid instillation may contribute to the loss of mechanical and thermal sensitivity. Recent results indicate that capsaicin induces a VR1 activity-dependent block of different ionic currents, including voltage-gated Na^+ currents in DRG cells innervating the rat colon and voltage-gated Ca^{2+} currents in cultured DRG cells [32]. As both Na^+ and Ca^{2+} currents play a crucial role in the generation of the action potential in nociceptive primary sensory neurones [18], the vanilloid-induced reduction of these currents may decrease the bladder sensory input to the spinal cord.

CONCLUSION

In conclusion, the present study showed that VR1-ir fibres are mainly peptidergic and form varicose plexuses in the mucosa and in the muscular layer in the base of the rat urinary bladder. The sub-epithelial localisation is consistent with the nociceptive function of VR1-expressing primary sensory neurons. As capsaicin-sensitive bladder afferents are involved in nociception and reflex micturition control, the numerous free terminal nerve endings expressing VR1 in the mucosa seem more adequate to accomplish the former function. However, the reduction in VR1-ir nerve densities in the aged bladder base suggests that the nociceptive function of VR1 has been affected with age. In the DRG VR1 was found to belong almost exclusively to the peptide-containing sub-population of primary afferents. However, the reduction with age in the VR1-ir DRG neurons could also have important implications for micturition control.

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