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CONCISE COMMUNICATION

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Design and validation of neuronal exocytosis blocking peptides as potential novel antiperspirants

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Abstract

Thermoregulation and heat dissipation by sweat production and evaporation are vital for human survival. However, hyperhidrosis or excessive perspiration might affect people's quality of life by causing discomfort and stress. The prolonged use of classical antiperspirants, anticholinergic medications or botulinum toxin injections for persistent hyperhidrosis might produce diverse side effects that limit their clinical use. Inspired by botox molecular mode of action, we used an in silico molecular modelling approach to design novel peptides to target neuronal acetylcholine exocytosis by interfering with the Snapin-SNARE complex formation. Our exhaustive design rendered the selection of 11 peptides that decreased calcium-dependent vesicle exocytosis in rat DRG neurons, reducing αCGRP release and TRPV1 inflammatory sensitization. The most potent peptides were palmitoylated peptides SPSR38-4.1 and SPSR98-9.1 that significantly suppressed acetylcholine release in vitro in human LAN-2 neuroblastoma cells. Noteworthy, local acute and chronic administration of SPSR38-4.1 peptide significantly decreased, in a dose-dependent manner, pilocarpine-induced sweating in an in vivo mouse model. Taken together, our in silico approach lead to the identification of active peptides able to attenuate excessive sweating by modulating neuronal acetylcholine exocytosis, and identified peptide SPSR38-4.1 as a promising new antihyperhidrosis candidate for clinical development.

KEYWORDS
peptides, Snapin, SNARE complex, sweat, TRPV1

1 | BACKGROUND

Sweat evaporation induces heat loss and thermoregulates human body. However, an excessive perspiration above the physiological necessary amount causes significant discomfort, emotional and social distress and interferes with daily living, affecting people's quality of life.[1]

Eccrine glands are the major responsible for thermoregulatory sweating. Eccrine sweating is controlled by the neurotransmitter acetylcholine (ACh) and binding to its cholinergic receptors.[2]

Neurotransmitter release requires vesicle transport, followed by membrane fusion and content release. This process named neuronal exocytosis is mediated by SNARE complex being constituted by one

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SNARE motif of synaptobrevin and syntaxin and two from SNAP-25.[3] Snapin is another SNAP-25 binding protein present in neurons and exclusively located on synaptic vesicle membranes[4] which mediates the interaction between SNARE and the Ca^{2+} sensor synaptotagmin. Therefore, regulation of Ca^{2+}-mediated exocytosis appears as a strategy to reduce excessive sweating. In addition, local skin application of botulinum toxin (BTX) completely blocks sweating by inhibiting ACh release through the cleavage of proteins involved in the formation of the neuronal exocytotic complex.[5–8] Although BTX injections are widely used to treat hyperhidrosis, they are costly and with marginal safety, requiring medical application due to diverse side effects and potent toxicity.

Disruption of protein–protein interactions required to form the SNARE complex and consequent vesicle exocytosis offers a therapeutic alternative for sweating control as demonstrated by peptides patterned after the SNAP-25 N-terminal or peptides containing the SNAP-25 binding sequence.[5–8]

1.1 | Questions addressed

We developed, through an in silico approach, peptides interfering with Snapin dimerization or Snapin-SNARE complex formation providing a therapeutic target for modulation of neuronal exocytosis. Peptides blocked in vitro Ca^{2+}-dependent ACh exocytosis and reduced in vivo pilocarpine-induced sweating in mice. Our approach represents a biotechnological solution to classical antihyperhidrosis formulations, whose prolonged use has been related to gland dysfunction and degeneration.[9] It is also a safe alternative to BTX injections, to which patients might develop treatment resistance and suffer of broad side effects along with pain sensation during administration.[10,11]

2 | EXPERIMENTAL DESIGN

2.1 | In silico molecular modelling and peptide design

Snapin dimmer was obtained modelling the SNAP-associated protein sequence from *Homo sapiens* (ref: NP_036569.1) with a BAG domain (Code 1HX1, chain B). Sequence alignment and homology modelling were performed with Yasara (http://www.yasara.org). The dimer was constructed with GRAMM-X.[12] The best solution was isolated in terms of interaction energy and orientation of the monomers. To model the Snapin-SNARE complex the Snapin dimeric model was docked against the neuronal synaptic fusion complex (Code 1SFC) in GRAMM-X.

Both complexes were used to derive peptide fragments for computational studies and protein interactions prediction. In the contact region of the complexes, a series of peptide fragments of different lengths were isolated and considered as ligands keeping only the interacting partner (Figure 1). The design started with an individual residue heat map calculation. The wild-type peptide was mutated to poly-Ala. Each individual position was then mutated to the 20 natural amino acids, maintaining the rest of positions as alanine. The fragment complex theoretical binding energy was determined.[13–15] The normalization of the binding energies produced position-specific scoring matrices (PSSM). PSSM were used to propose a list of peptide sequences that putatively could inhibit complex formation. In a second design step, the list of peptides was modelled, mutating all peptide positions at the same time, ensuring that incompatibilities between neighbour positions are considered.

2.2 | Acetylcholine release assay

After 4 days differentiation with Neurobasal-A medium, 1% N2 supplement, 0.5 μg/mL Leukaemia Inhibition factor, 0.5 mM Glutamax and 100 mM choline chloride,[16] LAN-2 cells were treated with peptides or control substances and then stimulated with 50 mM KCl—Hank's balanced salt solution (HBSS). Then, medium was collected and centrifuged (5000 xg, 5 min). Cells were used for BCA protein quantification. Supernatant was transferred to 96-well black plate, and released ACh was quantified.

2.3 | Animals

Primary culture of dorsal root ganglia (DRGs) was performed with 3–5 days neonatal Wistar rats obtained from the excess/remains of already isolated sensory neurons from the IDiBE at the University Miguel Hernandez of Elche. Sweat secretion assays were performed on 11–14 weeks old C57BL6/Rcc male mice, purchased form Harlan Laboratories. Animal procedures were approved by the University Miguel Hernández de Elche Institutional Animal and Ethical Committee in accordance with international guidelines.

2.4 | Primary culture from neonatal rat DRGs

Isolated neonatal Wistar rats DRGs underwent root removal prior to ganglia digestion with 0.25% (w/v) collagenase (type IA) in DMEM-Glutamax with 1% P/S (5000 U/mL) for 1 h. After digestion, DRGs were mechanically dissociated. Single-cell suspension was passed through a 100 μm cell strainer and washed with complete media. Cells were resuspended in complete media supplemented with 50 ng/mL 2.5S murine neural growth factor and 1.25 μg/mL cytosine arabinoside.[17,18]

2.5 | Regulated neuropeptide release assay

Sensory neurons from rat DRGs were seeded in 96-well plate coated with poly-L-lysine (8.33 μg/mL) and laminin (5 μg/mL). Forty-eight hours after seeding cells were incubated for 1 h with peptides at 20,

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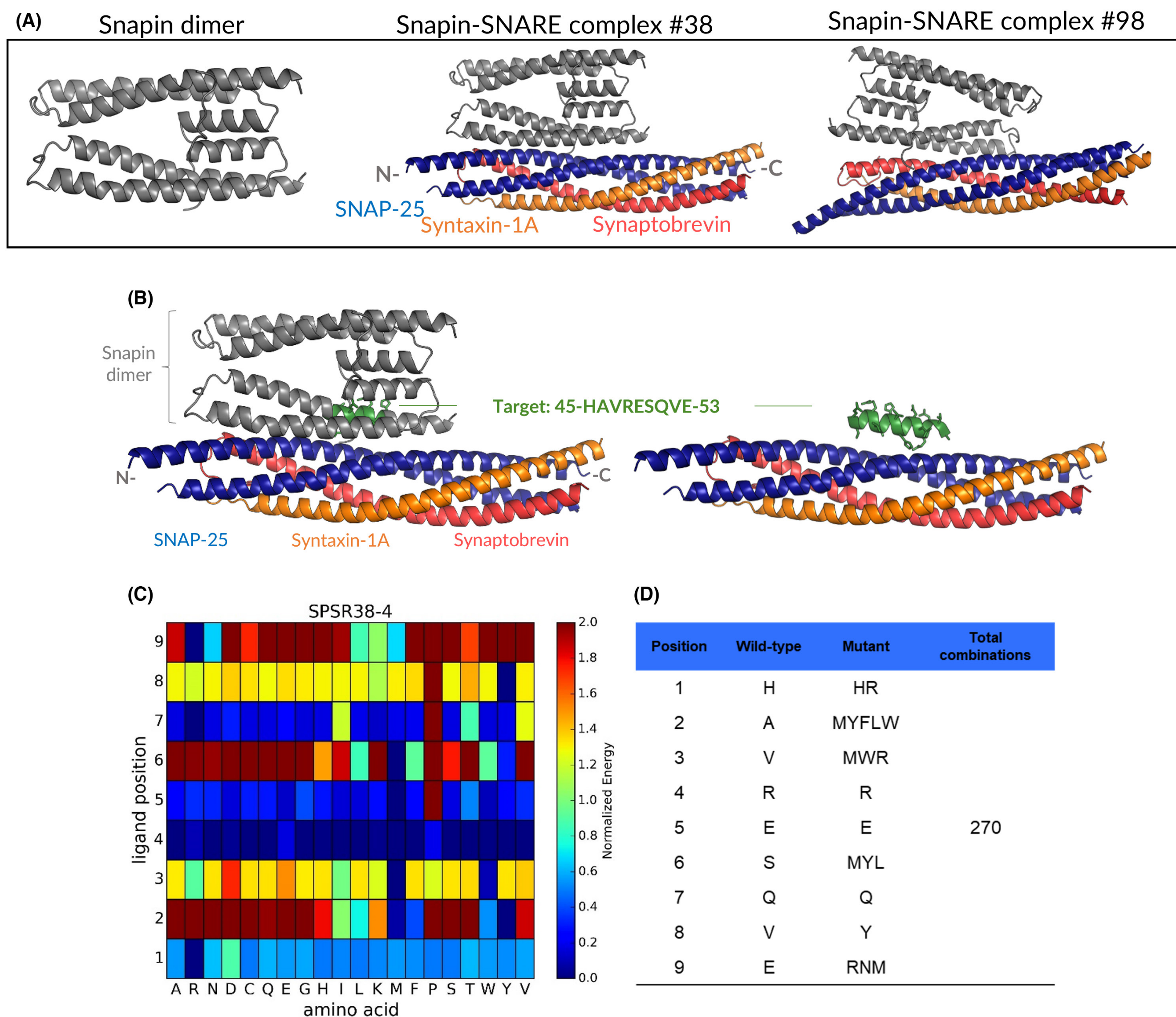

Position	Wild-type	Mutant	Total combinations
1	H	HR	
2	A	MYFLW	
3	V	MWR	
4	R	R	
5	E	E	270
6	S	MYL	
7	Q	Q	
8	V	Y	
9	E	RNM	

FIGURE 1 In silico complex, heat map and mutant peptides construction. (A) The Snapin dimer solution after rigid body docking and energy minimization is shown on the left. The best solution was selected based on Ser50 and Cys60 orientation in both monomers. The best solutions of the Snapin dimeric model docked against the neuronal synaptic fusion complex SNARE were #38 (centre) and #98 (right). SNARE complex is composed by two SNAP-25 motives, syntaxin-1A and synaptobrevin. The selection was based on the interaction energy between Snapin and SNARE, and its relative orientation. (B) Snapin-SNARE complex with target region referenced to 45-HAVRESQVE-53 sequence is shown in green (left). Isolated complex carrying selected peptide fragment is shown on the right. (C) Position-specific scoring matrix as a heat map for target sequence (9-mer peptide) shown in (B). It represents how the 20 natural amino acids fit, in terms of normalized interaction energy, in each ligand position.(1–9) Blue-coloured rectangles account for energetically favoured amino acid for a given position, while red-coloured ones for energetically disfavoured. (D) Selection of energetically favoured mutant amino acids per position. These were used for sequence search giving rise to a total of 270 possible combinations. Some positions were kept with their wild-type residue, as they are not considered key for the interaction.

50 or 100 μM, 1 mM acetyl hexapeptide-8 or 100 nM BTX. Then, cells were incubated with 1 μM capsaicin for 10 min. Calcitonin gene-related peptide (αCGRP) content was determined in supernatants using the commercially available αCGRP EIA Kit.[18]

2.6 | Multielectrode Array (MEA)

The electrical activity of primary sensory neurons was recorded by the MEA1060. MEA plates were previously coated with poly-L-lysine and laminin. TRPV1-mediated neuronal excitability was induced by three short 15 s applications of 500 nM capsaicin. Cells were washed with external solution for 3 min between P1 and P2. Between P2 and P3 cells were washed with external solution or potentiation cocktail for 8 min.

Mean spike frequency was obtained. Ratio P3/P1 was normalized to sensitization control conditions as percentage and it was compared between control cells, cells treated with pSPSR38-4.1 and acetyl hexapeptide-8. The average of registered electrodes was $n = 180–220$.

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2.7 | Sweat secretion assay

Sweat was induced by intraplantar (i.pl.) injection of 3 μg/paw pilocarpine. References and test substances were injected 30 min before sweating stimulation. Injected paws were painted with iodine solution followed by a coat starch solution. Dark sweating drops appeared 2–3 min after injection.[19] Photographs to quantify sweating drops were taken at the peak of sweating production. Glycopyrrolate (0.25 mg/kg) was injected subcutaneously on the back of the neck. Vehicle (saline solution) was injected i.pl. pSPSR38-4.1 was assessed at 10, 30 and 100 μg and injected i.pl., as well as the reference acetyl hexapeptide-8 at 100 μg. Group injected exclusively with pilocarpine was included to evaluate sweating level variability. Experimental groups were formed by 4–6 animals.

In chronic conditions, test peptide and acetyl hexapeptide-8 were administered three times a week in alternate days for 4 weeks. Sweat was assessed using pilocarpine once a week. pSPSR38-4.1 was assessed at 1, 10 and 100 μg and acetyl hexapeptide-8 at 100 μg. Sweat induction, monitoring and analysis were performed as in the acute assay. Experimental groups were formed by 3–6 animals.

2.8 | Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis consisted in one-way ANOVA with Dunnett's or Bonferroni's multiple comparison test, except for chronic sweat secretion assay where it was a two-way ANOVA followed by Dunnett's post hoc test, comparing each condition with sweating control within the same week. Significance was set to $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Exocytosis blocking peptides in silico design

Structural models of Snapin dimer or Snapin-SNARE interaction complex were built via molecular docking approach. The crucial structural regions were identified (Figure 1) and used to generate a total of eight peptide-receptor complexes of different lengths. Figure 1B depicts an example of Snapin-SNARE receptor complexed with a target ligand (encoded SPSR38-4) isolated and ready for mutagenesis and energy calculations.

Figure 1C,D show PSSM for the SPSR38-4 complexed to the receptor and depict all possible mutations likely to optimize the ligand–receptor interaction. All selected peptide-receptor complexes were treated following this procedure (Table S1).

A series of peptide sequences were constructed for each peptide-receptor complex, by combining the best amino acids selected at each position in the different complexes. The resulting combinations and differences in interaction energy between mutants and wild-type peptides were analysed (Table S2).

3.2 | Peptides inhibit αCGRP exocytosis from primary sensory neurons

A total of 11 peptides were selected for in vitro testing (Table S3). The ability of the selected peptides to inhibit regulated neuronal exocytosis was first screened on sensory neurons. Activation of TRPV1, a TRP ion channel involved in noxious thermal sensation, drives neuronal exocytosis of neuropeptides such as αCGRP. αCGRP release is fully dependent on the SNARE complex formation, thus providing a suitable assay for peptides targeting the exocytotic protein complex.[18] Thus, we evaluated the potency of the designed peptides on TRPV1 mediated αCGRP release in primary cultures of sensory neurons. As depicted in Table 1, N-acetylated peptide SPSR98-9.2 showed the strongest inhibitory effect with 77.4 ± 14.1% reduction of αCGRP release, followed by SPSR98-7.1 and SPSR38-4.1 with 55.3 ± 10.8 and 39.1 ± 12.5%, respectively. Lipopeptides SPSR38-4.1, SPSR98-7.1 and SPSR98-9.1 reduced neuropeptide release by 68.3 ± 14.2, 53.9 ± 14.6 and 74.8 ± 14.5% at 50 μM, respectively.

3.3 | Active peptides reduce inflammatory sensitization of TRPV1 in sensory neurons

TRPV1 inflammatory sensitization involves the exocytotic recruitment of a population of vesicular channels ready to be incorporated into the plasma membrane.[18,20] BTX and peptides that inhibit exocytosis exhibit anti-inflammatory activity by blocking the recruitment of TRPV1 channels to the neuronal surface.[8] Thus, we next determined SPSR38-4.1 effect on reducing the inflammatory potentiation of TRPV1 in sensory neurons. TRPV1 inflammatory sensitization was evaluated by monitoring capsaicin-induced generation of action potentials in MEA. As shown in Figure 2A, exposure to a pro-inflammatory cocktail composed by 1 μM bradykinin, 10 μM ATP and 50 μM histamine resulted in a TRPV1 desensitization reversal reflected by the increase of capsaicin-elicited electrical activity, consistent with TRPV1 responses potentiation (Figure 2A-top). Incubation of neurons with 10 μM SPSR38-4.1 resulted in an attenuation of capsaicin-evoked responses (Figure 2A-bottom, Figure 2B), suggesting a decrease in thermoTRP inflammatory sensitization. SPSR38-4.1 reduced TRPV1-mediated electrical activity being clearly more potent than acetyl hexapeptide-8 since it reached similar inhibitory extend but at a 1000-fold lower concentration.

3.4 | Peptides reduce acetylcholine release from human LAN-2 cells

The most active peptides in sensory neurons were also tested for their capacity to inhibit ACh release in LAN-2 cells. Acetylated and palmitoylated SPSR38-4.1, SPSR98-7.1 and SPSR98-9.1 and palmitoylated SPSR98-9.2 peptides were assayed (Table S4). The trifluoroacetic salt of acetylated SPSR38-4.1, SPSR98-7.1 and SPSR98-9.1 peptides exhibited strong inhibitory activity at 10 μM. Likewise, we

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TABLE 1 Inhibition of neuronal CGRP release. Sensory neurons incubated with acetylated at 20 and 100 μM or palmitoylated at 20 and 50 μM peptides for 1 h before CGRP release induction. As reference standard inhibitors were used 100 nM botulinum toxin or 1 mM acetyl hexapeptide-8. Neuropeptide release is normalized to CGRP release elicited by 1 μM capsaicin in nontreated cells. Data are expressed as mean ± SEM, $n=3$ biological replicates, $N=3$ independent experiments. Statistical analysis was performed with one-way ANOVA followed by Bonferroni's post hoc test. Significance was set to $p<0.05$.

Peptide code	Sequence	[μM]	% inhibition CGRP release	
			Acetylated	Palmitoylated
SNAPINM-1	RMTAMKRALLDLGM	100	−18.0 ± 15.6	-
		50	-	21.2 ± 6.2
		20	40.0 ± 37.5	15.7 ± 10.1
SPSR38-3.1	MQYRLRKLIDMDAT	100	−17.3 ± 20.0	-
		50	-	-
		20	43.0 ± 38.1	-
SPSR38-4.1	HYWRELQYR	100	39.1 ± 12.5*	-
		50	-	63.3 ± 14.2*
		20	10.1 ± 15.9	38.4 ± 12.5
SPSR38-4.2	RWWREYQYR	100	10.3 ± 13.3	-
		50	-	-
		20	−5.3 ± 18.7	-
SPSR38-5.1	YRLLRHRM	100	19.0 ± 14.8	-
		50	-	30.0 ± 9.1
		20	−1.2 ± 20.9	46.1 ± 14.5*
SPSR38-6.1	NHEYRVHWRRS	100	−10.6 ± 14.2	-
		50	-	29.4 ± 11.1
		20	3.8 ± 27.5	30.1 ± 12.6
SPSR98-7.1	MQVWLRMWIDYRAT	100	55.3 ± 10.8*	-
		50	-	53.9 ± 14.6*
		20	49.3 ± 18.1	35.5 ± 10.0
SPSR98-8.1	RRLLRRRL	100	−48.2 ± 18.3	-
		50	-	-
		20	−16.3 ± 34.5	-
SPSR98-8.2	WRLLRRRF	100	-	-
		50	-	-
		20	−39.2 ± 24.7	-
SPSR98-9.1	RRVVLVNNIL	100	-	-
		50	-	74.8 ± 14.5*
		20	-	34.0 ± 8.7
SPSR98-9.2	LRVQMVNMFL	100	77.4 ± 14.1*	-
		50	-	-
		20	14.2 ± 14.6	-
Botulinum toxin		0.1	46.1 ± 10.3*	-
Acetyl hexapeptide-8		1000	20.5 ± 6.0	-

*$p < 0.05$.

observed significant inhibitory activity of palmitoylated pSPSR38-4.1, pSPSR98-9.1 and pSPSR98-9.2 peptides blocking at 50 μM ≥75% of ACh release. Notably, the peptides inhibitory efficacy was like that exerted by 10 nM BTX, and stronger than that of 100 μM acetyl hexapeptide-8.

3.5 | SPSR38-4.1 peptide reduces pilocarpine-induced in vivo sweating

We also aimed to evaluate whether peptide SPSR38-4.1 exhibited antisweating activity in vivo. Pilocarpine-induced sweating model in the mouse paw was implemented[21,22] (Figure 3). Local administration of pilocarpine in the paw rapidly stimulated sweating that is sensitive to glycopyrrolate, an anticholinergic agent.[23] The weekly administration of pilocarpine induces a chronic-like model.

In the acute model, the three SPSR38-4.1 doses significantly diminished the sweating response by 34.5 ± 8.4%, 51.4 ± 9.6% and 58.2 ± 9.8% at 10, 30 and 100 μg compared to the vehicle group (Figure 3A). Glycopyrrolate completely inhibited the sweating response, and acetyl hexapeptide-8 reduced it 45.0 ± 8.6%. Thus, administration of peptides before pilocarpine significantly attenuates sweating.

In a longer-term assay, peptides were administered three days per week for four weeks, and the effect on pilocarpine-evoked sweating was evaluated weekly. SPSR38-4.1 peptide exhibited

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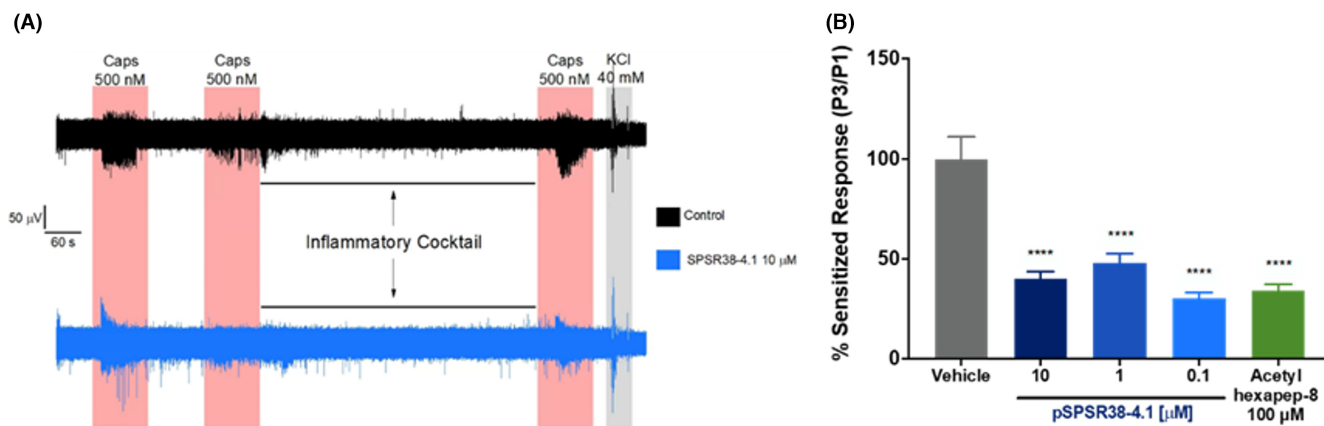

FIGURE 2 Sensitization inhibitory effect of peptide pSPSR38-4.1. (A) Representative recordings showing neuronal excitability induce by three short pulses of capsaicin 500 nM. Between second and third pulse, inflammatory cocktail was applied. At the end of the protocol, KCl 40 mM was applied to ensure the cell viability. (B) Percentage of sensitized response represented by normalizing the ratio P3/P1 of mean spike frequency from each compound to the control group. The percentage of sweating inhibition for each concentration of peptide was 59.7 ± 3.3% at 10 µM, 51.9 ± 4.7% at 1 µM and 69.7 ± 2.7% at 0.1 µM. The reference acetyl hexapeptide-8 at 100 µM inhibited 65.7 ± 2.9% of sweating production. All data are expressed as mean ± SEM, $N = 3$ independent experiments. Statistical analysis performed consisted in one-way ANOVA followed by Dunnett's post hoc test ****$p < 0.0001$.

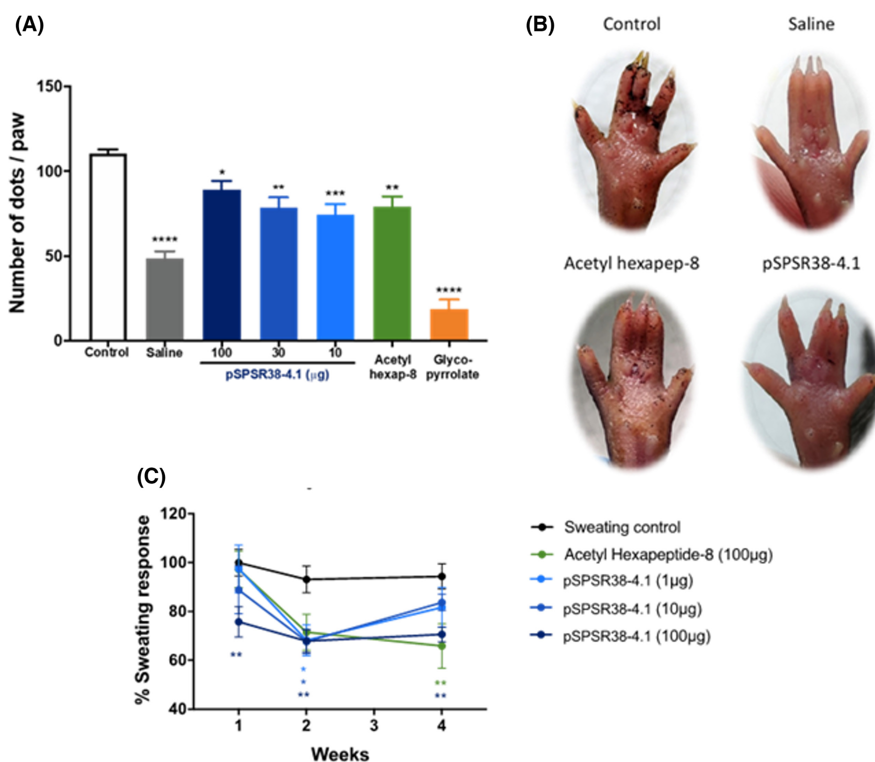

FIGURE 3 Sweating inhibition effect of pSPSR38-4.1 in acute and chronic application. Sweating was induced by local injection of 3 µg of pilocarpine. All treatments were administered i.pl. Thirty minutes before sweating stimulation. (A) Data represents number of sweat dots per paw on each condition. (B) Representative images of paws with dark sweating drops induced by pilocarpine for all tested conditions. (Basal): mice injected only with equivalent volume of saline solution but not pilocarpine. (Control): animals injected with saline followed by pilocarpine. (pSPSR38-4.1): peptide was assessed at 100, 30 and 10 µg/paw before sweat induction, showing an inhibition of 34.5 ± 8.4%, 51.4 ± 9.6% and 58.2 ± 9.8%, respectively. Acetyl hexapeptide-8 (100 µg, I.pl.) and Glycopyrrolate (0.25 mg/kg, sc) were used as reference standard controls for neuronal exocytosis blockade. Acetyl hexapeptide-8 decreased 45.0 ± 8.6% the sweating production and glycopyrrolate 31.9 ± 8.1% more than the basal sweating produced by saline injection. $n = 4$–6 animals per group in the acute study. (C) Data represents normalized values of sweating response on each condition. Peptide pSPSR38-4.1 (1, 10 and 100 µg, i.pl.) and acetyl hexapeptide-8 (100 µg, i.pl.) were administered 3 days/week for four weeks. Sweating was induced with 3 µg of pilocarpine once/week on first, second and fourth week. Data was normalized respect to sweating control on the first week for each experiment independently. $n = 3$–6 animals per group in the chronic study. Data represents mean ± SEM. Statistical analysis was one-way ANOVA followed by Dunnett's post hoc test, comparing each condition with the maximal sweat induction (control 100%) and the minimal basal levels (saline 0%), except for chronic model where it was two-way ANOVA followed by Dunnett's post hoc test, comparing each condition with sweating control within the same week.

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significant antisweating activity at the three tested doses (1, 10 and 100 µg, i.pl), reducing by >20% the sweating response akin to 100 µg (i.pl) acetyl hexapeptide-8 (Figure 3C and Table S5). These findings substantiate that SPSR38-4.1 peptide is an inhibitor of pilocarpine-induced sweating in mouse paws and pave de way to develop it as an antihyperhidrosis compound.

4 | CONCLUSIONS AND PERSPECTIVES

At variance with the large BTX-A protein, low molecular weight peptides can be tailored to have good therapeutic efficacy, skin permeability and low immunogenicity in sweat control. Thus, we focused on an in silico modelling strategy which resulted in the design of novel active peptides by targeting the assembly of Snapin-SNARE complex. The in vitro and in vivo pharmacological profile showed by peptide SPSR38-4.1 reveals it as a candidate for further clinical development as a topical antihyperhidrosis product.

AUTHOR CONTRIBUTION

LB conducted multielectrode assay and in vivo experiments, analysed the data, wrote and edited the first and subsequent drafts of the manuscript. MNK conducted in silico assays, analysed the data and wrote and edited the first and subsequent drafts of the manuscript. AS and VR performed neuropeptide and acetylcholine release assays. AE, EM, MV and AFC revised and edited the manuscript. GFB supervised and executed in silico experiments and revised and edited the manuscript. GM, SZ and LR conceptualized the project, supervised experiments, revised and edited the manuscript. AFM and ID supervised and designed experiments, conceptualized the project, wrote, revised and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

Within this work we report the identification of proprietary peptides (WO2019238683). Angelini Pharma S.p.A. founded this research. MV, EM, SZ, LR and FM are employees of Angelini Pharma. AFC, GFB and AFM are founders and shareholders of AntalGenics. ID, MNK, AE and AS are employees of Antalgenics. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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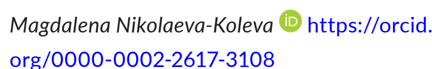
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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1.

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