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RESEARCH ARTICLE

# Antagonist of Chrna1 prevents the pathogenesis of primary focal hyperhidrosis

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## Abstract

**Background**: Primary focal hyperhidrosis (PFH) is an autonomic neurological disease in which exocrine glands are oversecreted due to autonomic dysfunction of the sympathetic nervous system. Chrna1 promotes the pathogenesis of PFH. We aimed to check if downregulating of Chrna1 by cisatracurium could alleviate the symptoms of PFH. **Methods**: The effect of cisatracurium in a hyperhidrosis mice model induced by pilocarpine hydrochloride was monitored for sweat gland secretion, and ultrastructural sweat secretory granules in sweat glands were analyzed. Meanwhile, markers of hyperhidrosis were checked, and release of Bdnf and Nrg1 from sympathetic ganglia axon was tested. Furthermore, the mechanism of cisatracurium function was evaluated in vitro using HEK293 expressing *Chrna1*. Finally, the effect of cisatracurium was determined in the hyperhidrosis mice model with overexpression or downregulation of Chrna1. **Results**: In hyperhidrosis mice, pretreatment with cisatracurium effectively inhibited sweat secretion, along with fewer particle secretion in sweat glands. The molecular markers of hyperhidrosis (Aqp5 and Cacna1c) were inhibited by cisatracurium, acetylcholine (Ach) level in serum was found decreased. Neurotrophic factors (Bdnf and Nrg1) secreted by sympathetic axon activation were also inhibited. At last, it was confirmed that cisatracurium could not alter the gene or protein expression level of Chrna1, but could block the ion channel. Overexpression of Chrna1 abolished the effect of cisatracurium on hyperhidrosis, while cisatracurium could not function more in si*Chrna1*-treated mice. **Conclusion**: Our results suggested that pretreatment of cisatracurium could alleviate hyperhidrosis in mice, probably through blocking the ion channel function of Chrna1.

## Introduction

Primary focal hyperhidrosis (PFH) refers to a state of unexplained hypersecretion of sweat glands in parts of the body.[1] It is an autonomic neurological disease in which exocrine glands are over-functioning due to autonomic dysfunction of the sympathetic nervous system. Sweating is commonly seen in the palms, soles, and armpits. Hyperhidrosis seriously affects the quality of life, study, and work for patients, whose long-term effect causes serious psychological diseases if patients stayed without treatment.[2] At present, the sympathetic nerve connection is mainly cutoff by minimal invasive surgery to treat PFH.[3] However, surgical operations always cause trauma and have a certain risk. Oral anticholinergic drugs, or topical aluminum chloride preparations, a mixed solution of 20–25% aluminum hydride and 70% ethanol is the first-line antiperspirant commonly used in clinical treatment of PFH.[4,5] It also includes iontophoresis, topical injecting *Bacillus* toxin A to treat PFH patients, but the effect gradually declines over time.[6] Besides, the latest laser treatment strategy remains controversial. Therefore, there is an urgent need to learn more about the mechanism of PFH and find a better treatment strategy.

At present, the pathogenesis of PFH is still unclear. Most studies believe that it is a disease in which sympathetic nervous overactivation leads to excessive secretion of sweat glands.[7] In patients with PFH, sweat glands are


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usually histologically and functionally normal, and it is more likely that there is a problem in the process of stimulating sweat glands. PFH may be related to the increased expression of acetylcholine (Ach) receptor subunits, choline acetyltransferase and vasoactive intestinal peptide in the thoracic sympathetic ganglion, and the thoracic sympathetic ganglia of PFH.[8,9] The increase in the number of myelin nerve fibers and the thickening of myelin sheath are also one of its pathogeneses. Family genetic studies have shown that *ACVR1* gene is overexpressed in sweat gland tissue of PFH patients,[10] and *CHRNA1* gene is significantly upregulated in thoracic sympathetic ganglia.[11] Studies have shown that CHRNA1 is the cholinergic receptor nicotinic alpha1 subunit, which is involved in the binding and gating of the Ach neurotransmitter.[12] If PFH model mice are induced and injected with siRNA of *Chrna1* gene, it can be seen that the siRNA can alleviate the symptoms of PFH and reduce the content of PFH biomarker molecules.[11] Our previous study suggested that the increased expression of Chrna1 is an important cause of excessive sweat secretion, and the use of small molecules to block the activity of Chrna1 may be an effective method for the treatment of PFH. Cisatracurium has been reported to inhibit Chrna1,[13] and it has been used clinically as a skeletal muscle relaxant in many situations.[14–16] Therefore, we sought to investigate the effect of cisatracurium in PFH and possible mechanisms in an animal model of PFH.

## Materials and Methods

### Animal model

Animal studies were approved by the First Affiliated Hospital of Fujian Medical University. Hyperhidrosis mice model was constructed by intraperitoneal injection of pilocarpine hydrochloride (5 mg/kg body weight). After 5 min for stimulation of perspiration, the black spots on paws were photographed to measure sweat secretion. Before stimulation, iodine solution (2.0% in EtOH) was applied to the hind paws. After drying, a starch suspension (0.5 g/mL in mineral oil) was used to cover the surface of the paw. Black spots were counted using ImageJ software.

For cisatracurium (CIS, Jiangsu Hengrui Pharmaceutical Company, Lianyungang, China) treatment, mice were treated with vehicle (saline) or 1 mg/kg cisatracurium (dissolved in saline) 6 h before hyperhidrosis induction. For virus treatment, mice were treated with virus packaged with empty vectors (EV) or Chrna1-expressing sequences 72 h before the administration of vehicle or 1 mg/kg cisatracurium 6 h before hyperhidrosis induction. For siRNA treatment, mice were treated with siControl (SiCtrl) or Si*Chrna1* 72 h before the administration of vehicle or 1 mg/kg cisatracurium 6 h before hyperhidrosis induction.

### Electron microscope

Samples were collected and prepared following a routine procedure. Briefly, tissues were fixed in glutaraldehyde and 2% $OsO_4$, then dehydrated, embedded in epoxy resin 618. Sections were made using a microtome (LKB-III) for ultra-thin (60 nm), stained with saturated uranyl acetate and 1% lead citrate, finally analyzed by electron microscope.

### Detection of protein and gene expression

The levels of acetylcholine (Ach) in serum were checked by ELISA using Acetylcholine ELISA Kit (OKEH02568; Aviva Systems Biology, San Diego, CA), following the manufacturer's manual. Protein levels of Aqp5, Cacna1c, Bdnf, and Nrg1 were detected by western blotting, following a standard protocol which is almost the same as indicated in our previous paper.[11] Antibodies used are as follows: Aqp5 (ab78486; Abcam, Cambridge, MA), Cacna1c (ab58552; Abcam), Bdnf (NB100-98683; Novus Biologicals, Centennial, CO), and Nrg1 (ab53104; Abcam), Chrna1 (MA3-043; Thermo Fisher, Waltham, MA), Gapdh (Cell Signaling Technologies, Danvers, MA).

Q-PCR followed the standard protocol described before.[11] Primers used are as follows:

*Chrna1*
Forward: 5′TCATCATTCCCTGCCTGCTCTTCT3′
Reverse: 5′TCTCTGCAATGTACTTCACGCCCT3′
*Aqp5*
Forward Primer: AGAAGGAGGTGTGTTCAGTTGC
Reverse Primer: GCCAGAGTAATGGCCGGAT
*Cacna1c*
Forward Primer: ATGAAAACACGAGGATGTACGTT
Reverse Primer: ACTGACGGTAGAGATGGTTGC
*Bdnf*
Forward Primer: TCATACTTCGGTTGCATGAAGG
Reverse Primer: AGACCTCTCGAACCTGCCC
*Nrg1*
Forward Primer: ATGGAGATTTATCCCCCAGACA
Reverse Primer: GTTGAGGCACCCTCTGAGAC

### Whole-cell patch-clamp on Chrna1-expressing HEK293 cells

Cell line with *Chrna1*-expressing HEK293 cells was established using pCMV plasmid carrying Chrna1-IRES-neo following the procedure as described before.[17] HEK293 cells were cultured in DMEM (L0091; Biowest, Bradenton,

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FL) supplemented with 10% fetal bovine serum (30–2020, ATCC, Manassas, VA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% $CO_2$/95% $O_2$ incubator. HEK293 cells were transfected stably with Lipofectamine™ 2000 (#11668019; Thermo Fisher) according to the manufacturer's protocol. After transfection, the positive cell clones were selected with G418. The transfected cells were then incubated for 24 h before the recordings were performed. Cells were treated with $10^{-5}$ mol/L acetylcholine (ACh) with or without $10^{-8}$ mol/L cisatracurium for 2 sec and followed the method for whole-cell patch-clamp described previously.[18]

### Overexpression or silencing for Chrna1

The full-length cDNA of murine Chrna1 or siRNAs were cloned into pAAV plasmid and sent to GenScript (Nanjing, China) for virus package. AAVs were injected subcutaneously to overexpress or knockdown *Chrna1* in sweat glands in mice. si*Chrna1* targets the mouse *Chrna1* sequence at 1213–1238. Scrambled RNA sequence (GGCAUAAGAUUAGCGGCAAGCAAU) was designated as si*Ctrl*.

### Statistical analysis

The significant difference was analyzed using one-way ANOVA with Tukey's multiple-comparisons test in GraphPad Prism 7 (San Diego, CA). Error bar represents mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, not significant.

## Results

### Sweat secretion was reduced by cisatracurium in hyperhidrosis mice

The mice were pretreated with different doses of cisatracurium 6 h before hyperhidrosis induction, and it was found that 1 and 3 mg/kg cisatracurium had better effects in inhibiting sweat gland secretion, and there was no significant difference between the two (Fig. 1A). Therefore, 1 mg/kg cisatracurium was used in the follow-up study. Then we checked the effect of processing time on hyperhidrosis. It was found that treatment with cisatracurium at 2, 6, and 12 h before induction can all have an inhibitory effect, but treatment with 6 h in advance had the best inhibiting effect (Fig. 1B). Therefore, follow-up studies all used 1 mg/kg cisatracurium to treat mice 6 h in advance. Notably, body weight, consumption of water and food, behavior including gait, etc., were normal in the cisatracurium-treated mice. These results suggested that cisatracurium may have a prevention effect on the excessive secretion of sweat glands. By using a transmission electron microscope, we detected sweat secretory granules in sweat glands (Fig. 2A), the ultrastructural differences of the sweat glands were compared, and the secretion particles, that is, sweat secretory granules, of the sweat gland duct epithelial cells were counted. It was found that cisatracurium treatment had an inhibitory effect for particle secretion (Fig. 2B). Thus, cisatracurium reduced the sweat secretory granules in sweat gland cells in hyperhidrosis mice.

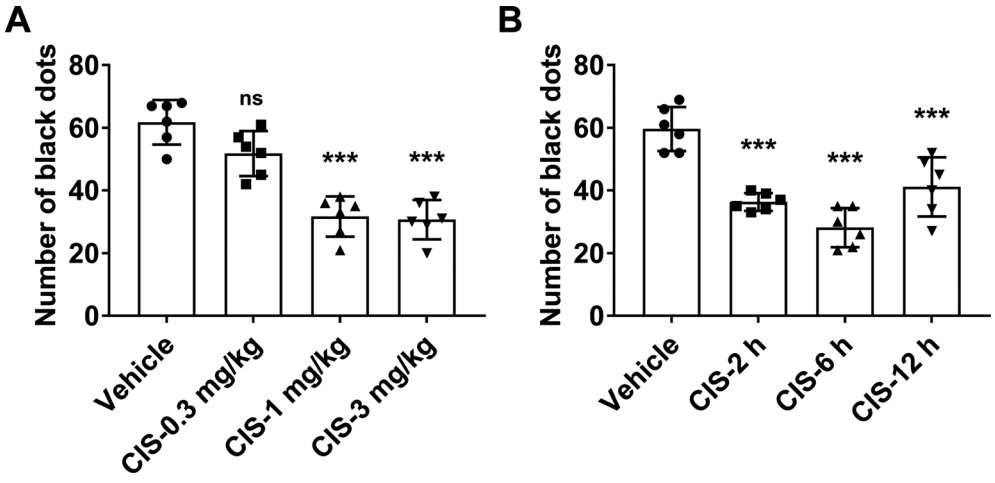


**Figure 1.** Cisatracurium reduced the sweat secretion in hyperhidrosis mice. Mice were administered vehicle, 0.3, 1, or 3 mg/kg cisatracurium (CIS) via tail vein 6 h before the induction of hyperhidrosis. (A) The number of black dots were calculated using ImageJ after pilocarpine injection. $n = 6$. (B) Mice were administered vehicle or 1 mg/kg cisatracurium via tail vein 2, 6, or 12 h before the induction of hyperhidrosis. The number of black dots was calculated using ImageJ after pilocarpine injection. $n = 6$. ***$p < 0.001$, ns, not significant.

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## Cisatracurium inhibited the markers of hyperhidrosis in mice

Next, we checked the molecular markers of hyperhidrosis. Aqp5 is a transmembrane protein that facilitates the transport of water molecules, it is highly expressed in PFH patients. *Cacna1c* is the gene encoding calcium channel proteins, and the upregulation of Cacna1c protein is a hallmark of hyperhidrosis. While treated with cisatracurium, after induction for hyperhidrosis in the mice, the level of acetylcholine (Ach) in serum was found decreased (Fig. 3A), compared to mice treated with vehicle, nearly to the level of control mice. mRNA levels of *Aqp5* and *Cacna1c* gene were similar as that in uninduced healthy control mice, while the expression was significantly increased in vehicle-treated hyperhidrosis mice (Fig. 3B and C). The proteins level showed the same trend (Fig. 3D–F).

## Cisatracurium inhibited the expression of Bdnf and Nrg1 in hyperhidrosis mice

Neurotrophic factors like Bdnf and Nrg1 can be triggered release from axons through stimulation, which can also

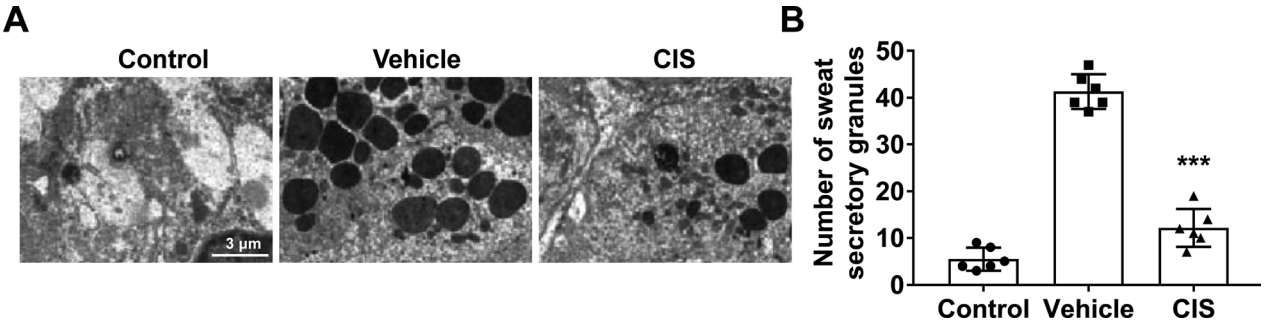


**Figure 2.** Cisatracurium reduced the sweat secretory granules in sweat gland cells in hyperhidrosis mice. (A) Representative images of transmission electron microscope from sweat gland tissues of control and hyperhidrosis mice treated with vehicle or cisatracurium (CIS) 6 h before hyperhidrosis induction. The sweat glands were taken 5 min after pilocarpine injection. (B) The number of sweat secretory granules were counted by using ImageJ. $n = 6$. ***$p < 0.001$.

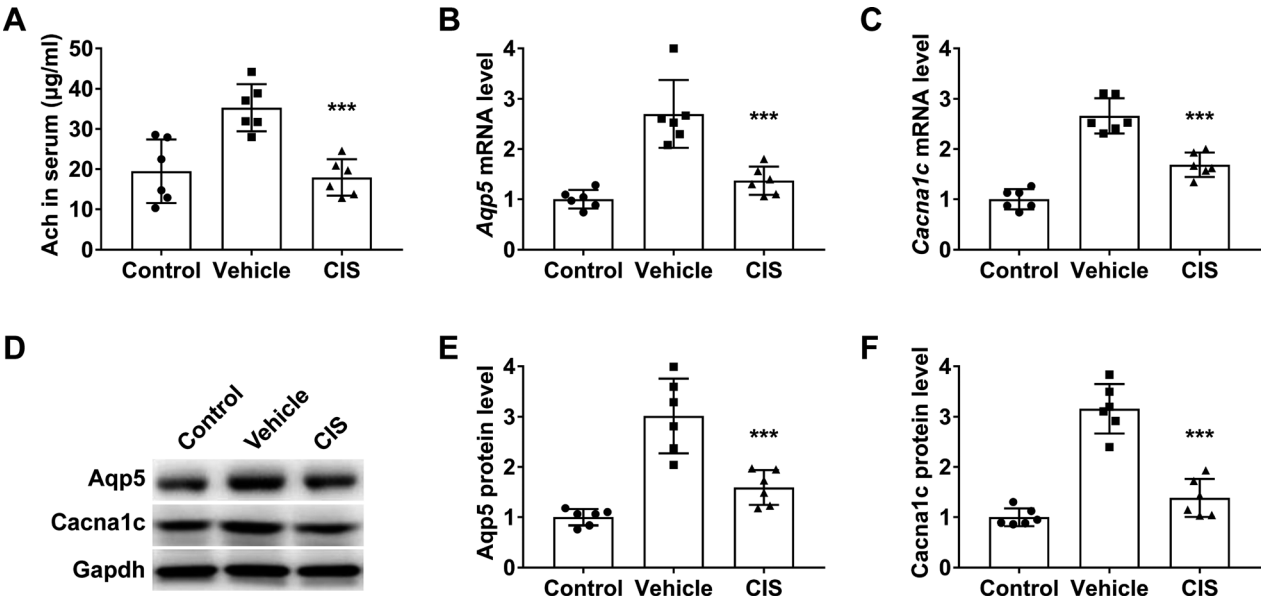


**Figure 3.** Cisatracurium inhibited the expression of markers of hyperhidrosis in hyperhidrosis mice. Mice were treated with vehicle or 1 mg/kg cisatracurium (CIS) 6 h before hyperhidrosis induction. (A) The concentration of acetylcholine (Ach) in serum was detected by ELISA. (B and C) The mRNA level of *Aqp5* (B) and *Cacna1c* (C) in sweat gland tissues. (D–F) The protein level of Aqp5 (D and E) and Cacna1c (D and F) in sweat gland tissues. $n = 6$. ***$p < 0.001$, ns, not significant.

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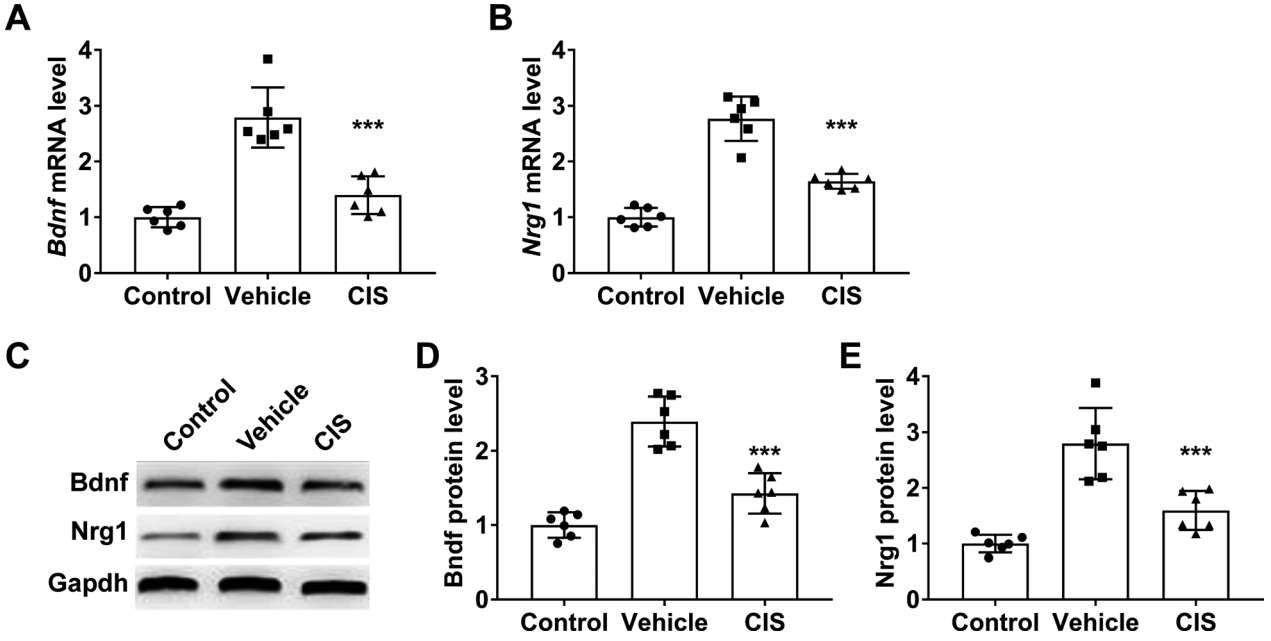


**Figure 4.** Cisatracurium inhibited the expression of Bdnf and Nrg1 in hyperhidrosis mice. Mice were treated with vehicle or 1 mg/kg cisatracurium (CIS) 6 h before hyperhidrosis induction. (A and B) The mRNA level of *Bdnf* (A) and *Nrg1* (B) in sympathetic ganglia axons. (C–E) The protein level of Bdnf (C and D) and Nrg1 (C and E) in sympathetic ganglia axons. $n = 6$. ***$p < 0.001$.

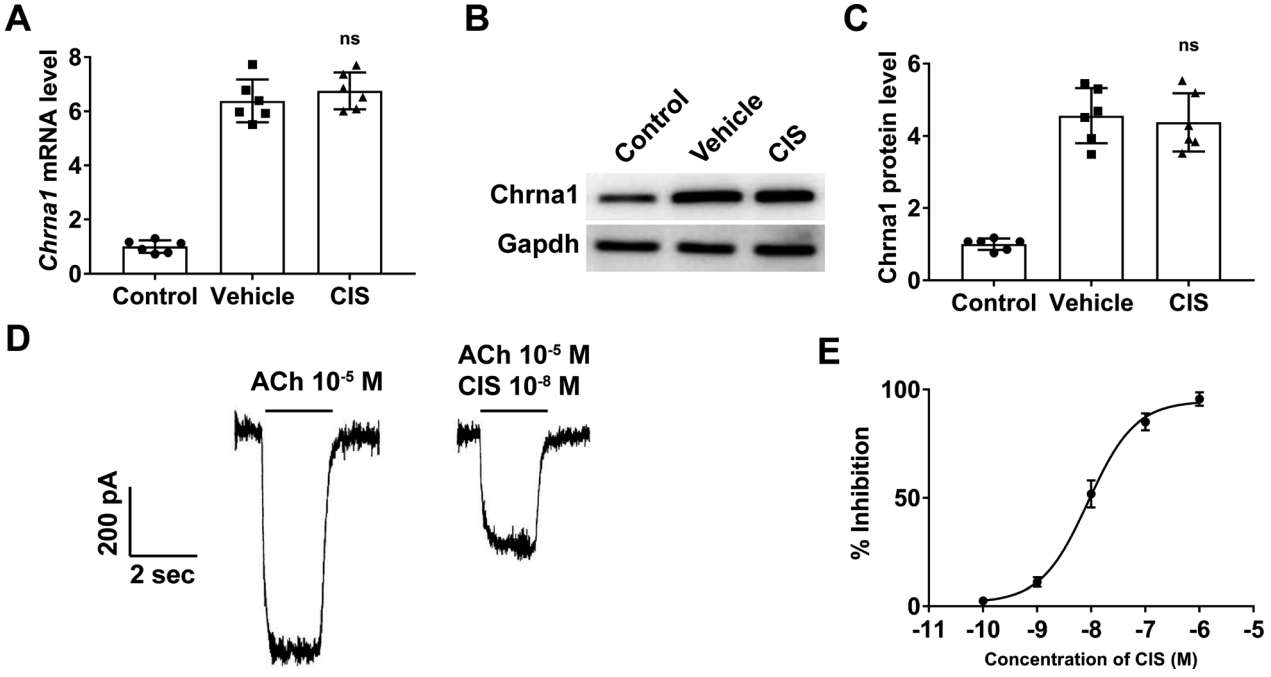


**Figure 5.** Cisatracurium blocked Chrna1. The mRNA level (A) and protein level (B and C) of *Chrna1* in sweat glands of control or hyperhidrosis mice treated with vehicle or 1 mg/kg cisatracurium (CIS) 6 h before hyperhidrosis induction. (D and E) Concentration-dependent effects of cisatracurium (CIS) on Chrna1-expressing HEK293 cells. (D) Representative raw currents observed during the application of $10^{-5}$ mol/L acetylcholine (ACh) with or without $10^{-8}$ mol/L cisatracurium (CIS) for 2 sec. (E) Concentration-dependent inhibition of cisatracurium (CIS) on Chrna1. Four wells per condition. ns, not significant.

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be reversed or blocked. Since in hyperhidrosis model, the sympathetic ganglia axon release Bdnf and Nrg1, which play an important role in stimulating sweat secretion. With the pretreatment of cisatracurium in hyperhidrosis mice model, the expression of *Bdnf and Nrg1* gene mRNA levels (Fig. 4A and B) and protein levels (Fig. 4C–E) in sympathetic ganglia axons were significantly decreased comparing with that were treated with vehicle, but similar to that of control normal mice. These results indicated there was an inhibition of sympathetic axon activity.

## Cisatracurium functions through Chrna1 in hyperhidrosis mice

Since Chrna1 was found to promote the pathogenesis of PFH, then we thought to investigate if cisatracurium function through Chrna1. There was no obvious change in mRNA level or protein level with cisatracurium pretreatment (Fig. 5A–C) in hyperhidrosis mice. However, while tested in Chrna1-expressing HEK293 cells, the ion channel was blocked by cisatracurium (Fig. 5D and E).

Next, we want to know if the overexpression of Chrna1 in hyperhidrosis model will overcome the prevention effect of cisatracurium. Mice were treated with virus packaged with empty vectors (EV) or *Chrna1*-expressing sequences 72 h before the administration of vehicle or 1 mg/kg cisatracurium 6 h before hyperhidrosis induction. Overexpression of Chrna1 was detected in sweat glands of hyperhidrosis mice both at mRNA level (Fig. 6A) and protein level (Fig. 6B and C), though cisatracurium did not affect the expression level of Chrna1. Overexpression of Chrna1 abolished the effect of cisatracurium on hyperhidrosis (Fig. 6D). On the contrary, we want to test how cisatracurium functions if Chrna1 is

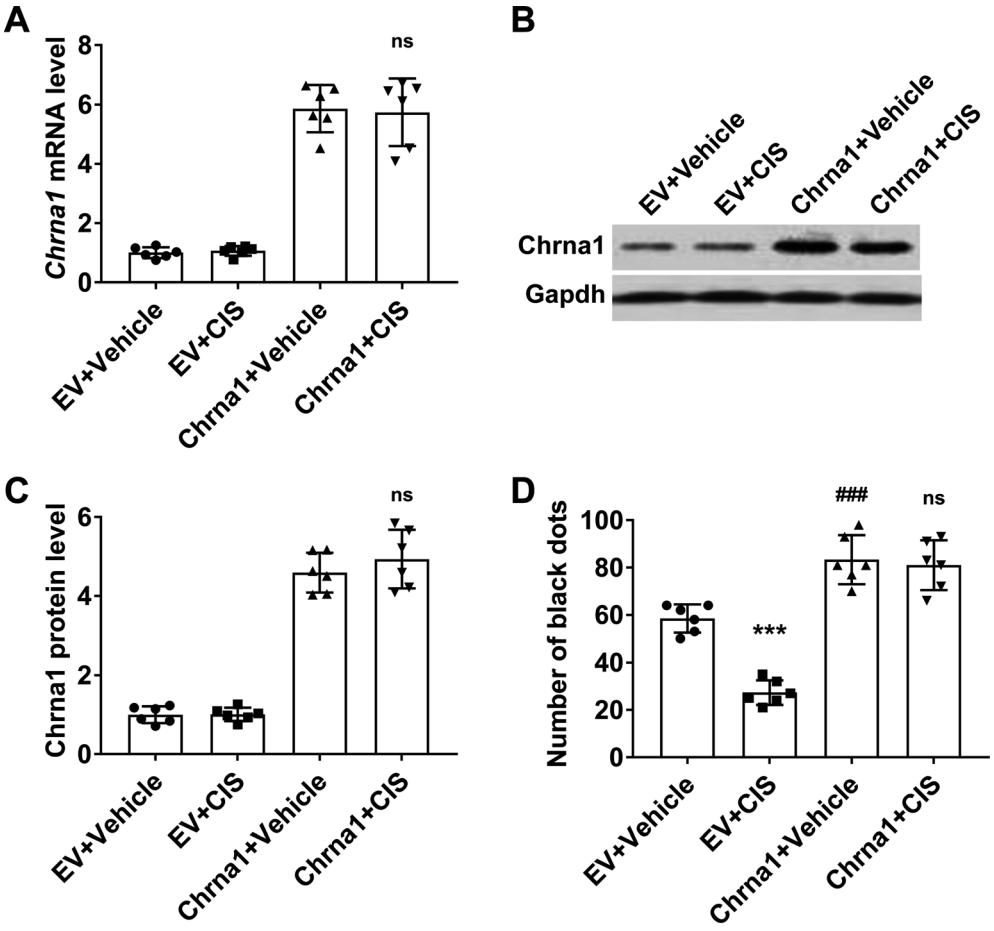


**Figure 6.** Overexpression of Chrna1 abolished the effect of cisatracurium on hyperhidrosis. Mice were treated with virus packaged with empty vectors (EV) or Chrna1-expressing sequences 72 h before the administration of vehicle or 1 mg/kg cisatracurium (CIS) 6 h before hyperhidrosis induction. (A) The mRNA level of *Chrna1* in sweat glands of hyperhidrosis mice. (B and C) The protein level of Chrna1 in sweat glands of hyperhidrosis mice. (D) The number of black dots were calculated using ImageJ after pilocarpine injection. $n = 6$. $^{###}p < 0.001$, $***p < 0.001$, ns, not significant.

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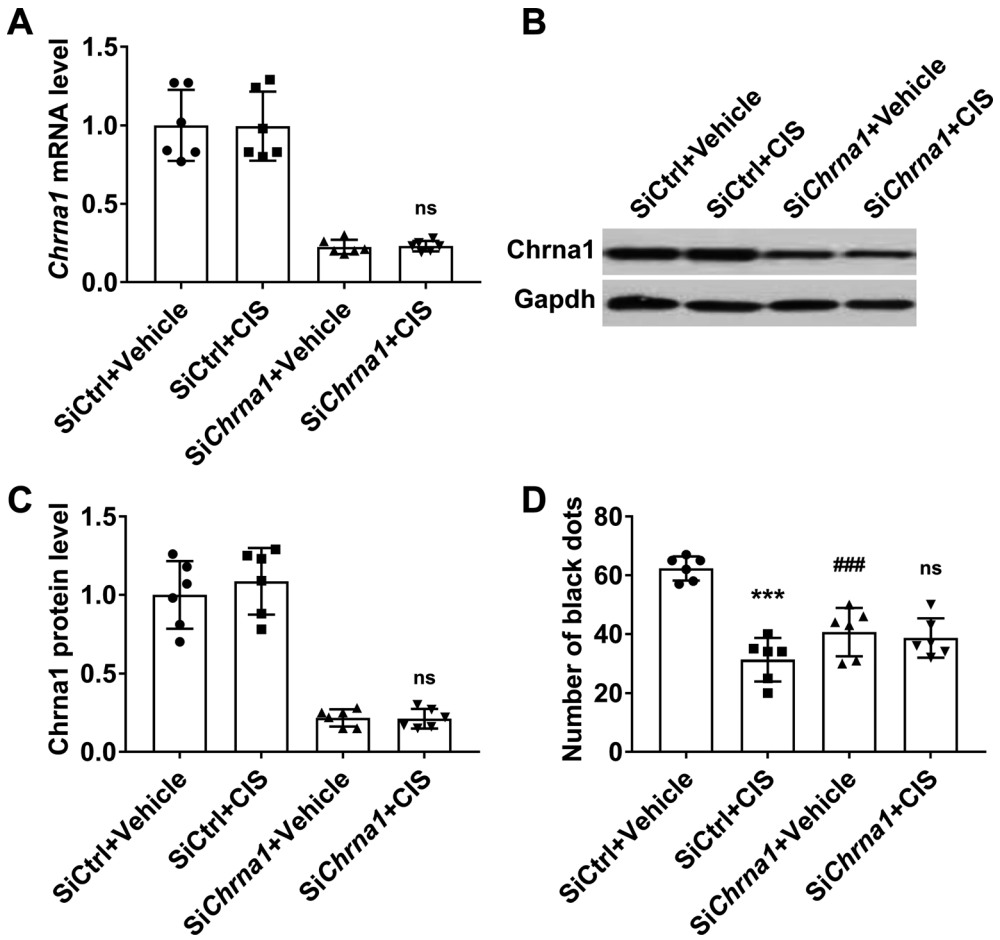


**Figure 7.** Cisatracurium could not function more in si*Chrna1*-treated mice. Mice were treated with siControl (SiCtrl) or Si*Chrna1* 72 h before the administration of vehicle or 1 mg/kg cisatracurium (CIS) 6 h before hyperhidrosis induction. (A) The mRNA level of *Chrna1* in sweat glands of hyperhidrosis mice. (B and C) The protein level of Chrna1 in sweat glands of hyperhidrosis mice. (D) The number of black dots were calculated using ImageJ after pilocarpine injection. $n = 6$. $^{###}p < 0.001$, $***p < 0.001$, ns, not significant.

knocked down. We checked the mRNA level (Fig. 7A) and protein level (Fig. 7B and C) and found that the expression of Chrnal1 was significantly decreased by knocking down with si*Chrna1*. Cisatracurium could not function better in si*Chrna1*-treated mice compared with those without cisatracurium treatment (Fig. 7D). These results further supported that cisatracurium functions through the inhibition of the Chrna1 activity, not by altering its expression.

## Discussion

In our previous study, we found that silencing *Chrna1* decreased secretion of sweat gland and the number of sweat secretory granules in hyperhidrosis mice.[11] Thus upregulation of Chrna1 protein level could be a potential biomarker of PFH, and Chrna1 could possibly be a therapeutic target to treat PFH. This study is a follow-up study with our previous findings. We sought to test that if pharmaceutically target Chrna1 could affect PFH, and in this investigation, we found that pretreatment with cisatracurium could alleviate the symptoms of PFH in hyperhidrosis mice model.

As the hypothesis is that prohibition of Chrna1 could reduce the symptoms of PFH via inhibition of the sympathetic system. The decrease in serum Ach level indicates that it has an inhibitory effect on neurosecretion. At the same time, it also has an inhibitory effect on the expression levels of Aqp5 and Cacna1c genes that are elevated in PFH. Furthermore, Bdnf and Nrg1 gene and protein expression levels have also recovered to normal mouse levels, indicating inhibition effect of cisatracurium on sympathetic axon activity. These results are consistence with previous finding that cisatracurium has neuron

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blockading effect.[18–20] As a commonly used neuromuscular blocking agent in the critical care due to its attractive pharmacokinetic profile,[21] cisatracurium has been widely used in clinic. Compared to other treatment, cisatracurium may lead to potential effect with minimal side effect, though it needs to be proved in clinical trial for PFH.

However, cisatracurium has no effect on Chrna1 gene and protein expression levels, both in mice overexpressing and silencing Chrna1. Experiments in HEK-293 cells indicate that cisatracurium directly inhibits the ion channel function of Chrna1 and how it works. Therefore, cisatracurium should act through a pharmacological mechanism acting on ion channels. The same dose of cisatracurium does not work in the mouse PFH model that expresses excessive Chrna1 protein, which further illustrates that cisatracurium works pharmacologically by blocking Chrna1 ion channel function, suggesting that cisatracurium or similar drugs can be optimized to target Chrna1 to treat or prevent PFH. However, cisatracurium did not have a better effect in the Chrna1-silencing mice, indicating that cisatracurium does act on Chrna1, so both cisatracurium and silencing Chrna1functions the same.

Although directly silencing *Chrna1* by siRNA has great potential for clinic treatment, but it needs a long period to optimize the delivery system, and to use it in clinic. A strategy to repurpose an already used drug in clinic targeting CHRNA1 would be an alternative way and could be used for patients sooner. Cisatracurium is widely used in clinic for providing muscle relaxation during surgery, medically assisted breathing, or insertion of a breathing tube.[22] It is a nondepolarizing skeletal muscle relaxant, that works by blocking the effects of Ach. Nevertheless, it is worth noting that the prevention effect is not good enough if the pretreatment time window is too long or too short. The time window should be controlled to achieve the best results if cisatracurium is used in clinical practice.

## Conclusion

Our study suggested that pretreatment of cisatracurium could alleviate hyperhidrosis in a mice model of PFH, probably through blocking the ion channel function of Chrna1. Thus, cisatracurium has the great potential usage in clinic for repurpose drug development, to alleviate PFH. However, further mechanism needs to be investigated as to better understand how cisatracurium functions to promote its clinical usage in treating PFH patients.

## Conflict of Interest

None declared.

## Author Contributions

Data curation, analysis: Jian-Bo Lin, Nan-Long Lin, Xu Li, and Ming-Qiang Kang; Drafting of the manuscript: Jian-Bo Lin and Ming-Qiang Kang; Concept, design of the study: Jian-Bo Lin and Ming-Qiang Kang. All authors approved the publication the manuscript.

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