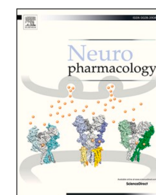
Neuropharmacology 195 (2021) 108632

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



Therapeutic concentrations of varenicline increases exocytotic release of catecholamines from human and rat adrenal chromaffin cells in the presence of nicotine

Amanda Jiménez-Pompa [a,b], Sara Sanz-Lázaro [a], Arik J. Hone [a,g,1], Lola Rueda-Ruzafa [a,**], José Medina-Polo [c], Carmen González-Enguita [d], Jesús Blázquez [e], Cristóbal de los Ríos [b], J. Michael McIntosh [g,1], Almudena Albillos [a,f,*]

[a] Departamento de Farmacología y Terapéutica, Universidad Autónoma de Madrid, Madrid, Spain
[b] Instituto-Fundación Teófilo Hernando, Universidad Autónoma de Madrid, Madrid, Spain
[c] Servicio de Urología, Hospital Universitario 12 de Octubre, Instituto de Investigación I+12, Madrid, Spain
[d] Servicio de Urología y Unidad de Trasplante Renal, Fundación Jiménez Díaz, Madrid, Spain
[e] Servicio de Urología, Hospital Clínico San Carlos, Madrid, Spain
[f] Instituto Ramón y Cajal de Investigación Biosanitaria (IRYCIS), Madrid, Spain
[g] School of Biological Sciences and Department of Psychiatry, University of Utah, Salt Lake City, Utah, USA

ARTICLE INFO

Keywords:
Varenicline
Nicotine
Exocytosis
Chromaffin cell
Nicotinic receptor
Human

Chemical compounds used in this article:
Varenicline (PubChem CID:170361)
Nicotine (PubChem CID: 89594)
Acetylcholine (PubChem CID: 187)
Dimethylphenylpiperazinium (PubChem CID: 1316)
Cytisine (PubChem CID: 597)
Dihydro-β-erythroidine (PubChem CID: 31762)

ABSTRACT

Cardiovascular side effects of varenicline and a case report of a hypertensive crisis in a varenicline-prescribed patient with pheochromocytoma have been reported. The goal of the present study was to determine whether such side effects might derive, in part, from increased exocytosis of secretory vesicles and subsequent catecholamine release triggered by varenicline in human chromaffin cells of the adrenal gland. In this study, we performed electrophysiological plasma membrane capacitance and carbon fiber amperometry experiments to evaluate the effect of varenicline on exocytosis and catecholamine release, respectively, at concentrations reached during varenicline therapy (100 nM). Experiments were conducted in the absence or presence of nicotine, at plasma concentrations achieved right after smoking (250 nM) or steady-state concentrations (110 nM), in chromaffin cells of the adrenal gland obtained from human organ donors. Cells were stimulated with short pulses (10 ms) of acetylcholine (ACh; 300 μM) applied at 0.2 Hz, in order to closer mimic the physiological situation at the splanchnic nerve-chromaffin cell synapse. In addition, rat chromaffin cells were used to compare the effects obtained in cells from a more readily available species. Varenicline increased the exocytosis of secretory vesicles in human and rat chromaffin cells in the presence of nicotine, effects that were not due to an increase of plasma membrane capacitance or currents triggered by the nicotinic agonists alone. These results should be considered in nicotine addiction therapies when varenicline is used.

1. Introduction

Varenicline is a nicotinic acetylcholine receptor (nAChR) agonist successfully used in the treatment of nicotine addiction. Its mechanism of action as a smoking cessation aid is mediated by partial agonism of the α4β2 subtype. Through this mechanism, varenicline stimulates α4β2 nAChR-mediated dopamine release in the ventral tegmental area sufficiently to reduce craving when abstinent from smoking, but inhibits the action of nicotine on α4β2 nAChRs when smoking. Furthermore, this drug exhibits additional effects on other nicotine addiction related

Abbreviations: nAChRs, Nicotinic acetylcholine receptors; ACh, acetylcholine; DMPP, Dimethylphenylpiperazinium; DhβE, Dihydro-β-erythroidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's Modified Eagle's Medium.

* Corresponding author. Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, c/ Arzobispo Morcillo 4, 28029 Madrid, Spain.

E-mail address: almudena.albillos@uam.es (A. Albillos).

[1] George E. Whalen Veterans Affairs Medical Center, Salt Lake City, Utah, USA (A.J.H.: Present address).

[**] Centro de Investigación Biomédica (CINBIO), Universidad de Vigo, Vigo, Spain (L.R.R.: Present address).

https://doi.org/10.1016/j.neuropharm.2021.108632
Received 17 March 2021; Received in revised form 30 April 2021; Accepted 20 May 2021
Available online 4 June 2021
0028-3908/© 2021 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

nAChR subtypes including α6β2, α3β4, and α7 (Mihalak et al., 2006; Capelli et al., 2011; Tammimäki et al., 2012). However, although varenicline is an efficacious drug for smoking cessation, some adverse cardiovascular side-effects have been reported in humans (Chelladurai and Sing, 2014; Gershon et al., 2018; Munarini et al., 2015; Prochaska and Hilton, 2012; Rigotti et al., 2010; Singh et al., 2011) and rats (Selçuk et al., 2015). Also, a case report of varenicline-triggered pheochromocytoma crisis in a smoker has been reported (Hukkanen et al., 2010). The US Food and Drug Administration reviewed a randomized clinical trial of 700 smokers with cardiovascular disease who were treated with varenicline or placebo. In this trial, adverse cardiovascular events were infrequent overall, but certain serious events, including heart attacks, were reported more frequently in patients treated with varenicline than in patients treated with placebo (Faessel et al., 2006).

Because the action of varenicline on the peripheral α3-containing subtypes expressed in human chromaffin cells (Hone et al., 2015, 2017) might contribute to the cardiovascular side effects of this drug by triggering the release of catecholamines, we previously investigated whether varenicline might increase the firing of actions potentials in these cells. We reported that varenicline in the presence of nicotine alters cell ability to fire action potentials in response to acetylcholine (ACh) at therapeutically relevant concentrations and, in addition, is capable of evoking actions potentials in the absence of ACh stimulation (Hone et al., 2017). These results prompted us to investigate whether varenicline together with nicotine could enhance exocytosis of secretory vesicles and catecholamine release in human chromaffin cells of the adrenal gland. The situation in which both drugs are administered together occurs during therapeutic treatment with varenicline in smokers who are in the process of quitting smoking, or during the use of varenicline in conjunction with a nicotine replacement therapy (Chang et al., 2015). This study was also conducted using rat and bovine chromaffin cells due to the limitations of obtaining and working with human cells. The main nAChR subtypes expressed in rat chromaffin cells are, as in humans, α3β4* (Di Angelantonio et al., 2003; Hone et al., 2020) and α7 (Hone et al., 2020) but an α3β2β4* subtype (not investigated in human chromaffin cells) has also been reported (Hone et al., 2020).

2. Materials and Methods

2.1. Human, rat and bovine adrenal chromaffin cell isolation and culture

Human adrenal glands were obtained from organ donors from three hospitals in Madrid (12 de Octubre, Fundación Jiménez Díaz and Clínico San Carlos). Written consent was signed by the relatives of donors and the use of human tissue was approved by the Universidad Autónoma de Madrid institutional Ethics Committee and by the review boards of each of the hospitals. The age of the male donors was 68.7 ± 3.3 (n = 3) years and 53.3 ± 10.7 (n = 3) years old for the female donors. Human chromaffin cells were isolated and cultured according to procedures described by Hone and colleagues (2015), and maintained in culture for up to 7 days. Bovine adrenal glands were obtained from a local abattoir, and chromaffin cells were isolated using the method reported by Moro and colleagues (1990).

Rat adrenal glands were harvested from male PD30-60 Sprague Dawley rats. All animal care and experimental procedures were approved by the Universidad Autónoma de Madrid institutional Ethics Committee. Experiments were carried out in accordance with the EU Directive 2010/63/EU and the ARRIVE guidelines. Adrenal medulla were dissected from the adrenal glands and cut into 5–7 pieces using fine iridectomy scissors. The medullary pieces were then incubated in 2 ml of a saline solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM D-glucose, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); pH was adjusted to 7.4 with NaOH containing 0.25% (vol/vol) Trypsin (Sigma, Aldrich) and 0.1% (wt/vol) collagenase type I for 60 min at 37 °C. After enzymatic digestion, the cells were homogenized with a glass Pasteur pipette to obtain a single cell suspension after which 8 ml of saline were added. The cells were centrifuged at 200×*g* for 10 min, the supernatant aspirated, and the cells suspended in 200–300 μl of culture medium composed of Dulbecco's Modified Eagle's Medium (DMEM), 500 μM Glutamax, 10% Fetal bovine serum (FBS), penicillin/streptomycin and plated on glass coverslips that had been treated with poly-D-lysine. Cells were maintained in DMEM culture medium at 37 °C in an incubator under an atmosphere of 95% air and 5% CO_2. Half of the rat chromaffin cells coverslips were treated with nicotine 110 nM from one day after the culture was performed. Experiments were conducted within 4 days after isolation.

2.2. Patch-clamp electrophysiology

To conduct electrophysiology experiments, the cells were gravity perfused with extracellular solution composed of (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES. The osmolarity was 315 mOsM and the pH was adjusted to 7.4 with NaOH. The flow rate was 1.5 ml/min and the solution was delivered by means of a polyethylene tube with an inner diameter of 0.58 mm. Patch electrodes were pulled from borosilicate glass capillaries (Kimbal Chase, cat. #3400–99) using a P97 pipette puller (Sutter Instruments, Novato CA, USA). The electrodes had resistances between 1.5 and 3.0 MΩ when filled with an internal electrode solution (in mM: 145 K-glutamate, 10 NaCl, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES; pH adjusted to 7.2 with KOH; observed osmolarity 322 mOsM). To initiate whole-cell recordings, a stock solution of 0.5 mg/ml amphotericin B was prepared daily in dimethysulfoxide. Five μl of this stock solution was added to 500 μl of intracellular solution, ultrasonicated, and used to back fill the patch electrodes. Experiments were performed under a sodium lamp for light at 25-27 °C.

A HEKA EPC10 amplifier (HEKA Electronik, Lambrect, Germany) controlled by PatchMaster software was used to record agonist-evoked responses. The signals were sampled at 10 kHz and filtered at 1 kHz through a Bessel filter. The average plasma membrane capacitance (C_m) of the cells was 5.7 ± 0.7 pF (n = 13) for human and 5.6 ± 0.3 pF (n = 26) for rat chromaffin cells, both compensated electronically. Series resistances ranged between 7 and 15 MΩ and were compensated electronically by 60–80% in the voltage-clamp mode. In the current-clamp mode, the resistance was compensated by ≥ 80% using the bridge balance feature of PatchMaster.

Agonists were applied in two ways depending on the type of experiment. For current-clamp experiments, a Picospritzer III (General Valve Corp., Fairfield, NJ, USA) was used to apply ACh to the cell by means of pressure ejection (15 psi) through a glass capillary pipette (World Precision Instruments, Sarasota, FL, USA; cat. 1B200F-4) with 1–2 μm diameter tips and filled with extracellular solution containing 300 μM ACh. For agonist studies in the voltage-clamp mode, a multi barrel pipette was constructed using polyethylene tubing with an inner diameter of 0.4 mm. A single polyethylene tube with an inner diameter of 0.28 mm was used for the outlet. The flow rate of the perfusion system was approximately 850 μl/min. The agonist pulses were controlled by a valve controller triggered by the amplifier. For the agonist concentration-response experiments, 500 ms pulses of agonist were applied every 3 min. The cells were first stimulated with 300 μM ACh until steady baseline responses were achieved, and then different agonists of interest were applied in ascending concentrations. Agonist responses were normalized to those obtained by 300 μM ACh in the same cell.

In the triple-step protocol and the recording of nicotinic currents, each condition was repeated three times and the average value was obtained. Also, agonists were applied in a different order each time.

2.3. Carbon fiber amperometry

Carbon fiber electrodes were prepared by cannulating a 10 μm-diameter carbon fiber in polyethylene tubing (diameter: outer, 1 mm;

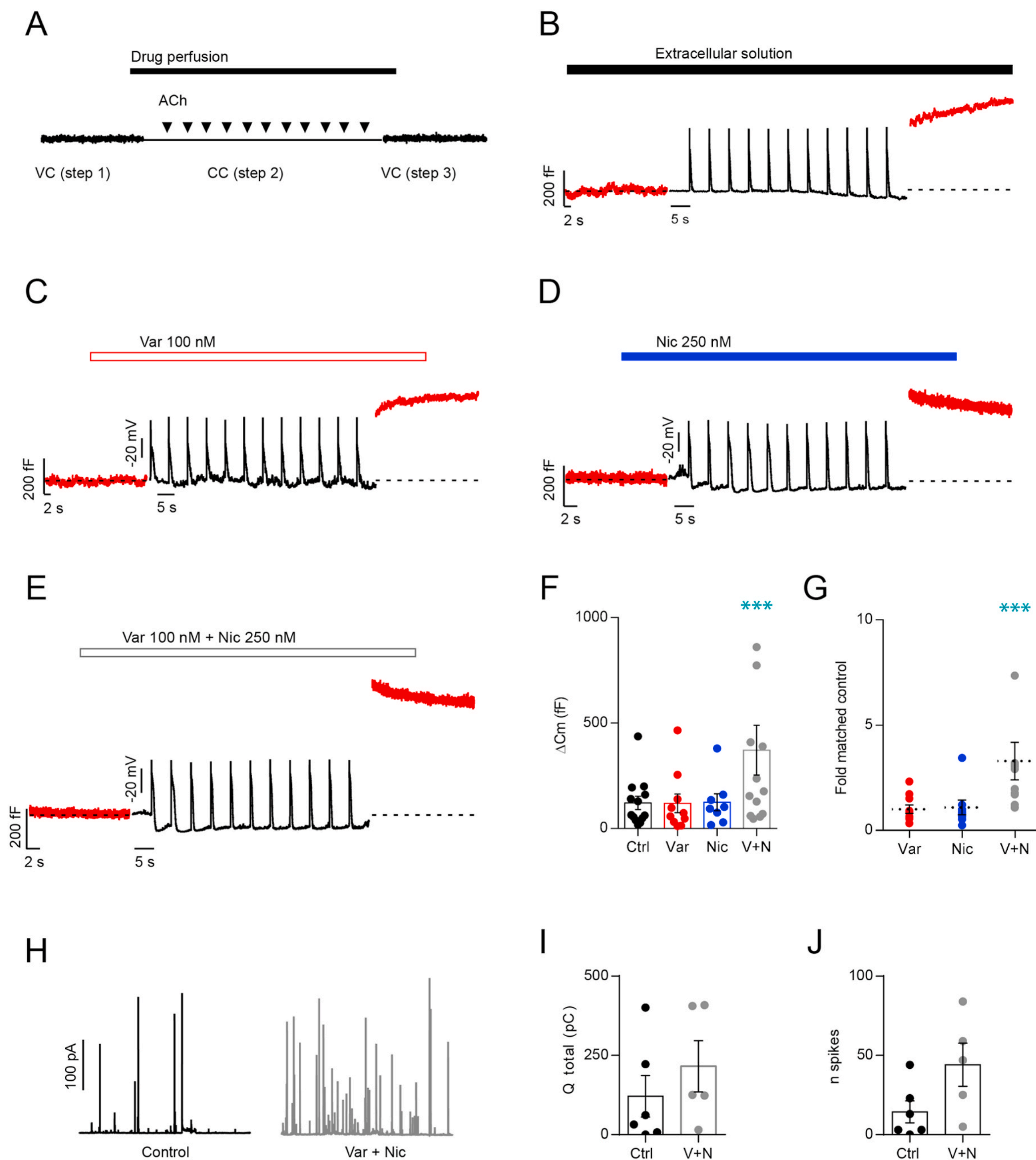


Fig. 1. Exocytosis is increased in the presence of varenicline and nicotine in human chromaffin cells. A) Scheme of the triple step protocol in which C_m is recorded in Steps 1 and 3, and the membrane potential in Step 2. Pulses (10 ms) of 300 μM ACh are applied at 0.2 Hz in the second step. Drug perfusion takes place 15 s before and after, and during the 1 min duration of this step. B-E) Representative recordings of the triple step protocol under control conditions, in the presence of varenicline (100 nM), nicotine (250 nM) and both nicotinic agonists together, respectively. F**)** C_m increments represented as a dot plot graph (*mean ± SEM, n = 8-13 from six organ donors, 2-tailed Wilcoxon matched-pairs signed rank test*). **G)** Fold times of C_m increment of each value normalized with respect to its control in each cell (*mean ± SEM; n = 8-13, 2-tailed Wilcoxon matched-pairs signed rank test*). H) Representative amperometric recordings obtained under control conditions (left panel) and varenicline (100 nM) and nicotine (250 nM) (right panel) perfusion. I) Total charge and number of spikes (J) of the amperometric recordings obtained under control and varenicline plus nicotine conditions (*n = 5, from one organ donor, no statistical tests could be run*).

inner, 0.5 mm). The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp headstage, and back filled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. Amperometric currents were recorded using an EPC10 USB amplifier and PatchMaster software running on a PC. The sampling rate was 14.5 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments using 50 μM adrenaline as standard solution. Only fibers that rendered 200–300 pA of current increment after 50 μM adrenaline pulse were used for the experiments. The bath solution was the same used in the patch-clamp electrophysiology experiments.

2.4. Data and statistical analysis

Statistical analysis was conducted with GraphPad Prism. Data are given as mean ± SEM for the number (n) of cells. For each result, the t value, degree of freedom and exact p value are provided. The normality test Kolmogorov–Smirnov was first performed, and then data were compared using the corresponding two tailed paired tests. In some

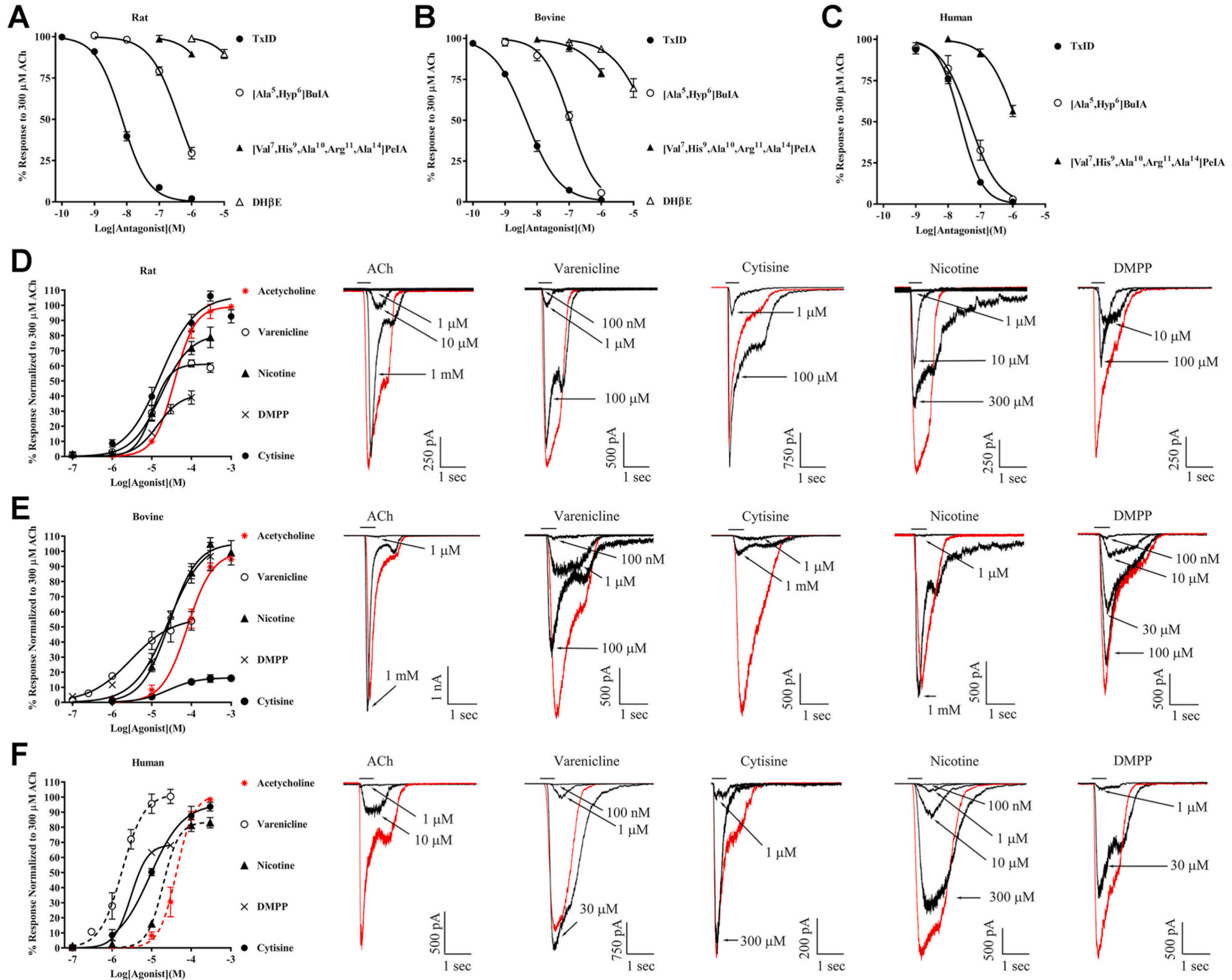

A
Rat
B
Bovine
C
Human
% Response to 300 μM ACh
Log[Antagonist](M)
TxID
[Ala5,Hyp6]BuIA
[Val7,His9,Ala10,Arg11,Ala14]PeIA
DHβE
D
E
F
% Response Normalized to 300 μM ACh
Log[Agonist](M)
Acetycholine
Varenicline
Nicotine
DMPP
Cytisine
ACh
Varenicline
Cytisine
Nicotine
DMPP
1 μM
10 μM
1 mM
100 nM
100 μM
300 μM
30 μM
250 pA
500 pA
750 pA
200 pA
1 nA
1 sec

Fig. 2. Non-α7 nAChRs in rat, bovine, and human adrenal chromaffin cells are mainly of the α3β4* subtype. A-C) Concentration-response curves were generated for a number of antagonists in order to probe the expression of various nAChR subtypes in rat (A), bovine (B) and human (C) chromaffin cells. The cells were subjected to patch-clamp electrophysiology as described in Methods. Briefly, cells were incubated in extracellular solution containing α-Bgtx (1 μM) for 5 min to irreversibly inhibit the α7 subtype, and then they were placed in the electrophysiology chamber. Cells were stimulated with 200 ms pulses of 300 μM ACh until a steady baseline was achieved, then perfused with extracellular solution containing increasing concentrations of antagonists to determine the pIC_{50} values. They are provided in Table 1. The responses to ACh were nearly completely inhibited by TxID (1 μM), suggesting that essentially all of the response was mediated by α3β4* nAChRs. The error bars denote the SEM from at least 4 cells for each experimental determination. Data for human adrenal chromaffin cells were adapted from Hone et al. (2015) and Hone et al. (2017). D-F) Concentration-response curves for selected nAChR agonists in rat (D), bovine (E) and human (F) chromaffin cells. Adrenal chromaffin cells were stimulated with 500 ms pulses of increasing concentrations of agonists and the data fit to the Hill equation to determine the pEC_{50} values for activation; potency and efficacy values are provided in Tables 2 and 3 All agonists responses were normalized to the responses evoked by 300 μM ACh obtained in the same cell. The error bars denote the SEM from 3 to 8 cells. All experiments in rat and bovine adrenal chromaffin cells were performed in cells that had been treated with 1 μM α-Bgtx for 5 min prior to being placed in the electrophysiology chamber. Experiments in human cells were performed in the sustained presence of 100 nM [Leu^{11},Asp^{16}]ArIB. Dashed lines in the graph of human data indicate data adapted from Hone et al. (2017).

experiments, each drug value was normalized with respect to the corresponding matched control value due to the high variability of the responses. In this case, data are given as X-fold matched control. In the case of nonparametric tests, Wilcoxon matched-pairs signed rank test is reported as the sum of signed ranks (W), number of pairs and exact p value. Individual data points are shown for every experiment, except for concentrations-response curves, for which data tables are provided.

Antagonist concentration-response curves were generated according to the following procedures: the peak amplitudes of three control responses were averaged and the level of inhibition by the antagonist was determined by dividing the peak amplitude of the response in the presence of the antagonist by the averaged control response to obtain a percent response value. In the case of the agonists, EC_{50} and efficacy values were obtained as follows: for the ACh curves, the current amplitudes for ACh-evoked responses for each individual cell were fit to the Hill equation to obtain the calculated plateau value for activation. The observed ACh-evoked responses were then normalized to the calculated plateau value to obtain a percent response. All other non-ACh agonist responses were normalized to those obtained by 300 μM ACh in the same cell. Data used to obtain concentration-response relationships for agonists were analyzed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and Igor Pro 4.08 (Wavemetrics, Lake Oswego, OR, USA).

In the amperometry experiments, data came from a single donor due to the low number of transplants performed during the coronavirus pandemic situation. In this case, no statistical analysis was performed, and data are reported as tendencies. The analysis of the amperometry data was performed using IGOR Pro software and macros that allow the analysis of single events and the rejection of overlapping spikes (Mosharov and Sulzer, 2005). A threshold of 4.5 times the first derivative of the noise standard deviation was calculated to clearly detect amperometry events. Among the events whose first derivative was above this threshold, only those showing one peak and one rising and falling phase were considered as single spikes. To minimize variability among cells, the overall mean of average spike values recorded in several single cells was used.

2.5. Peptides

Solid-phase Fmoc peptide chemistry was used to synthesize the α-conopeptides as described previously (Whiteaker et al., 2007) or using an AAPPTec Apex 396 automated peptide synthesizer (AAPPTec, Louisville, KY, USA) (Hone al. 2013).

2.6. Reagents

ACh chloride, (−)-nicotine tartrate, dimethylpiperazine (DMPP), cytisine, HEPES, amphotericin B, penicillin/streptomycin, protease type XIV, collagenase Type I, poly-D-lysine hydrobromide, Red Blood Cell Lysis solution, dimethylsulfoxide, and all salts were purchase from Sigma-Aldrich (St. Louis, MO, USA). Varenicline tartrate was purchased from Tocris Bioscience (Abindgon, UK). DMEM and Glutamax were purchased from Life Technologies (Carlsbad, CA, USA). FBS was from LabClinics (Barcelona, Spain) and the D-glucose from Panreac (Barcelona, Spain).

3. Results

3.1. Exocytosis and catecholamine release elicited by ACh was increased by varenicline and nicotine in human chromaffin cells

In the present study, we investigated the effect of varenicline, a drug successfully used in the treatment of tobacco addiction, on the exocytosis elicited by ACh in human chromaffin cells obtained from organ donors. At the beginning of the treatment with varenicline, patients often continue smoking for at least 2 weeks. Thus, here we investigated the effect of 100 nM varenicline alone and in combination with nicotine (250 nM or 110 nM nicotine were used to investigate its acute and chronic consumption, respectively) on the exocytotic process that leads to catecholamine secretion. The concentration of varenicline (100 nM) was the maximum plasma concentration achieved in patients consuming a therapeutic dose of 1 mg twice a day (Faessel et al., 2006; Ravva et al., 2009; Kikkawa et al., 2011). Nicotine at 250 nM was the arterial plasma concentration achieved the first 10 min after smoking 1–3 cigarettes, one puff/min for 10 min (Gourlay and Benowitz, 1997). The chronic treatment of nicotine was performed using 110 nM concentration of the drug because this was the average steady-state plasma concentration achieved by healthy adult smokers (Faessel et al., 2006).

In this study, a modification of the triple-step protocol reported by Pérez-Alvarez and Albillos (2007) was employed to measure the agonist-induced exocytosis by means of C_m changes. This method combines the voltage-clamp and the current-clamp configurations of the patch-clamp technique. First, the potential of the cell was fixed at −60 mV in human cells and at −55 mV in rat cells. The initial C_m was recorded in the voltage-clamp configuration (Step 1) for 60 s. Then by switching to the current-clamp configuration, 11 pulses (10 ms) of 300 μM ACh at 0.2 Hz were applied to closer mimic the physiological stimulation in which the ACh released by the splanchnic nerve basally stimulates chromaffin cells (Hone et al., 2017) (Step 2). Drugs were perfused 15 s before, during and 15 s after the current-clamp part of the protocol. Finally, by returning to the voltage-clamp mode, the increase produced in C_m could be recorded, reflecting the overall exocytosis evoked by the agonist (Step 3) (Fig. 1A).

Exocytosis was determined as the difference in the C_m values obtained between the Steps 3 and 1 in human chromaffin cells obtained from adrenal glands of six donors (three males and three females). Representative recordings obtained under control conditions (Fig. 1B), or after the different treatments with 100 nM varenicline or 250 nM nicotine applied separately (Fig. 1C and D, respectively), or both together (Fig. 1E), are displayed. Absolute average values and the corresponding normalized values with respect to matched controls are plotted in Fig. 1F and G, respectively. The exocytosis achieved in control

Table 1
Antagonist potencies for α3β4* nAChRs expressed by adrenal chromaffin cells.

Antagonist	Rat		Bovine		Human	
	pIC_{50}	n	pIC_{50}	n	pIC_{50}	n
TxID	8.14 ± 0.02	4	8.34 ± 0.03	6	7.62 ± 0.03	4
[Val[7],His[9],Ala[10],Arg[11],Ala[14]]PeIA	5.04 ± 0.65	4	5.18 ± 0.27	5	5.75 ± 0.08	4
[Ala[5],Hyp[6]]BuIA	6.39 ± 0.04	6	6.98 ± 0.04	5	5.04 ± 0.06	7
DHβE	<5.00	6	<5.00	6	ND	

Antagonist potencies were determined by fitment of the data with the Hill equation; ± denotes the SEM; 'n' denotes the number of cells.

Table 2
Agonist potencies for α3β4* nAChRs expressed by adrenal chromaffin cells.

Agonist	Rat		Bovine		Human	
	pEC_{50}	n	pEC_{50}	n	pEC_{50}	n
Acetylcholine	4.43 ± 0.06	4	4.10 ± 0.06	5	4.37 ± 0.04	8
Varenicline	4.98 ± 0.06	4	5.53 ± 0.24	5	5.75 ± 0.08	8
Cytisine	4.78 ± 0.12	6	4.58 ± 0.26	5	5.04 ± 0.06	4
Nicotine	4.77 ± 0.14	6	4.56 ± 0.08	5	4.71 ± 0.11	8
DMPP	4.85 ± 0.11	5	4.58 ± 0.14	6	5.52 ± 0.05	4

Agonist potencies were determined by fitment of the data with the Hill equation; '±' denotes the SEM, 'n' denotes the number of cells.

cells was 121.2 ± 31.6 fF (n = 13), ranging from 17.5 fF to 437 fF. Varenicline alone evoked 119.3 ± 44.9 fF and nicotine elicited 124.2 ± 40.4 fF, which represented a 1 ± 0.2 and 1.1 ± 0.4-fold increment with respect to the matched control values, respectively. However, 100 nM varenicline in the presence of 250 nM nicotine triggered a C_m increase of 371.7 ± 118 fF (W(13) = -91, p = 0.0002). These values were normalized, and represented a 3.3 ± 0.9-fold increase with respect to the control condition (W (13) = -91, p = 0.0002).

The membrane potential of the cells did not change after perfusing the different drugs. Initially, under control conditions, it exhibited a value of −57.8 mV ± 1.4 mV (n = 13). Then it amounted to −58.3 ± 5.1 mV (n = 10), −58.5 ± 1 mV (n = 8), and −53.3 ± 2.3 mV (n = 13) with varenicline and nicotine alone, and in the presence of both drugs, respectively.

Additionally, a series of experiments to determine the release of catecholamines triggered by varenicline (100 nM) and nicotine (250 nM) was performed. Amperometry carbon fiber electrodes were used in human chromaffin cells obtained from one donor (26 year old, female). The protocol consisted of 11 pulses of 300 μM ACh applied at 0.2 Hz. Drugs were perfused 15 s before, during and 15 s after the ACh pulses were applied. Representative original recordings of the amperometry spikes recorded under control and nicotinic stimulation conditions are shown in Fig. 1H. The total charge of the catecholamines released by varenicline in the presence of nicotine (Fig. 1I), and the number of spikes triggered (Fig. 1J), showed a tendency to increase with respect to the control condition.

3.2. Rat and bovine chromaffin cells were tested as alternative species to further investigate the effect of varenicline and nicotine: antagonists and agonists effects on rat, bovine and human chromaffin cells

Due to the scarce availability of human cells, we also investigated the effect of varenicline, nicotine and other nAChR agonists on rat and bovine chromaffin cells in order to find a non-human species in which these compounds elicit the most similar results to human. This would allow further investigation of this and related issues without the limits of cell availability.

Table 3
Agonist efficacies for α3β4* nAChRs expressed by adrenal chromaffin cells.

Agonist	Rat		Bovine		Human	
	% Relative Efficacy	Hill Slope	% Relative Efficacy	Hill Slope	% Relative Efficacy	Hill Slope
Acetylcholine	99 ± 3	2.4 ± 0.3	100 ± 5	2.0 ± 0.2	101 ± 3	2.0 ± 0.2
Varenicline	61 ± 3	2.0 ± 1.8	57 ± 8	0.8 ± 0.3	103 ± 7	2.6 ± 0.4
Cytisine	106 ± 6	1.0 ± 0.2	16 ± 2	2.2 ± 0.6	94 ± 3	2.2 ± 0.3
Nicotine	81 ± 7	2.7 ± 0.4	105 ± 5	2.2 ± 0.5	83 ± 3	2.2 ± 0.7
DMPP	41 ± 5	2.5 ± 0.5	107 ± 12	1.0 ± 0.2	69 ± 2	2.1 ± 0.2

Percent relative efficacy is defined as the estimated plateau value determined by fitment of the concentration-response data with the Hill equation; ± denotes the SEM.

First, we characterized the non-α7 nAChR in these species by testing different antagonists in cells pretreated with α-bungarotoxin (α-BgTx) to inhibit α7 nAChR. These compounds were α-conotoxins (α-CTxs) TxID, [Ala[5],Hyp[6]]BuIA and [Val[7],His[9],Ala[10],Arg[11],Ala[14]] PeIA, which target human (Hone et al., 2015, 2017) and rat (Luo et al., 2013) α3β4, β4*-, and α6*-containing nAChRs, respectively, and DHβE, which targets human and rat α4β2 and α4β4 nAChRs (Chavez-Noriega et al., 1997).

The activities of the antagonists were determined by applying 200 ms pulses of ACh once every 2 min until a steady baseline response was achieved. The control solution was then switched to one containing the antagonist of interest and perfused until a steady state level of inhibition was observed. The curves obtained for rat and cow are shown in Fig. 2A and B. The α-Ctxs [Ala[5],Hyp[6]]BuIA, [Val[7],His[9],Ala[10],Arg[11],Ala[14]] PeIA and TxID were investigated previously in human chromaffin cells in our lab (Hone et al., 2015, 2017). Data were plotted here for comparison with the other species (Fig. 2C). The IC_{50} values obtained for the various antagonists are shown in Table 1. In the presence of 1 μM TxID, the ACh-evoked responses were inhibited by 98.0 ± 0.5% (n = 4) in rat (A) and 98.7 ± 0.6% (n = 6) in cow (B). They were inhibited by 98.8 ± 0.3% in human (Hone et al., 2017). Very little inhibition was observed with DHβE at concentrations expected to inhibit β2-containing nAChRs. These results indicated that the predominant non-α7 nAChR subtype expressed by all three species is α3β4*.

In a different set of experiments, increasing concentrations of various nicotinic agonists (ACh, varenicline, nicotine, DMPP and cytisine) were tested in chromaffin cells of the three species. The concentration-response curves of the different agonists and the representative recordings of the nicotinic currents elicited in the three species by them are plotted in Fig. 2D–F. The data of ACh, varenicline and nicotine were previously reported in human cells (Hone et al., 2017), and showed here for ease of comparison with the other species curves. Agonist potencies and efficacies are summarized in Tables 2 and 3, respectively. Potency and efficacy values of ACh, varenicline and nicotine in the three species do not differ substantially to justify to choose rat or bovine. Therefore, the greater availability of rats as a model organism was the final criteria to select this species to perform the rest of the experiments of this study.

3.3. Exocytosis elicited by ACh was increased by varenicline, nicotine or by both agonists together in rat chromaffin cells

The final goal of the use of rat chromaffin cells was to investigate whether the chronic treatment of the cells with nicotine, which will mimic the situation of a chronic smoker, would increase exocytosis. In a latter step, we investigated whether the effects of these drugs on exocytosis could be due to an effect on the nicotinic currents. First, we

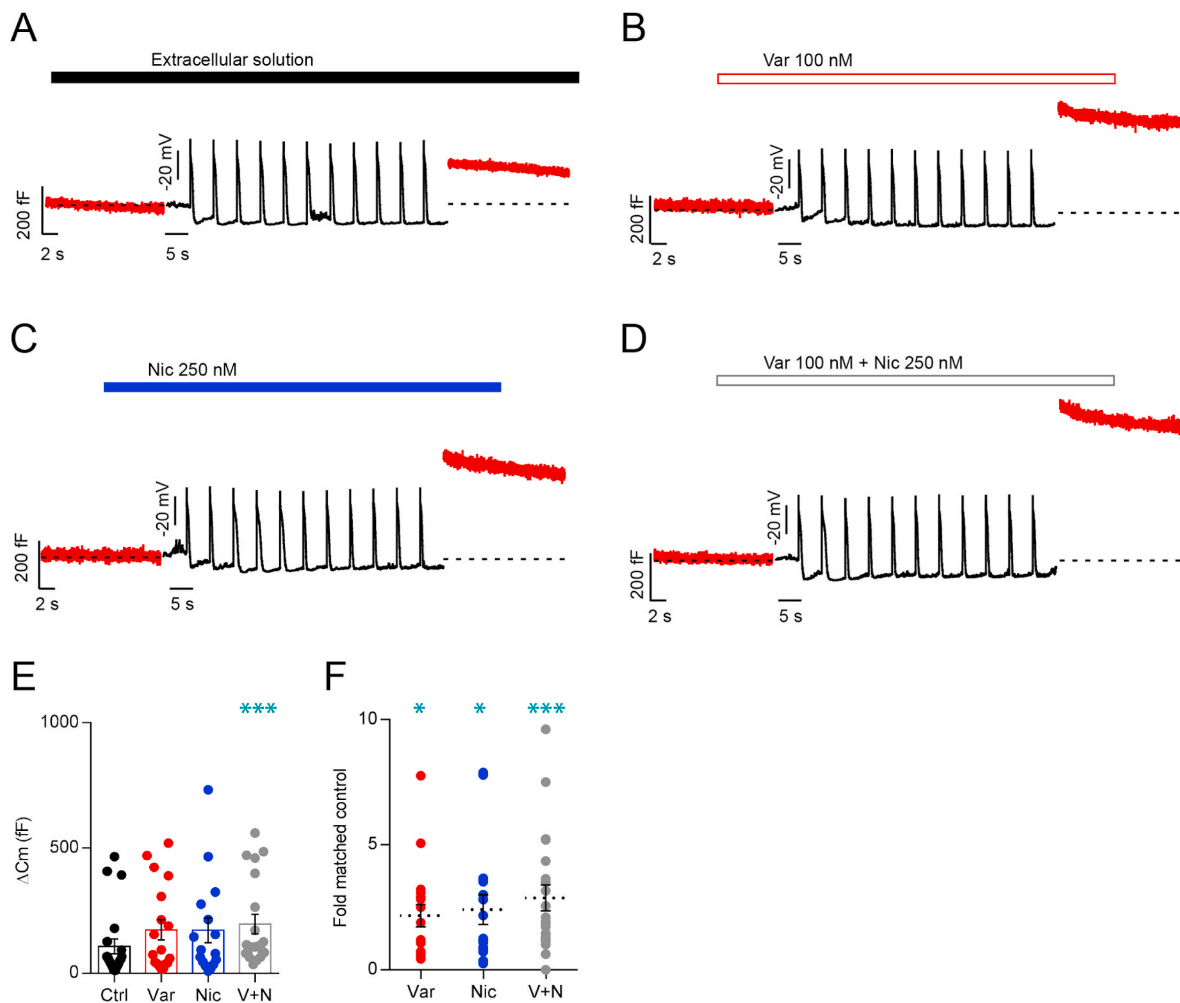


Fig. 3. Exocytosis is increased in the presence of varenicline, nicotine and both drugs together in rat chromaffin cells. A-D) Representative recordings of the triple step protocol under control (A), varenicline (100 nM) (B), nicotine (250 nM) (C) and varenicline plus nicotine (D) conditions in rat chromaffin cells. E) C_m increments are represented as a dot plot graph (*mean ± SEM, n = 16-21 from ten rats, 2-tailed Wilcoxon matched-pairs signed rank test*). F) Fold times of C_m increment of each agonist with respect to its control in each cell (*mean ± SEM; n = 16-21 from ten rats, 2-tailed Wilcoxon matched-pairs signed rank test*).

investigated whether previous results obtained in Fig. 1 were reproducible in rat chromaffin cells. We performed the same protocol described in Fig. 1 in chromaffin cells of ten rats. Representative recordings under the control condition, varenicline, nicotine or both drugs together are shown in Fig. 3A–D, respectively. Exocytosis elicited under control conditions amounted to 107.5 ± 30.1 fF (n = 21). The same protocol was tested with 100 nM varenicline or 250 nM nicotine, eliciting 173.6 ± 40.6 fF (n = 18) and 171.8 ± 49.2 fF (n = 16) of exocytosis respectively. Varenicline and nicotine evoked 196.5 ± 38.9 fF (W(20) = -186, p = 0.0001) (n = 20) of exocytosis. There was a wash-out period of 5 min between the different treatments. Average values are shown in Fig. 3E.

In order to compare these responses and due to the high variability of the control responses (from 24 fF to 466 fF), each result was compared and normalized with its own control. Drugs had a significantly higher response when applied separately. Varenicline increased the response to 2.2 ± 0.4-fold (W(18) = 93, p = 0.0432) (n = 18) and nicotine increased it to 2.4 ± 0.6 fold (W(16) = 76, p = 0.0507) (n = 16). Both drugs perfused together increased by 2.9 ± 0.5-fold the exocytotic response (W (20) = 197, p = 0.0002) (n = 20) (Fig. 3F).

There was no change in the membrane potential when the different drugs were perfused during the Step 2. Under control conditions, the membrane potential was −56.4 ± 2 mV (n = 21), and it reached the values of −57.3 ± 2.7 mV (n = 18), −57 ± 2.6 mV (n = 16) and −58 ± 2 mV (n = 20) in the presence of varenicline, nicotine and varenicline perfused together with nicotine, respectively.

3.4. Exocytosis elicited by ACh was increased by varenicline, nicotine and by both together in rat chromaffin cells treated chronically with nicotine

In a different set of experiments, chromaffin cells from six rats were incubated with 110 nM nicotine for 24 h and then perfused during the whole experiment. The same protocol as in Fig. 1 was performed. Representative original C_m traces are drawn in Fig. 4A–D for control conditions, and in the presence of varenicline, nicotine and both drugs applied together, respectively. The exocytosis achieved in control cells amounted to 58.2 ± 6.1 fF (n=8). Varenicline alone evoked 120.7 ± 20.7 fF (W(6) = -21, p = 0.0313) (n = 6) and nicotine triggered 160.9 ± 28.5 fF (W(6) = -21, p = 0.0313) (n = 6). Varenicline and nicotine elicited 186.5 ± 32.3 fF (t(7) = 4.031, p = 0.0050) (n = 8). The average values of C_m are displayed in Fig. 4E and the normalized values with respect to its own control are shown in Fig. 4F. Varenicline alone exhibited an increase in the exocytotic value of 2.4 ± 0.5 fold (W(6) = 21, p = 0.0313) (n = 6), and nicotine triggered 3.1 ± 0.6 fold more exocytosis with respect to control (W(6) = 21, p = 0.0313) (n = 6). The response elicited by varenicline together with nicotine was 3.4 ± 0.7-fold with respect to the control response (t(7) = 3.475, p = 0.0103) (n = 8). Thus, exocytotic responses were also increased by varenicline alone

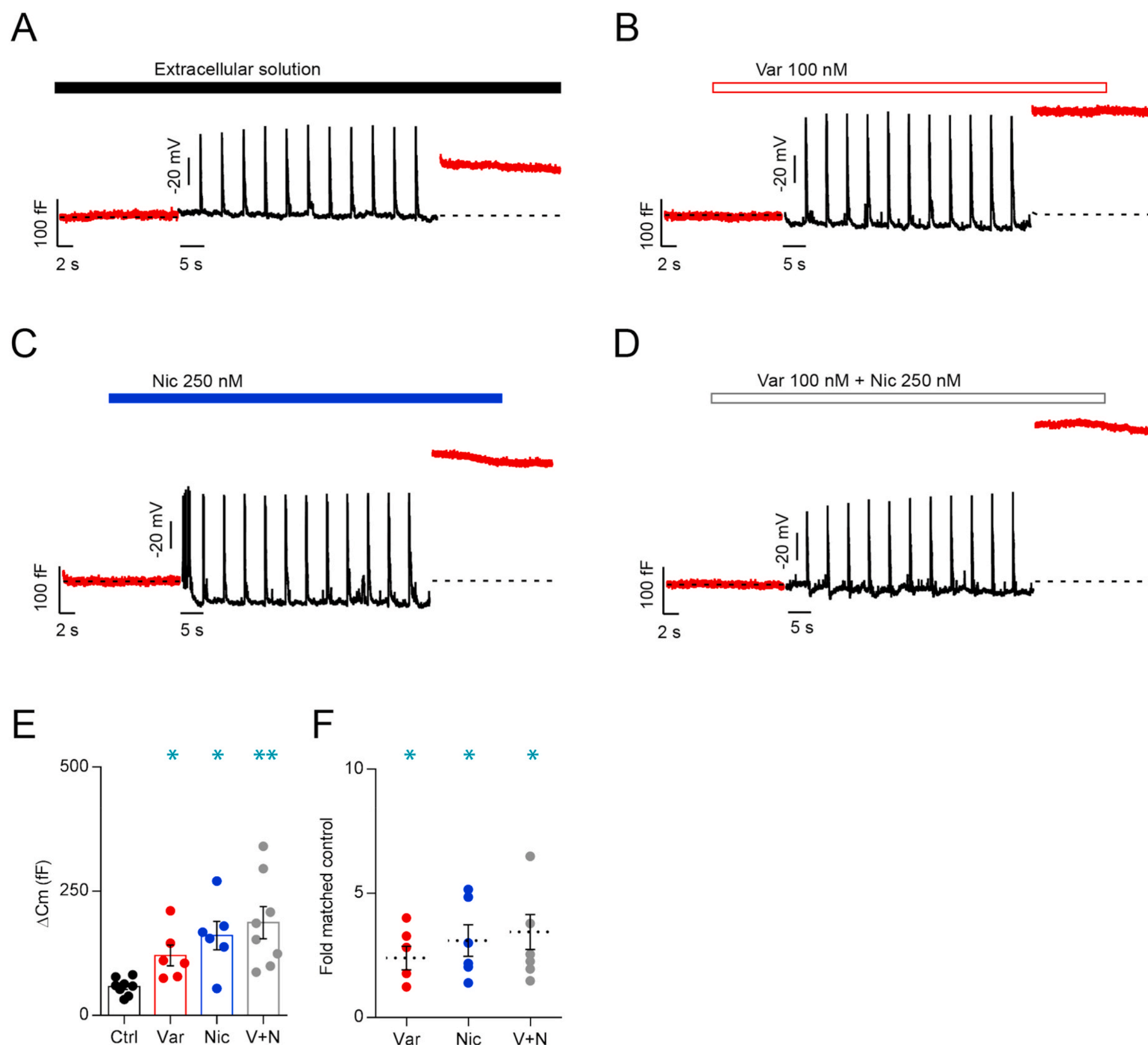


Fig. 4. The chronic treatment with nicotine does not lead to further increase of exocytosis. Cells were treated during 24 h with nicotine 110 nM and then the different agonists were applied on top of the continuous application of nicotine 110 nM. Representative recordings of the triple step protocol under control (A), varenicline (100 nM) (B), nicotine (250 nM) (C) and varenicline plus nicotine (D) conditions in rat chromaffin cells. **E)** C_m increments are represented as a dot plot graph (*mean ± SEM, n = 6-8 cells from six rats, 2-tailed Wilcoxon matched-pairs signed rank test*). **F)** Fold times of C_m increment of each agonist with respect to its control in each cell (*mean ± SEM; n = 6-8 from six rats, 2-tailed Wilcoxon matched-pairs signed rank test*).

or in the presence of nicotine, when the chronic nicotine treatment was applied.

The values achieved for the membrane potential in these cells were −62.2 ± 1.3 mV (n=8), −57.8 ± 7.5 mV (n = 6), −55.2 ± 3.4 mV (n = 6) and −51.5 ± 2.6 mV (t(6) = 4.307, p = 0.0051) (n = 8), for control conditions, varenicline and nicotine alone, and both drugs perfused together, respectively. This reflects that nicotine, in the acute and chronic treatment, may depolarize the cell membrane in the presence of varenicline.

3.5. *Effect of varenicline and nicotine as agonists on nicotinic currents and evoked exocytosis and on the ACh-evoked currents in rat chromaffin cells*

In order to determine whether the increased exocytosis observed might be due to the agonist effect of the drugs, they were applied in 500 ms pulses in chromaffin cells obtained from six rats. Representative original recordings of the currents elicited by each agonist are shown in Fig. 5A. ACh triggered a current of 671.1 ± 127.3 pA (n = 11), while varenicline elicited currents that amounted to 316.8 ± 143.6 pA (W(11) = 56, p = 0.0098) (n = 11), nicotine to 70.5 ± 24.5 pA (t(8) = 5.35, p = 0.0007) (n = 9), and varenicline in the presence of nicotine to 81.5 ± 33.7 pA (t(8) = 4.955, p = 0.0011) (n = 9) (Fig. 5B). Thus, varenicline, nicotine and both drugs applied together evoked smaller currents with respect to ACh. In order to diminish the variability among data, values were normalized, being 0.1 ± 0.9 (t(8) = 19.87, p < 0.0001) (t(10) = 4.998, p = 0.0005) and 0.4 ± 1.3 (t(8) = 40.56, p < 0.0001) and 0.1 ± 0.02 fold with respect to the matched control condition for each value in the presence of both drugs perfused together, and varenicline and nicotine alone, respectively.

These currents elicited exocytosis as shown by the C_m recordings of Fig. 5C. ACh evoked 121.3 ± 34 fF (n = 11), varenicline triggered 147.5 ± 51.1 fF (n = 11), nicotine evoked 114 ± 46.6 fF (n=9) and both together elicited 54.6 ± 20.2 fF (n = 9) (Fig. 5D). These values were normalized with respect to the corresponding matched control, amounting to 1.5 ± 0.5 and 1 ± 0.1- fold for varenicline and nicotine perfused separately, and 0.4 ± 0.2- fold (W(9) = 39, p = 0.0195) for varenicline and nicotine perfused together, respectively.

Finally, the ACh-elicited currents obtained from ten rats were recorded while perfusing varenicline or/and nicotine. Drugs inhibited nicotinic currents as shown in the representative recordings displayed in Fig. 5E. ACh evoked a current of 1242 ± 141.7 pA (n = 20), that was

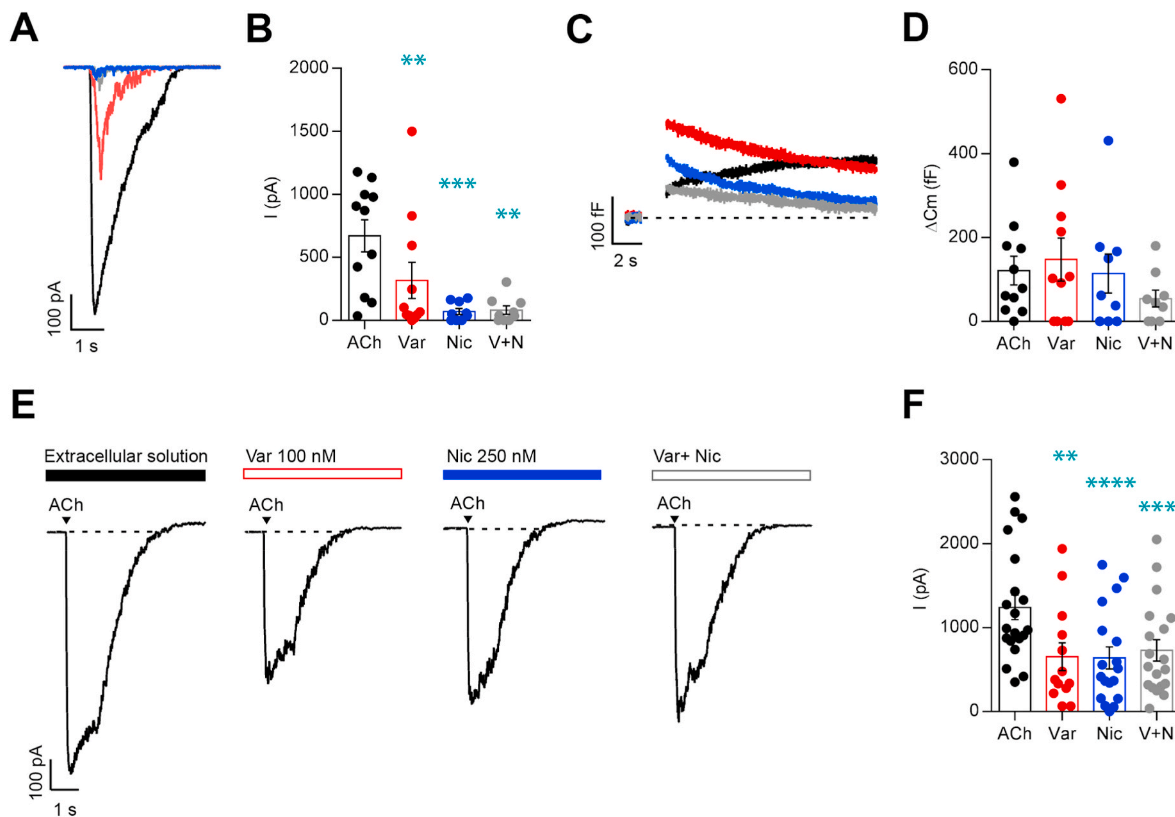


Fig. 5. Nicotinic currents and exocytosis elicited by the nicotinic agonists in the absence and presence of ACh in rat chromaffin cells. A) Representative current traces elicited by 500 ms pulses of ACh (black), varenicline 110 nM (red), 250 nM nicotine (blue), and varenicline and nicotine applied together (grey). **B)** Dot plot of the currents achieved with the different agonists *(mean ± SEM, n = 10-12 from six rats, 2-tailed paired t-test)*. **C)** Representative C_m recordings corresponding to the currents evoked by the agonists applied in A. **D)** Dot plot of the C_m increment achieved with the different agonists *(mean ± SEM, n = 10-12 from six rats, 2-tailed paired test)*. **E)** Representative current traces elicited by 500 ms ACh pulses in the presence of the agonists. **F)** Dot plot of the peak currents achieved by ACh in the presence of varenicline, nicotine or both agonists together *(mean ± SEM, n = 14-22 from 10 rats, 2-tailed Wilcoxon matched-pairs signed rank test)*.

reduced to 653.7 ± 164.6 pA in the presence of varenicline (W(13) = 81, p = 0.0024) (n = 13), to 639.7 ± 131.6 pA with nicotine (W(18) = 163, p < 0.0001) (n = 18) and to 729.8 ± 127.4 pA when both drugs were applied together (W(19) = 160, p = 0.0005) (n = 19). These values represented 0.6 ± 0.1 (W(13) = 81, p = 0.0024), 0.5 ± 0.1 (W(18) = 165, p < 0.0001) and 0.6 ± 0.1 (W(19) = 170, p = 0.0002) fold with respect to their control matched currents, respectively (Fig. 5F).

4. Discussion

Varenicline is a drug widely used in smoking-cessation therapy. It inhibits the reinforcing effects of nicotine and reduces craving and withdrawal symptoms by modulating dopaminergic function through partial agonism of α4β2* nAChRs (Coe et al., 2005; Gonzales et al., 2006; Jorenby et al., 2006; Rollema et al., 2007). However, varenicline therapy is associated with increased frequency of some cardiovascular side effects such as heart attack. Because the drug acts also on α3β4* and α7 nAChRs, the main receptors expressed in human chromaffin cells (Hone et al., 2015; Pérez-Alvarez et al., 2012a,b), it is possible that varenicline potentiates the release of catecholamines from the adrenal gland, contributing to those undesirable effects. Two previous studies support this idea. First, therapeutic concentrations of varenicline (100 nM) increase the firing of action potentials in chromaffin cells in the presence of nicotine (Hone et al., 2017). Second, a case of varenicline-triggered pheochromocytoma crisis in a smoking subject has been reported (Hukkanen et al., 2010). Varenicline is 10-fold more potent and a full agonist relative to ACh, and nicotine is 2-fold more potent and behaved as a partial agonist with respect to ACh (Hone et al., 2017). This might explain why varenicline could trigger pheochromocytoma crisis in the case report (Hukkanen et al., 2010), whereas nicotine derived from smoking did not. Moreover, the patient of the case report had no other known crisis triggers, and she had a documented visit to the emergency department for palpitations six years before the admission, suggesting that she may have suffered pheochromocytoma for years without crisis. Furthermore, two 0.5 mg doses of varenicline results in plasma concentrations of about 75 nM (Kikkawa et al., 2011), which increases the electrical activity when nicotine is present (Hone et al., 2017) and the exocytosis and catecholamine release in human chromaffin cells (present study), something that happens in a smoker that begin the smoking cessation therapy.

In this study, we confirmed our hypothesis by measuring C_m as an index of exocytosis elicited by very short pulses of ACh applied at 0.2 Hz in the presence of varenicline, nicotine or both drugs together. We determined that varenicline, at concentrations that could be achieved in smoking cessation therapy, increased exocytosis in human chromaffin cells obtained from organ donors when it was perfused together with nicotine at concentrations that would be achieved in the plasma after smoking, or/and during chronic consumption of tobacco. Furthermore, this enhanced exocytosis corresponds to an increase in the release of catecholamines as determined by carbon-fiber amperometry. Although these last data were obtained from only a single donor, it shows a tendency to increase catecholamine release by varenicline and nicotine. This is shown by the rise in the total charge and the number of amperometric spikes elicited by the application of both drugs (each spike reflects the content of a single secretory vesicle being released as shown by Wightman et al., 1991).

Next, we wanted to investigate whether chronic administration of nicotine (in order to mimic the situation of a smoker) could enhance the

exocytosis elicited by varenicline. Also, we wanted to know whether varenicline and nicotine could be increasing the nicotinic currents, explaining the increase in exocytosis. However, the COVID-19 pandemic has severely limited the number of organ transplants preventing the continuation of this study in human cells. Therefore, we aimed at investigating these issues in non-human species such as rat or cow. We first characterized the non-α7 nAChRs in these species using a panel of agonist and antagonist ligands. We performed experiments with different nAChR antagonists that confirmed the presence of an α3β4* nAChR subtype in the three species. Also, the DHβE and the PeIA analog responses suggest very few α4-and α6-containing receptors in the three species. Thus, as the differences found were not large among these species, we performed concentration-response curves with different agonists that would help us to determine which species was the most similar to the human one in relation to the nicotinic response. We found that the α3β4* nAChR subtypes of the three species also exhibited similar sensitivity to agonists. Therefore, we chose the rat species to continue with the experiments due to the greater availability of rats.

We first performed the same protocol in rat chromaffin cells to that conducted in human chromaffin cells, to determine the effects of the drugs on exocytosis. We found that varenicline in the presence of nicotine, as well as varenicline and nicotine separately, increased exocytosis. Chronic treatment with nicotine at a concentration achieved in plasma by smokers (110 nM) (Faessel et al., 2006) also resulted in an increase in the exocytosis elicited with varenicline or nicotine applied independently or together. Differences with human cells might be due to the fact that young rats were used in the experiments (one month and a half), while the experiments in human were performed in 50–70 years old subjects. Functional differences might also account for the different activity of varenicline and nicotine on rat and human nAChR auxiliary subunits, reflecting that nAChR subtypes of human and rat chromaffin cells are not identical.

On the other hand, varenicline elicited smaller currents than ACh at the concentrations used in the present study (100 nM varenicline, 300 μM ACh), which would not explain the increase in exocytosis. Neither the effect of varenicline or nicotine perfusion while pulses of ACh were applied could explain the increase in exocytosis obtained, because these agonists inhibited the ACh-evoked currents. Further research will enable understanding of the mechanisms by which these agonists increased the exocytotic response and whether those processes depend on age.

5. Conclusions

The present study reports that the combination of varenicline and nicotine increases the exocytosis of chromaffin vesicles in human and rat chromaffin cells. This finding might help to explain the increased number of heart attacks reported in subjects taking varenicline. It should be considered when administering this drug to patients with cardiovascular risk.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Amanda Jiménez-Pompa: Methodology, Project administration, Formal analysis, Writing – review & editing. **Sara Sanz-Lázaro:** Investigation. **Arik J. Hone:** Investigation, Writing – review & editing, Formal analysis. **Lola Rueda-Ruzafa:** Investigation. **José Medina-Polo:** Resources. **Carmen González-Enguita:** Resources. **Jesús Blázquez:** Resources. **Cristóbal de los Ríos:** Resources. **J. Michael McIntosh:** Resources, Writing – review & editing, Funding acquisition. **Almudena Albillos:** Methodology, Project administration, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition.

Acknowledgements

The authors thank Dr. Luis Gandía and Dr. Antonio García for the supply of bovine adrenal glands. This work was supported by grants from the Spanish Ministry of Science and Innovation [grant number BFU2015-69092 to A.A.] and the U.S. National Institutes of Health [GM136430 and GM103801 to J.M.M].

References

Capelli, A.M., Castelletti, L., Chen, Y.H., Van der Keyl, H., Pucci, L., Oliosi, B., Powell, A., 2011. Stable expression and functional characterization of a human nicotinic acetylcholine receptor with α6β2 properties: discovery of selective antagonists. Br. J. Pharmacol. 163 (2), 313–329.

Chang, P., Chiang, C., Ho, W., Wu, P., Tsai, J., Guo, F., 2015. Combination therapy of varenicline with nicotine replacement therapy is better than varenicline alone: a systematic review and meta-analysis of randomized controlled trials. BMC Publ. Health 15 (1), 1–8.

Chavez-Noriega, L.E., Crona, J.H., Washburn, M.S., Urrutia, A., Elliott, K.J., Johnson, E. C., 1997. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 expressed in xenopus oocytes. J. Pharmacol. Exp. Therapeut. 280 (1), 346–356.

Coe, J.W., Brooks, P.R., Vetelino, M.G., Wirtz, M.C., Arnold, E.P., Huang, J., Fox, C.B., 2005. Varenicline: an α4β2 nicotinic receptor partial agonist for smoking cessation. J. Med. Chem. 48 (10), 3474–3477.

Di Angelantonio, S., Matteoni, C., Fabbretti, E., Nistri, A., 2003. Molecular biology and electrophysiology of neuronal nicotinic receptors of rat chromaffin cells. Eur. J. Neurosci. 17 (11), 2313–2322.

Faessel, H.M., Gibbs, M.A., Clark, D.J., Rohrbacher, K., Stolar, M., Burstein, A.H., 2006. Multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy smokers. J. Clin. Pharmacol. 46 (12), 1439–1448.

Gershon, A.S., Campitelli, M.A., Hawken, S., Victor, C., Sproule, B.A., Kurdyak, P., Selby, P., 2018. Cardiovascular and neuropsychiatric events after varenicline use for smoking cessation. Am. J. Respir. Crit. Care Med. 197 (7), 913–922.

Gonzales, D., Rennard, S.I., Nides, M., Oncken, C., Azoulay, S., Billing, C.B., Reeves, K.R., 2006. Varenicline, an α4β2 nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking cessation: a randomized controlled trial. Jama 296 (1), 47–55.

Gourlay, S.G., Benowitz, N.L., 1997. Arteriovenous differences in plasma concentration of nicotine and catecholamines and related cardiovascular effects after smoking, nicotine nasal spray, and intravenous nicotine. Clin. Pharmacol. Ther. 62 (4), 453–463.

Hone, A.J., Ruiz, M., Scadden, M., Christensen, S., Gajewiak, J., Azam, L., McIntosh, J. M., 2013. Positional scanning mutagenesis of alpha-conotoxin PeIA identifies critical residues that confer potency and selectivity for alpha6/alpha3beta2beta3 and alpha3beta2 nicotinic acetylcholine receptors. J. Biol. Chem. 288 (35), 25428–25439.

Hone, A.J., McIntosh, J.M., Azam, L., Lindstrom, J., Lucero, L., Whiteaker, P., Albillos, A., 2015. A-conotoxins identify the α3β4* subtype as the predominant nicotinic acetylcholine receptor expressed in human adrenal chromaffin cells. Mol. Pharmacol. 88 (5), 881–893.

Hone, A.J., Michael McIntosh, J., Rueda-Ruzafa, L., Passas, J., de Castro-Guerín, C., Blázquez, J., Albillos, A., 2017. Therapeutic concentrations of varenicline in the presence of nicotine increase action potential firing in human adrenal chromaffin cells. J. Neurochem. 140 (1), 37–52.

Hone, A.J., Rueda-Ruzafa, L., Gordon, T.J., Gajewiak, J., Christensen, S., Dyhring, T., McIntosh, J.M., 2020. Expression of α3β2β4 nicotinic acetylcholine receptors by rat adrenal chromaffin cells determined using novel conopeptide antagonists. J. Neurochem.

Hukkanen, J., Ukkola, O., Benowitz, N.L., 2010. Varenicline and pheochromocytoma. Ann. Intern. Med. 152 (5), 335–336.

Jorenby, D.E., Hays, J.T., Rigotti, N.A., Azoulay, S., Watsky, E.J., Williams, K.E., Varenicline Phase 3 Study Group, 2006. Efficacy of varenicline, an α4β2 nicotinic acetylcholine receptor partial agonist, vs placebo or sustained-release bupropion for smoking cessation: a randomized controlled trial. Jama 296 (1), 56–63.

Kikkawa, H., Maruyama, N., Fujimoto, Y., Hasunuma, T., 2011. Single-and multiple-dose pharmacokinetics of the selective nicotinic receptor partial agonist, varenicline, in healthy Japanese adult smokers. J. Clin. Pharmacol. 51 (4), 527–537.

Luo, S., Zhangsun, D., Zhu, X., Wu, Y., Hu, Y., Christensen, S., McIntosh, J.M., 2013. Characterization of a novel α-conotoxin TxID from conus textile that potently blocks rat α3β4 nicotinic acetylcholine receptors. J. Med. Chem. 56 (23), 9655–9663.

Mihalak, K.B., Carroll, F.I., Luetje, C.W., 2006. Varenicline is a partial agonist at α4β2 and a full agonist at α7 neuronal nicotinic receptors. Mol. Pharmacol. 70 (3), 801–805.

Moro, M.A., López, M.G., Gandía, L., Michelena, P., Garcia, A.G., 1990. Separation and culture of living adrenaline-and noradrenaline-containing cells from bovine adrenal medullae. Anal. Biochem. 185 (2), 243–248.

Mosharov, E.V., Sulzer, D., 2005. Analysis of exocytotic events recorded by amperometry. Nat. Methods 2 (9), 651–658.

Munarini, E., Marabelli, C., Pozzi, P., Boffi, R., 2015. Extended varenicline treatment in a severe cardiopathic cigarette smoker: a case report. J. Med. Case Rep. 9 (1), 1–4.

Pérez-Alvarez, A., Albillos, A., 2007. Key role of the nicotinic receptor in neurotransmitter exocytosis in human chromaffin cells. J. Neurochem. 103 (6), 2281–2290.

Pérez-Alvarez, A., Hernández-Vivanco, A., Alonso y Gregorio, S., Tabernero, A., McIntosh, J.M., Albillos, A., 2012a. Pharmacological characterization of native α7 nicotinic ACh receptors and their contribution to depolarization-elicited exocytosis in human chromaffin cells. Br. J. Pharmacol. 165 (4), 908–921.

Pérez-Alvarez, A., Hernández-Vivanco, A., McIntosh, J.M., Albillos, A., 2012b. Native α6β4* nicotinic receptors control exocytosis in human chromaffin cells of the adrenal gland. Faseb. J. 26 (1), 346–354.

Prochaska, J.J., Hilton, J.F., 2012. Risk of cardiovascular serious adverse events associated with varenicline use for tobacco cessation: systematic review and meta-analysis. BMJ 344, e2856.

Ravva, P., Gastonguay, M.R., Tensfeldt, T.G., Faessel, H.M., 2009. Population pharmacokinetic analysis of varenicline in adult smokers. Br. J. Clin. Pharmacol. 68 (5), 669–681.

Rigotti, N.A., Pipe, A.L., Benowitz, N.L., Arteaga, C., Garza, D., Tonstad, S., 2010. Efficacy and safety of varenicline for smoking cessation in patients with cardiovascular disease: a randomized trial. Circulation 121, 221–9.

Rollema, H., Chambers, L.K., Coe, J.W., Glowa, J., Hurst, R.S., Lebel, L.A., Rovetti, C.C., 2007. Pharmacological profile of the α4β2 nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. Neuropharmacology 52 (3), 985–994.

Selçuk, E.B., Sungu, M., Parlakpinar, H., Ermiş, N., Taslıdere, E., Vardı, N., Karatas, M., 2015. Evaluation of the cardiovascular effects of varenicline in rats. Drug Des. Dev. Ther. 9, 5705.

Singh, S., Loke, Y.K., Spangler, J.G., Furberg, C.D., 2011. Risk of serious adverse cardiovascular events associated with varenicline: a systematic review and meta-analysis. CMAJ (Can. Med. Assoc. J.) 183 (12), 1359–1366.

Tammimäki, A., Herder, P., Li, P., Esch, C., Laughlin, J.R., Akk, G., Stitzel, J.A., 2012. Impact of human D398N single nucleotide polymorphism on intracellular calcium response mediated by α3β4α5 nicotinic acetylcholine receptors. Neuropharmacology 63 (6), 1002–1011.

Wightman, R.M., Jankowski, J.A., Kennedy, R.T., Kawagoe, K.T., Schroeder, T.J., Leszczyszyn, D.J., Near, J.A., Diliberto Jr., E.J., Viveros, O.H., 1991. Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. Proc. Natl. Acad. Sci. U. S. A. 88 (23), 10754–10758.

Whiteaker, P., Christensen, S., Yoshikami, D., Dowell, C., Watkins, M., Gulyas, J., Rivier, J., Olivera, B.M., McIntosh, J.M., 2007. Discovery, synthesis, and structure activity of a highly selective alpha7 nicotinic acetylcholine receptor antagonist. Biochemistry 46 (22), 6628–6638.