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## The opiorphin gene (*ProL1*) and its homologues function in erectile physiology

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### Abstract

**OBJECTIVE**—To determine if *ProL1*, a member of the opiorphin family of genes, can modulate erectile physiology, as it encodes a peptide which acts as a neutral endopeptidase inhibitor, other examples of which (*Vcsa1*, *hSMR3A*) modulate erectile physiology.

**MATERIALS AND METHODS**—We cloned members of the opiorphin family of genes into the same mammalian expression backbone (pVAX); 100 µg of these plasmids (pVAX-*Vcsa1*, -*hSMR3A*, -*hSMR3B* and -*ProL1*) were injected intracorporally into retired breeder rats and the affect on erectile physiology assessed visually, by histology and by measuring the intracavernous pressure (ICP) and blood pressure (BP). As a positive control, rats were treated with pVAX-*hSlo* (expressing the MaxiK potassium channel) and as a negative control the empty backbone plasmid was injected (pVAX). We also compared the level of expression of *ProL1* in corporal tissue of patients not reporting erectile dysfunction (ED), ED associated with diabetes and ED not caused by diabetes.

**RESULTS**—Gene transfer of plasmids expressing all members of the opiorphin family had a similar and significant effect on erectile physiology. At the concentration used in these experiments (100 µg) they resulted in higher resting ICP, and histological and visual analysis showed evidence of a priapiclike condition. After electrostimulation of the cavernous nerve, rats had significantly better ICP/BP than the negative control (pVAX). Gene transfer of pVAX-*hSlo* increased the ICP/BP ratio to a similar extent to the opiorphin homologues, but with no evidence for a priapic-like condition. Corpora cavernosa tissue samples obtained from men with ED, regardless of underlying causes, had significant down-regulation of both *hSMR3A* and *ProL1*.

**CONCLUSION**—All members of the human opiorphin family of genes can potentially modulate erectile physiology. Both *hSMR3* and *ProL1* are down-regulated in the corpora of men with ED, and therefore both genes can potentially act as markers of ED.

### Keywords

*Vcsa1*; *hSMR3*; *ProL1*; opiorphin; erectile dysfunction; priapism

## INTRODUCTION

We reported recently that the gene *hSMR3A* is down-regulated in patients with erectile dysfunction (ED) [1]. Gene transfer of plasmids expressing *hSMR3A* into the corporal tissue of ageing rats improved erectile function at lower doses and caused priapism at higher doses, suggesting a direct involvement of this gene in erectile physiology. *hSMR3A* belongs to a family of genes which include *Vcsa1* (in the rat) and *ProL1* (in humans), which encode the

pentapeptides sialorphin and opiorphin, respectively (Table 1). *hSMR3A* has a close homologue called *hSMR3B*, which does not affect the region that encodes the hypothetical pentapeptide product (hSMR3).

The expression of this family of genes appears to be restricted to relatively few tissues. They were first isolated in the submanibular gland, but subsequently have been shown to be expressed in corporal tissue [2,3]. There is accumulating evidence to suggest the opiorphins are important in mammalian sexual function. There is a marked genderspecific expression, with male rats expressing 1000 times more of the *Vcsal* transcript in the submandibular glands than female rats [4]. Male rats injected with sialorphin 45 min before being paired with sexually receptive females had a dose-dependent ability to modulate, at doses related to physiological circulating levels, the male rat mating pattern, exerting a dual facilitative or inhibitory dose-dependent effect on the sexual performance, while stimulating the apparent sexual arousal or motivation [5]. Expression of both *Vcsal* in rats and *hSMR3* in humans is down-regulated in response to ED [1,3]. Intracorporal injection with a plasmid expressing *hSMR3*, *Vcsal* or the peptide, sialorphin, can increase erectile function (as determined by an increase in intracorporal pressure/blood pressure (ICP/BP) in retired breeder rats after electrostimulation) [1,3,6]. If large amounts of the plasmid encoding sialorphin or hSMR3 are injected into the rats they will cause a 'priapic-like' state in the rat penis.

Sialorphin acts as a potent neutral endopeptidase (NEP) inhibitor in rats, and human opiorphin has been shown to have similar activity [7,8]. NEPs are located at the surface of cells in neural and systemic tissues where they have important functions as ectoenzymes, catalysing the postsecretory processing or metabolism of several signalling peptides, and their inhibition can prolong the binding of peptide agonists to their receptors [9]. Signal peptides are involved in the receptor-dependent control of many physiological functions, e.g. central pain perception and depression, and peripheral inflammatory phenomena, arterial tone and mineral homeostasis. Particularly relevant to erectile physiology is the observation that inhibitors of NEP can cause relaxation of smooth muscle tissue [10,11]. Relaxation of corporal smooth muscle tissue is a prerequisite for erectile function in male animals [12,13]. Sialorphin appears to function in erectile physiology through its action as a NEP inhibitor, by modulating the tone of corporal smooth muscle tissue [6].

Opiorphin, encoded by *ProL1*, was recently shown to also act as a NEP inhibitor [8]. Therefore we determined if intracorporal gene transfer of plasmids expressing *ProL1* (pVAX-ProL1) had a similar effect on erectile physiology as plasmids expressing *Vcsal* and *hSMR3A*. Because the gene encoding hSMR3 is down-regulated in human patients with ED we also determined if *ProL1* could also act as a marker for ED.

## MATERIALS AND METHODS

Experiments to determine the function of the opiorphin homologues in erectile physiology were carried out in 30 9- to 10-month-old (old) retired breeder male Sprague-Dawley rats (>500 g). Vectors/plasmids were microinjected into rat corporal tissue essentially as previously described [1,3]. Briefly, the rats were anaesthetized by an i.p. injection with pentobarbital sodium (35 mg/kg). The perineum was shaved using an electric shaver, cleaned with betadine solution (povidoneiodine, 10%) and 1-cm incision was made at the base of the corpora to expose the left crura. All micro-injections consisted of a single bolus of naked plasmid DNA into the left crura, using an insulin syringe (1/2 mL 28 G1/2). The total volume of all microinjections was 150  $\mu$ L. The plasmid pVAX was obtained commercially (Invitrogen, Carlsbad, CA, USA). Construction of the plasmid pVAX-hSlo, pVAX-Vcsal, and pVAX-hSMR3B was described previously [1,3,14]. To construct pVAX-ProL1, the pVAX vector and pCMV6-XL5-PROL1 vector (OriGene, Rockville, MD, USA) were digested with NotI/EcoRI.

The NotI/EcoRI fragment from pCMV6-XL5-PROL1 vector was then ligated to the NotI/EcoRI site of pVAX1 vector. To construct pVAX-hSMR3A vector, pCMV6-XL5-SMR3A vector (OriGene) was digested with NotI/XbaI. The hSMR3A fragment was then cloned in the NotI/XbaI-digested pVAX vector. The plasmids pVAX1-hSMR3A and pVAX1-ProL1 were verified by DNA sequencing.

At 1 week after gene transfer the rats were anaesthetized by i.p. injection with pentobarbital sodium (35 mg/kg) and the ICP/BP determined before and during electrostimulation of the cavernous nerve, using the method described previously [1,3]. Briefly, an incision was made in the perineum, and a window was made in the ischiocavernosus muscle to expose the crus of the corpus cavernosum. The cavernous nerves were identified on the posterior lateral surface of the prostate gland. The cavernous nerve was electrostimulated directly using a delicate stainless-steel bipolar hook electrode attached to the multijointed clamp. Each probe was 0.2 mm in diameter; the two poles were separated by 1 mm. Monophasic rectangular pulses were delivered by the signal generator (custom-made and with built-in constant current amplifier; Grass Instruments, Astro-Medical Inc., Quincy, MA, USA). Stimulation parameters were 20 Hz, pulse width 0.22 ms and duration 1 min; current was applied at 0.75 and 4 mA. The changes in the ICP and BP were recorded at each level of neurostimulation, and compared with the negative control (pVAX, the empty parent vector). The mean (SD) ICP/BP and ANOVA were calculated for each of the treatment groups; differences from the negative control of  $P < 0.05$  were considered statistically significant.

Human corporal tissue was procured from 10 patients during penile prosthetic implant surgery, according to protocols approved by the AECOM/Montefiore Hospital Internal Review Board. The conditions and ages of the patients were reported previously [1]. Briefly five patients had ED associated with diabetes, five had ED and were not diabetic and three control patients did not report ED.

For quantitative reverse-transcriptase (RT) PCR of *ProL1* and *hSMR3*, total RNA was extracted from human frozen tissue, RNA prepared and quantitative RT-PCR performed as previously described [1]. The expression of transcripts was analysed using the comparative crossing threshold ( $C_t$ ) method (also known as the  $2^{-\Delta\Delta C_t}$  method). The primers used for normalization (glyceraldehyde phosphate dehydrogenase) and *hSMR3* were previously described. The primers used for *ProL1* determination were: forward primer: 5'-GCTCTTATTTTCATGTTTCACACCCAG-3'; reverse primer: 5'-TAACCCGGAAAGAGTCGAGGT-3'.

## RESULTS

We previously reported that plasmids expressing *Vcsa1* and *hSMR3A* result in improved erectile function at low doses and also cause priapism at higher doses [1,3]. We also reported that gene transfer of pVAX-hSlo improves erectile function with no evidence of priapism, even at doses of up to 1000  $\mu$ g [15]. We investigated if a plasmid expressing the gene encoding *ProL1* or *hSMR3B* (a splice variant of *hSMR3A*, in which the region of the gene encoding the hypothetical pentapeptide is unaltered) would modulate erectile physiology in a similar manner to *Vcsa1* and *hSMR3A*. At 1 week after intracorporal injection with the plasmids (pVAX, pVAX-hSlo, pVAX-hSMR3A, pVAX-hSMR3B, pVAX-ProL1 and pVAX-Vcsa1) the rats were examined visually for signs of priapism (postmortem, Table 2) and the basal ICP/BP was determined in the rats (Fig. 1).

Evidence for priapic episodes were either visually apparent penile tumescence or vasocongestion of corporal tissue confirmed by postmortem histological confirmation. None of the rats in the control groups (treated with pVAX and pVAX-hSlo) had any evidence of

priapism (Table 2). In all groups treated with the opiorphin homologues, at least two of five rats had visual evidence of priapism. This was previously reported for *Vcsa1* and *hSMR3A* [1,3], but not for *ProL1* or *hSMR3B*.

The visual observation of priapism was supported by the basal ICP/BP. All the genes expressing opiorphin homologues (*hSMR3A*, *hSMR3B*, *ProL1* and *Vcsa1*) caused a higher basal ICP than the empty vector controls. pVAX-hSlo, which we previously showed to be effective in improving ED in ageing rats, but has never caused priapism, gave no significant difference in resting basal ICP/BP from the empty vector controls.

After measuring the basal ICP/BP the cavernous nerve of the rats was electrostimulated with 0.75 and 4 mA, and the effect on erectile physiology monitored visually (Table 2) and by measuring the ICP/BP. In the negative control rats (treated with pVAX, the empty vector) none at the lower or higher level of stimulation had an observable full erection. With pVAX-hSlo, three of five at 0.75 mA and all five rats at 4mA had a full erection. The rats treated with plasmids expressing the opiorphin homologues had visually better erections than had control rats treated with pVAX after electrostimulation of the cavernous nerve.

The visual effects on erectile function were confirmed by measuring the ICP/BP response after electrostimulation (Fig. 2). The plasmid pVAX-hSlo (hMaxiK) is presently being evaluated in clinical trials for treating ED, and we have several reports showing that the plasmid can improve erectile function in rats. This plasmid, when transferred into rats, gave a better erectile function than the negative control (pVAX) at both stimulations. In addition, despite evidence of priapism, there was a significant increase in the ICP/BP ratio with plasmids expressing opiorphin and its homologues (pVAX-*Vcsa1*, pVAX-*SMR3A*, pVAX-*SMR3B* and pVAX-*ProL1*) after stimulation. Interestingly, although rats treated with pVAX-*ProL1* developed an ICP/BP ratio normally indicative of a full erection, these rats were unable to maintain a full erection and were recorded visually as having only a partial erection.

Visual postmortem examination of the corpora of rats that had intracorporal injection of pVAX-*ProL1* had evidence of vascular congestion of the corporal tissue, suggesting they had had a 'priapic-like' episode. This was confirmed through histopathological examination of rats injected with 100 µg pVAX-*ProL1*. Trabecular interstitial oedema, blood clot formation within the cavernosa, and destruction of the endothelial lining was apparent with microscopy, suggesting that priapism had been severe in the 1 week between injection of the plasmid and termination of the experiment. As previously reported, this observation is similar to the effects of treating rats with pVAX-*Vcsa1* or pVAX-*hSMR3A* [1,3]. By contrast, the plasmid pVAX-hSlo has never been observed to cause priapism after intracorporal injection, even at doses as high as 1000 µg [15]. In addition, rats treated with pVAX-*ProL1* had marked hypertrophy and hyperplasia, and minimal inflammation in the region of the hyperplastic smooth muscle, which was interpreted to be the result of minimal degeneration of the hypertrophic cells. Neither negative control rats nor rats injected with the same dose of pVAX-hSlo showed any signs of priapism (Fig. 3).

We previously showed that *hSMR3* expression is down-regulated in patients with ED. Using the same tissue samples described in [1] we analysed the expression of *ProL1* and compared the changes in expression to those previously reported for *hSMR3* [1]. In patients with ED there was a statistically significant nearly 10-fold decrease in the levels of *ProL1* expression similar to the effect of ED on *hSMR3* expression (Fig. 4). The effect on the gene expression was independent of whether the ED aetiology was diabetes-associated or not. These results suggest that all members of the opiorphin family of genes are potential markers of ED in patients.

## DISCUSSION

The major conclusion of this study is that a third member of the opiorphin family of genes (*ProLI*) can modulate erectile physiology. We previously showed that changes in the expression of another human member of this gene family (*hSMR3A*) and a rat homologue (*Vcsa1*) modify normal erectile physiology [1,3]. In addition we now provide evidence that not only *hSMR3* [1], but also *ProLI* can act as a marker for ED in human patients.

The rat homologue of the opiorphin family of genes, *Vcsa1*, was previously shown to be down-regulated in several aetiological models of ED, a rat model for ageing, a neurogenic model (cavernous nerve transection) and streptozotocin-induced diabetes [3,16]. In humans reporting ED, *hSMR3* is down-regulated compared to patients without ED, and the present study shows that *ProLI* is also down-regulated in patients with ED. In the rat submandibular gland, transcriptional regulation of rat *Vcsa1* expression is under adrenergic control [2,4] and the release of the mature pentapeptide product, sialorphin, is regulated by adrenaline [17]. It is possible that in humans both *hSMR3* and *ProLI* are under the same regulatory mechanisms in corporal smooth muscle tissue. The expression of opiorphin family genes in corporal tissue might therefore be secondary to other factors that result in ED, e.g. the down-regulation of androgens that occur with ageing and diabetes [18] or decreases in the levels of neurotransmitters that occur with nerve damage or transection.

It was reported that the mature pentapeptide gene products of *ProLI* and *Vcsa1* (opiorphin and sialorphin, respectively) act as NEP inhibitors [7,8]. We showed that sialorphin can relax corporal smooth muscle tissue in organ-bath studies by a mechanism involving its action as an NEP inhibitor [6]. Given the similarity in the peptides generated by the opiorphin homologues it seems likely that all the members of the opiorphin family of genes exert their effect through a mechanism which involves inhibition of NEP. We recently noted that *Vcsa1* is involved in the regulation of G-protein-coupled receptor expression, and therefore the involvement of the opiorphin family of genes in ED might involve changes in the expression of this family of genes.

Overall, these studies suggest that all members of the opiorphin family of genes will be useful markers for organic ED. Identifying such a marker, which would distinguish between organic and psychogenic ED, would have several medical applications. It could act as an objective assessment of the severity of erectile disease, and the efficacy of treatments, which would then lead to more appropriate dosage regimens for the pharmacotherapy of ED. In addition, given the association between ED and cardiovascular disease, such a marker might enable the early identification of patients at risk of cardiovascular disease and the early initiation of preventative measures. This report adds to the accumulating evidence that the opiorphins are important in male sexual function, as well as suggesting that they, and the downstream pathways which they regulate, might represent novel therapeutic targets for treating ED.

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## Abbreviations

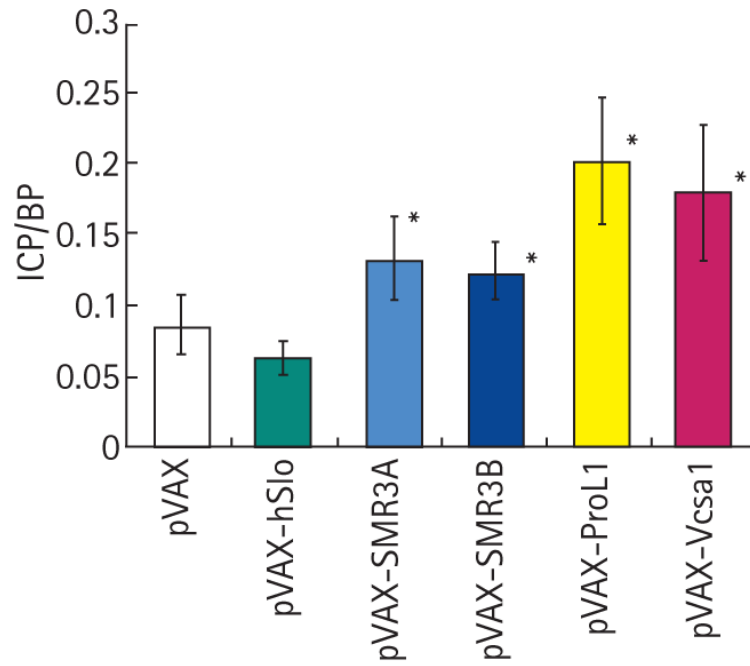
ED	erectile dysfunction
ICP/BP	intracorporal pressure/blood pressure
NEP	neutral endopeptidase



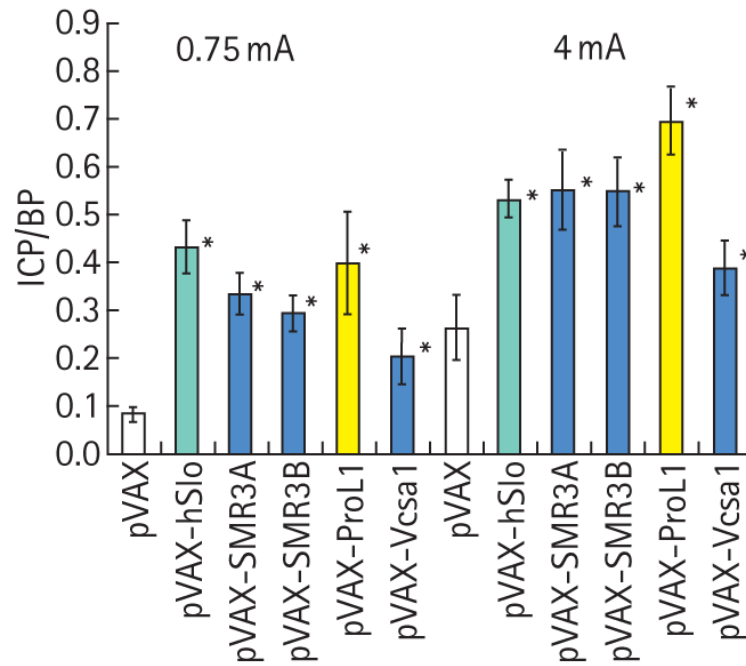
RT reverse-transcriptase.

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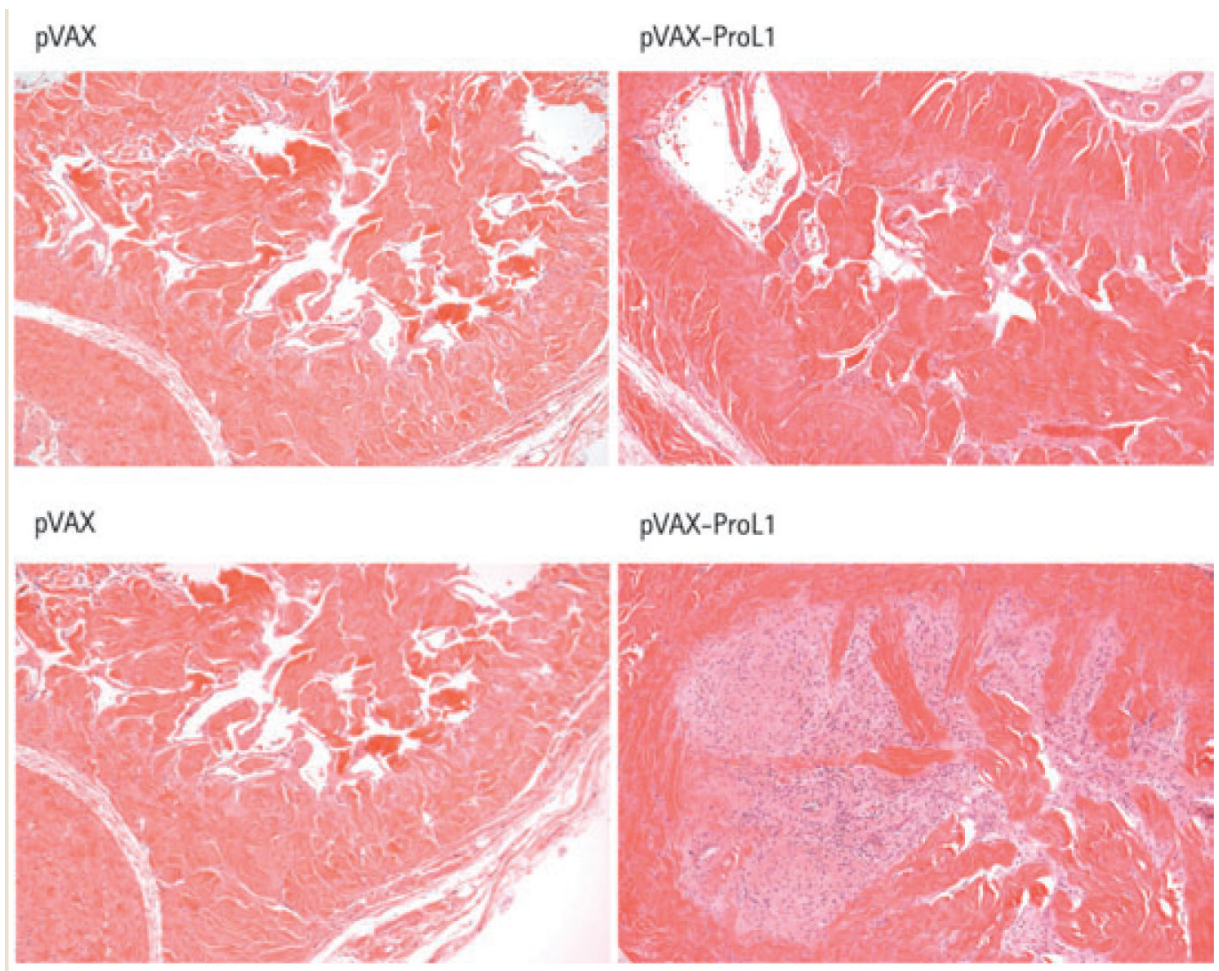


**FIG. 1.** Comparison of the basal ICP at 1 week after intracorporal injection with 100 µg plasmids expressing hSlo, SMR3A, SMR3B, ProL1 or Vcsa1 with control (rats injected with the parent vector, pVAX). \*P < 0.05 vs pVAX control.

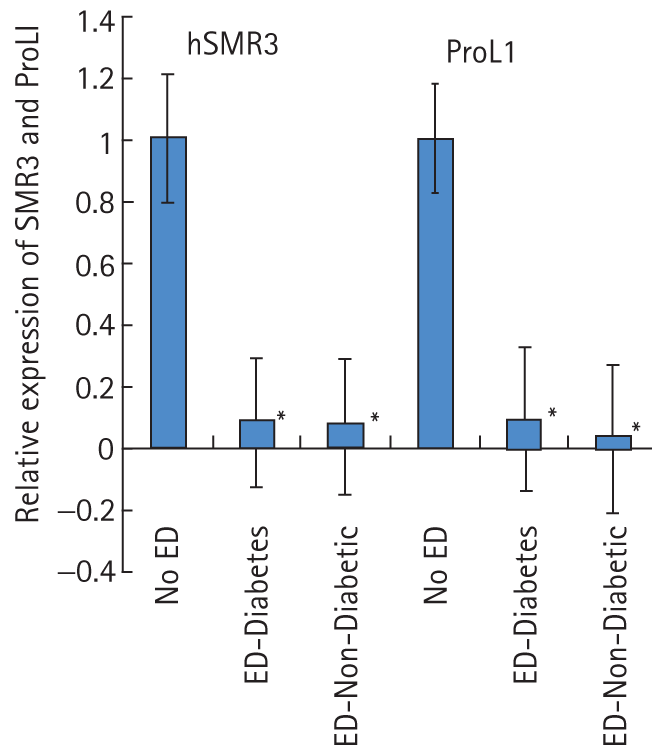


**FIG. 2.** ICP/BP ratio after electrostimulation of the cavernous nerve in rats intracorporally injected with 100 µg of pVAX, pVAX-hSlo, pVAX-hSMR3A, pVAX-hSMR3B, pVAX-ProL1 and pVAX-Vcsa1. \*Significantly different ( $P < 0.05$ ) from negative control (pVAX).





**FIG. 3.** Histological analysis of corporal tissue sections 1 week after intracorporal injection of 100  $\mu\text{g}$  pVAX-ProL1 compared with control tissue (treated with pVAX). Two different sections of corpora from the same rat are shown.  $\times 10$ .



**FIG. 4.** Comparison of the expression of SMR3 and ProL1 in patients without ED or with ED associated with a diabetic or non-diabetic aetiology.

TABLE 1

Pentapeptides encoded by the opiorphin family of genes

Gene	Pentapeptide	Species	Amino-acid sequence
<i>Vesal</i>	Sialorphin	Rat	QHNP
<i>ProL1</i>	Opiorphin	Human	QRFSR
<i>hSMR3A/B</i>	hSMR3	Human	QRGPR (hypothetical peptide)
		Consensus	QrpR
			Qr.pR

Visual assessment of erectile function after intracorporal gene transfer of the empty vector plasmid or plasmids expressing opiorphin homologues (Vcsa1, hSMR3A/B and ProL1) or hSlo

**TABLE 2**

Plasmid	No. of rats	Priapism*	Erection at 0.75 mA			Erection at 4 mA		
			None	Partial	Full	None	Partial	Full
pVAX	5	0	5			4	1	
pVAX-hSlo	5	0		2	3			5
pVAX-Vcsa1	5	4	1	4			2	3
pVAX-hSMR3A	5	2		4	1		1	4
pVAX-hSMR3B	5	3		5			1	4
pVAX-ProL1	5	4		3	2			5

\* Visual evidence of priapism.

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**CONFLICT OF INTEREST** None declared.