γEpithelial Na+ Channel and the Acid-Sensing Ion Channel 1 expression in the urothelium of patients with neurogenic detrusor

γEpithelial Na^+ Channel and the Acid-Sensing Ion Channel 1 expression in the urothelium of patients with neurogenic detrusor overactivity.

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Objective

To investigate the expression of two types of cation channels such as the γEpithelial Na^+ Channel (γENaC) and the Acid-Sensing Ion Channel1 (ASIC1) in the urothelium of controls and in patients affected by neurogenic detrusor overactivity (NDO). In parallel, the urodynamic parameters were collected and correlated to the immunohistochemical (IHC) results.

Subjects and Methods

Four controls and 12 patients with a clinical diagnosis of NDO and suprasacral spinal cord lesion underwent to urodynamic measurements and cystoscopy. Cold cup biopsies were frozen and processed for immunohistochemistry and western blots. Spearman's correlation coefficient between morphological and urodynamic data was applied. One-way ANOVA followed by Newman–Keuls multiple comparison post-hoc test was applied for western blot results.

Results

In the controls, γENaC and ASIC1 were expressed in the urothelium with differences in their cell distribution and intensity. In NDO patients, both markers showed consistent changes either in cell distribution and labeling intensity compared to controls. A significant correlation between the higher intensity of the γENaC expression in urothelium of NDO patients and the lower values of bladder compliance was detected.

Conclusion

The present findings show important changes in the expression of γENaC and ASIC1 in NDO human urothelium. Of note, while the changes in γENaC might impair the mechanosensory function

of urothelium, the increase of the ASIC1 might represent an attempt to compensate excess in local sensitivity.

Keywords

Degenerin family. Spinal cord lesion. Cystoscopy. Urodynamic parameters. Immunohistochemistry. Western blot.

Introduction

The Degenerin/Epithelial Na^+ Channel (Deg/ENaC) family represents a new class of cation channels discovered at the early 2000s [1]. These channels are characterized by amiloride-sensitivity, and are either constitutively active or activated by mechanical stimuli and/or by ligands such as peptides or protons. Functionally, they are implicated in various sensory modalities [2]. An increasing body of evidence implicates the Deg/ENaC family in the control of bladder afferent excitability under physiological and pathological conditions [3].

In particular, ENaC behaves as a mechanosensitive receptor, having the ability to change its sodium transport properties following changes in hydrostatic pressure [3,4]. Three subunits (α or δ, β and γ) are required to form a constitutively active channel of ENaC [3,4]; however, channels with only the β and/or γ subunit have some physiologic functions [1]. In the rat bladder epithelium ENaC is implicated in the mechanosensory transduction by controlling stretch-evoked ATP release [4]. In the normal human bladder, the expression of ENaC was found extremely low [5], but was up-regulated in outlet obstruction, when it was characterized by detrusor instability.

The Acid-Sensing Ion Channels (ASICs), an H^+-gated subgroup of the Deg/ENaC family, are encoded by at least three different subunit genes, ASIC1, ASIC2 and ASIC3, and the protein subunits form homo- and hetero-multimeric channels, which differ in their pH sensitivity and other

pharmacological properties [1,6]. In the central and peripheral nervous system, ASICs have emerged as key receptors for extracellular protons and recent studies suggest diverse roles for these channels in the physiology of mechanosensation and the pathophysiology of bladder pain syndrome [7]. Kobayashi et al. [8] reported the presence of ASIC1 and ASIC2 in the urothelium and detrusor muscle of rat and mouse bladder; besides, in the rat urothelium the ASIC1 was the dominant subunit. In summary, the literature indicates that among the different ASIC proteins, ASIC1 seems to be the most represented in the urothelium. Furthermore, in the rat bladder, the expressions of ASIC2 and ASIC3 increased in the urothelium and suburothelial nerve plexus in cyclophospamide-induced cystitis, while ASIC1 expression was not modified [9]. It has been suggested that acid-induced Ca^{2+} influx and ATP release in the rat urothelium is partly due to ASIC activation [10]. Briefly, the distribution and role of ASIC in human bladder is not well understood at present. For all these reasons we presently focus our attention on ASIC1.

Neurogenic detrusor overactivity (NDO) is one of the most common disabilities reported in patients with spinal cord lesions (SCL). NDO is characterized by involuntary detrusor contractions during the filling phase and the symptoms include urinary frequency, urgency and incontinence [11,12]. Keeping into account the importance of the urothelium in micturition reflex [13], the possibility that changes in the expression of degenerin receptors in this tissue could participate to pathophysiology of NDO deserves to be investigated.

The aim of this work is to investigate the γENaC and ASIC1 expression in normal human urothelium and patients suffering of NDO.

Subjects and Methods

Patients and samples collection

Bladder samples from 4 controls (people suffering of episodic asymptomatic microscopic haematuria clinically negative for LUTS and for bladder cancer. When they undergone to urodynamic measurements showed normal results, see Table 2) and 12 patients with diagnosis of NDO associated with distal urethral sphincter dyssynergia by at least 5 years, were obtained by multiple cold cup biopsies during cystoscopy preceded by clinical and urodynamic evaluations. The NDO patients received antimuscarinic therapy and periodic (mean=8 months) intradetrusorial injections of 300 UI BoNT/A and underwent to intermittent catheterization. Biopsy was done when the BoNT/A treatment had exhausted its effectiveness, immediately before re-treatment. All the patients gave written consent and a local ethical committee approved the study after the study protocol was submitted to the competent authority. Cystoscopy and microscopic examination (by Haematoxylin-eosin staining, **Fig. 1**) of the bladder mucosa did not show alterations. No urinary infection was documented.

Morphological studies

The samples were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) pH 7.4, for 4–6 h at 4°C, cryoprotected in 30% sucrose at 4°C overnight (ON), washed in PBS, embedded

in killik cryostat medium (Bio-Optica, Milan, Italy) and frozen at –80°C. Further, sections, 8 µm thick, were obtained with a cryostat (Leica CM, Leica Microsystems, Milan, Italy), collected on polylysine coated slides. For western blot analysis, the samples were quickly frozen and stored at -80°C. The primary antibodies presently used were indicated either for immunohistochemical or western Blot investigations.

Immunohistochemistry (IHC). Sections were pre-incubated in 1,5% bovine serum albumin (BSA, Sigma Aldrich, Milan, Italy) in PBS with 0.5% Triton X-100 for 20 min at room temperature (RT), then incubated ON at 4°C with the primary antibodies diluted following the data sheet instructions in 0.5% Triton, 1.5% BSA in PBS. Next day, the sections were washed for 3x5 min in PBS and incubated for 2h at RT in the presence of secondary antibodies diluted in 0.5% Triton and 1.5% BSA in PBS. At the end of incubation, the sections were washed and mounted in an aqueous medium (Fluoremount, Sigma-Aldrich) (Antibodies are listed in Table 1). The immunoreaction products were observed under an epifluorescence Zeiss 6Axioskop microscope (Mannheim, Germany). Negative controls were performed omitting the primary antibodies.

Western blot (WB) Specimens from controls (n=3) and NDO patients (n=3) were minced and homogenized with a tissue homogenizer (Ing. Terzano, Milan, Italy) in cold-lysis buffer composed of: 10 mM Tris/HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 2 mM Na_2EDTA, 1% Triton X-100, added with 1X Sigmafast Protease Inhibitor cocktail tables (Sigma-Aldrich). Upon centrifugation at 13,000 *g* for 30 min at 4°C, the supernatants were collected and the protein content was measured spectrophotometrically using micro-BCA™ Protein Assay kit (Pierce IL, USA). The samples, each containing 70 µg of proteins and appropriate molecular-weight markers (Bio-Rad, Hercules, CA, USA), were electrophoresed by SDS-PAGE (200 V, 1h) using a denaturating 7.6% polyacrylamide gel and blotted onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, Italy; 150 V, 1 h). After washings in PBS containing 0.1% Tween (PBS-T, Sigma-Aldrich), the membranes were treated with T-PBS containing 5% BSA (Sigma-Aldrich) at RT for 2h and

incubated ON at 4°C while being stirring, with the primary antibodies diluted following the data sheet instructions. Immune reaction products were revealed by incubating membranes in peroxidase-conjugated anti-rabbit and anti-goat for 1 h at RT (Table 1). Immunoreaction was detected by ECL chemiluminescence reagent (Immun-Star^{TM} HRP Chemiluminescent Kit, Bio Rad). The WB runs were stripped for 20 min at RT with Stripping buffer (Thermo scientific, Rockford, IL, USA) and then immunostained with rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) assumed as the control housekeeping protein. The immunoreactive bands were revealed using ImageQuant 350 Imager (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

The presence of a correlation between morphological and urodynamic data was evaluated by using the Spearman's correlation coefficient (GraphPad Prism, Version 4.0 San Diego, CA, USA). Under fluorescence microscope two of the authors (MGV and CT), blind to each other, made a semi-quantitative analysis of the γENaC and ASIC1 labeling. Five slices and ten different fields/slice under a 40x objective for each control (n=4) and patient (n=12) were evaluated. The intensity of the labeling was expressed as follow: +-- = low intensity, ++- = medium intensity, +++ = high intensity and the evaluation made by the two observers were averaged. WB quantitative analysis was performed by computer-assisted densitometry, each band corresponding to a single specimen (n=3 each group), using the QuantityOne analysis software (Bio-Rad). Statistical significance among the experimental groups was evaluated by one-way ANOVA followed by Newman–Keuls multiple comparison post hoc test (Prism 3.0, GraphPad Software, Inc. S. Diego, CA, USA); $p < 0.05$ was considered significant.

Results

Clinical and urodynamic results

Clinical data of controls and NDO patients are summarized in Table 2. The urodynamic values of controls and NDO patients are reported in Table 3. In 11 patients the mean reflex volume was 255 ml (range: 130-450 ml); mean maximum detrusor pressure 36.9 cm H_2O (range: 9-50); mean maximum cystometric capacity 375 ml (range: 200-460). Although half of the NDO patients actually had normal cystometric capacity, we decided to re-treat patients according to the following variables: > 1 urinary incontinence episodes within 2 days, low compliance and reduction reflex volume. One patient (Pz3), in spite of the subjective symptoms reported, showed urodynamic results similar to controls and, indeed, he was not re-treated with BoNT/A.

Immunohistochemistry

γENaC-immunoreactivity (IR). (Fig.2A-E) In the controls (**A**), the IR was very faint and detectable only on the luminal portion of the dome cells. The labeling was made by small granules either scattered in the cytoplasm or distributed along the plasma membrane. In the NDO patients (**B**), the labeling was detected also in the more superficial club cells and in some patients (see Table 3) the IR appeared more intense, especially in the dome cells (**C**). In the patient with the diagnosis of multiple sclerosis (**D**), the IR was very intense and distributed in the cytoplasm of all the cell types, being particularly intense in the dome cells. Some IR nerve fibers were sporadically visible in the deepest layers of the urothelium (**A,D**). When the primary antibody was omitted, any specific labeling was detected (**E**).

ASIC1-IR. (Fig.2F-L) In the controls (**F**), the IR was present in all the urothelial cell types. The club cells were particularly rich in the labeling that appeared as a thin ring along the plasmalemma made by brilliant small granules or short bars. Few and lighter IR granules were also scattered in the cytoplasm of these cells as well as in the basal ones. In the dome cells the labeling was barely

detectable and appeared as small granules accumulated on the luminal cytoplasmic portion. In NDO patients (**G**), the IR had a cell distribution similar to controls but extended also to the dome cells. In some patients (see Table 3), the IR appeared more intense, especially in the dome cells, where it was distributed either along the plasma membrane or in the cytoplasm (**H**). In the patient with a diagnosis of multiple sclerosis (**I**), the labeling showed intensity and distribution similar to the other patients. Some IR nerve fibers were sporadically recognizable in the deepest layers of the urothelium (**E**,**F**). When the primary antibody was omitted, any specific labeling was detected (**L**).

Western blotting

Western blot analysis in controls as well as in patients, ASIC1 polyclonal antibody identified a single band at 54 kDa and the γENaC polyclonal antibody recognized a single band at 82 kDa (**Fig.3A**); both molecular weights corresponded to those expected compared with the literature data. Quantification of the results, by measuring the optical density of each band/group (3 specimens/each group), showed no change between control and NDO patients (**Fig.3B**).

γENaC and ASIC1 expression and urodynamic data

The Spearman's correlation coefficient method showed a significant correlation between the higher intensity of the γENaC expression in urothelium of NDO patients and the lower values of bladder compliance ($r = 0.75$, $p<0.01$). On the contrary, no significant correlation was seen when the γENaC and ASIC1 expression changes were evaluated vs the other urodynamic parameters.

Discussion

Our findings confirmed the presence of both γENaC and ASIC1 in normal human urothelium and, for the first time, showed changes in their cellular distribution in patients with urodynamically proved neurogenic detrusor overactivity. Moreover, the changes in γENaC labeling intensity correlate with the bladder compliance impairment. Finally, WB analysis proved antibody specificity.

Presently, γENaC labelling in controls was faint and limited to the dome cells where it appeared as small cytoplasmic granules mostly concentrated in the portion facing the bladder lumen. This finding is in agreement with literature data in laboratory mammals. In rat bladder epithelium and in other organs, ENaCs labelling was seen localized in the cells facing the lumen, along the apical membrane or in the sub-apical cytoplasm corresponding to a population of intracellular vesicles [14,15]. In particular, in the epithelium of collecting ducts of rat kidney, immune electron microscopy showed γENaC subunit in these vesicles [16]. Conversely, Araki et al. [5], in human bladder urothelium found a labelling only for the βsubunit. This discrepancy could be due to the different procedure for specimen preparation and/or to the different antibody used. In NDO patients the γENaC labelling was increased in the dome cells and its distribution was extended also to the immediately underlying club cells. Interestingly, the changes in the γENaC labelling intensity significantly correlated with the lower bladder compliance recorded in NDO patients. Araki et al. [5] reported an increase in all ENaC subunits expression in the urothelium of human urinary bladder affected by detrusor overactivity (DO) due to bladder outlet obstruction [5] and interpreted this over-expression as the cause for the DO. This possibility could also be taken into consideration for the present findings in NDO; however, it remains unclear which is/are the mechanism(s) implicated. Taken into account the important role played by this cation channel in the micturition reflex, it is likely that its over-expression could compromise this reflex by lowering the

threshold to the stretching during filling as well as to the hydrostatic pressure [17]. This hypothesis is in agreement with a role of ENaCs as mechanosensors in the urinary bladder [13]. Interestingly, the sensitivity to hydrostatic pressure involves mainly if not only the dome cells [17], the cells where we reported the most remarkable changes in the γENaC expression.

In controls, ASIC1 expression showed notable differences in cell distribution and intensity compared to γENaC, since its labelling was present and intense in the basal and club cells and barely appreciable in the dome cells. Studies in rodents [8] and humans [7] showed that ASIC1 was the prominent isoform expressed in the urothelium and ASIC1 expression in mouse urinary bladder was higher in males compared to females [8]. Interestingly, in the NDO patients the ASIC1 expression was consistently increased in the dome cells and, in some cases, the labelling was more intense than in the other cell types. At variance, in other diseases affecting the bladder sensitivity such as the bladder pain syndrome (BPS)[7] and a rat model of CYP-induced cystitis [9] it has been reported that the ASIC1 expression in the urothelium was unchanged. This discrepancy might depend on the different pathogenesis that characterized the NDO and the BPS. In particular, NDO is commonly associated with detrusor hypertrophy a condition very rare in BPS patients. Intriguingly, studies on gastrointestinal sensation indicate that the disruption of ASIC1 increases the mechanical sensitivity [18,19] suggesting that ASIC1 serves as a suppressor in visceral sensation. In regard, the lower sensitivity to intravesical acidic irritation observed in male mice might depend on the greater expression of ASIC1 in their bladder epithelium [8,20]. Thus, ASIC1 in the bladder epithelium might be implicated in sensory transduction mediating an increase in the threshold to local changes in hydrostatic pressure and/or urine acidity and the increase in ASIC1 expression in NDO patients might represent an attempt to avoid an excessive lowering of this threshold. The possibility that the BoNT/A treatment might affect the expression of the receptors presently investigated cannot be excluded *a priori*. In a recent review it was reported that either short or long term BoNT/A treatment did not alter the histological properties of the bladder wall [21]. Studies in a animal model

of detrusor overactivity (DO) [22] as well as in humans affected by NDO or idiopathic overactivity (IDO) (23) demonstrated that BoNT/A treatment prevented the changes in TPVR1, M1 and M3 expression, and this action was related to the beneficial effects of the toxin. Therefore, we can suppose that when the drug benefits are extinguished, as in our patients, also the potential effects of BoNT/A on the receptor expression are lacking.

Nevertheless, this study may pose some limitations. We focused only on ASIC1 and not on ASIC2 and 3. The choice was made on the basis of the literature data [8,9] and we are aware that the present study represents the first step in this field of investigations. Furthermore, the biopsies used for WB were not dissected in order to separate the urothelium from the detrusor. Thus WB results cannot be claimed to be specific for the urothelium as we cannot exclude the presence of few smooth muscle cells of the detrusor. Finally, although immunohistochemically we could verify that there were also positive nerve fibers to both the antibodies, they were quite few. Therefore, because of the scarcity of the contribution of the nerve/muscle component, we considered also the WB results reliable.

In conclusion, the present study represents the first report on the expression of the Degenerin channels in neurological human bladder urothelium. The changes in intensity and distribution of the two channels in NDO likely interfere with the local generation of afferent outputs. Noteworthy, while the changes in γENaC expression might cause a lowering of the urothelium threshold to local stimuli (i.e. hydrostatic pressure, pH), therefore worsening the sensory function, the increase of the ASIC1 in the dome cells might represent an attempt of the urothelium to compensate the local hypersensitivity.

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Conflict of interest: The authors declare that they do not have any conflict of interest.

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Legends to illustrations

Figure 1. Hematosylin-eosin (H&E) staining shows the typical organization of the transition epithelium in basal, club and dome cells. Arrows indicate some plasmacells. V = vessels, Bar = 20 μm.

Figure 2. γEpithelial sodium channel (γENaC)-immunoreactivity (IR) and Acid sensing ion channel 1 (ASIC1)-IR in the urothelium of controls (**A,F**) and NDO patients (**B-E,G-L**). In controls, **γENaC-IR** (**A**) is faint and limited to the dome cells where it appears as small granules in the proximity of the plasma membrane facing the lumen (arrows). In the NDO patients (**B),** the IR extends to the neighbor club cells (arrows) with a distribution similar to controls. In some patients (**C**), the IR is more intense compared to the other patients, especially in the dome cells where it is distributed in the entire cytoplasm (arrows). In multiple sclerosis (**D**), the **γENaC-IR** is located in the cytoplasm of all cell types, has a granular aspect and is intense, especially in the dome cells (arrows). Arrowheads indicate IR nerve fibers. When the primary antibody is omitted no labeling is detectable (**E**). In controls, **ASIC1-IR** (**F**) is mainly detectable in the club and basal cells and appears as small brilliant granules and short bars located along the plasma membrane; besides, small and fainter granules are scattered in the cytoplasm. The dome cells are barely labeled and the IR is present on the luminal portion of the cytoplasm (asterisks). In the majority of the patients (**G),** the IR is similar in intensity and cell distribution to controls, but it can be detected in all epithelial cells, dome cells included (arrows). In some patients (**H**), the IR is more intense and located also in the cytoplasm. In multiple sclerosis (**I**), the **ASIC1-IR** is intense, located in all cell types and distributed either along the plasma membrane or in the cytoplasm. When the primary antibody is omitted no labeling is detectable (**L**). dc=dome cells; cc=club cells; bc=basal cells. Bar=20 μm.

Figure 3. Western blot of Acid sensing ion channel 1 (ASIC1) and γEpithelial sodium channel (γENaC). In controls and NDO patients, each antibody recognizes a single band with a molecular weight (mw) of 54 (ASIC1) and 82 (γENaC) kDa, respectively (**A**). Both mw are in agreement with the literature data. No differences in the band intensity are appreciable between controls and NDO patients. Quantitative analysis (**B**) of the bands, expressed as % of the GAPDH band, showed no significant change in band density in NDO patients compared to controls, $p>0.05$, (One way Anova followed by Newman-Keuls multiple comparison test).

Table 1. List of primary and secondary antibodies.

Primary antibodies	**Host**	**Dilution IHC-WB**	**Producer**
Anti-ASIC1(H-70)	Rabbit	1:250 – 1:2000	Santa Cruz Biotechnology, Dallas, USA
Anti-γENaC	Goat	1:250 – 1:200	Santa Cruz Biotechnology
Anti-GAPDH	Rabbit	1:3000	Santa Cruz Biotechnology
Secondary antibodies	**Host**	**Dilution IHC**	**Producer**
Anti-Rabbit	Goat	1:333	Invitrogen, San Diego, CA, USA
Anti-Goat	Donkey	1:333	Invitrogen
Secondary antibodies	**Host**	**Dilution WB**	**Producer**
Anti-Rabbit	Goat	1:20 000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Anti-Goat	Donkey	1:5 000	Santa Cruz Biotechnology

Table 2 Clinical data

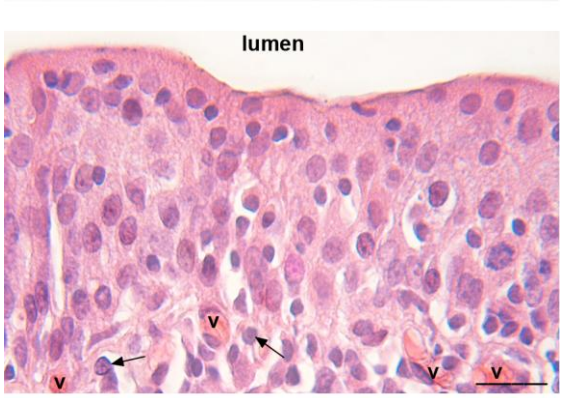
Subjects	age	sex	Pathology	Site	Extension	NDO	Treatment
Crtl 1	63	female	-	-	-	-	-
Crtl 2	73	female	-	-	-	-	-
Crtl 3	78	male	-	-	-	-	-
Crtl 4	80	male	-	-	-	-	-
Pz 1	44	male	SCL	D6	complete	yes	BoNT/A
Pz 2	27	male	SCL	D4-D5	complete	yes	BoNT/A
Pz 3	57	male	SCL	D4-D5	complete	yes	BoNT/A
Pz 4	40	male	SCL	D4-D5	complete	yes	BoNT/A
Pz 5	69	male	SCL	D12	incomplete	yes	BoNT/A
Pz 6	21	male	SCL	C6	complete	yes	BoNT/A
Pz 7	63	female	MS	Suprapontine Suprasacral	EDSS 5	yes	BoNT/A
Pz 8	24	male	SCL	D10-L2	complete	yes	BoNT/A
Pz 9	53	male	SCL	D2-D3	complete	yes	BoNT/A
Pz 10	22	male	SCL	C5-C6	complete	yes	BoNT/A
Pz 11	46	male	SCL	D5-D6	complete	yes	BoNT/A
Pz 12	26	male	SCL	D12	complete	yes	BoNT/A

SUI= stress urinary incontinence SCL= spinal cord lesion; MS= multiple sclerosis

Table 3. Urodynamic parameters and IR intensity.

Subjects	CC ml	Compliance ml/cm H_2O	Reflex volume (ml)	MDP cm H_2O	ASIC1-IR intensity	γENaC-IR intensity
Ctrl 1	404	101	N/A	4	+ + -	+ - -
Ctrl 2	380	47,1	N/A	8	+ + -	+ - -
Ctrl 3	450	75	N/A	6	+ + -	+ - -
Ctrl 4	400	82	N/A	5	+ + -	+ - -
Pz 1	250	12	180	15	+ + -	+ - -
Pz 2	500	62,5	N/A	8	+ + -	+ - -
Pz 3	370	7,5	300	40	+ + -	+ - -
Pz 4	200	2,6	130	50	+ + +	+ + -
Pz 5	500	30	450	40	+ + +	+ - -
Pz 6	300	5	250	50	+ + +	+ + -
Pz 7	250	6,6	200	30	+ + +	+ + +
Pz 8	420	5,5	190	35	+ + -	+ + -
Pz 9	400	6,25	250	40	+ + -	+ - -
Pz 10	250	5	200	40	+ + -	+ - -
Pz 11	350	22.2	200	9	+ + -	+ - -
Pz 12	460	10	200	20	+ - -	+ - -

CC=cistometry capacity; MDP=maximal detrusor pressure; N/A= not applicable.



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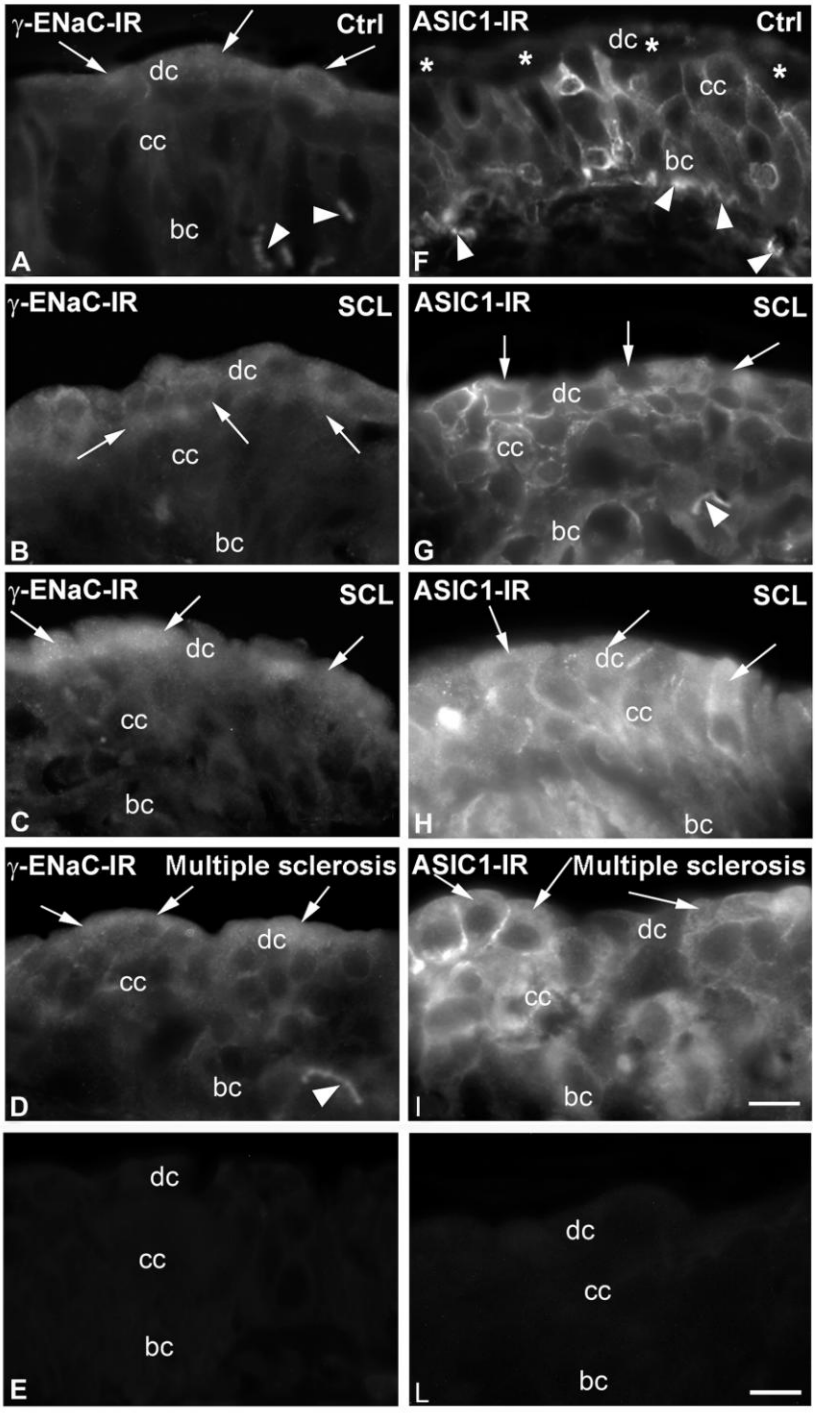

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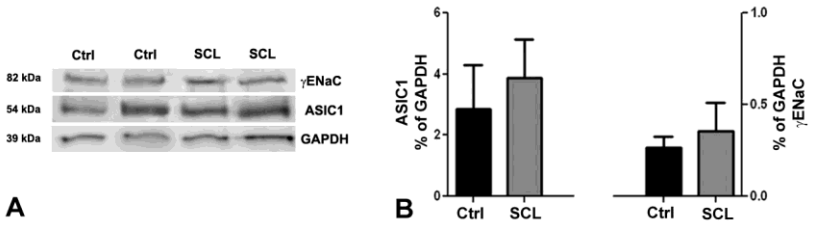

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