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1 **The alpha-7 nicotinic acetylcholine receptor is involved in a direct inhibitory effect of nicotine on**
2 **GnRH release: in vitro studies**

3 Running title: Effect of nicotine on GnRH neurons

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5 Elio Messi[a], Federica Pimpinelli[a], Valentina Andrè[a], Chiara Rigobello[a], Cecilia Gotti[b,c] and Roberto
6 Maggi[a,c]

7 [a]*Dept. of Pharmacological and Biomedical Sciences, Università degli Studi di Milano, Milan, Italy*

8 [b]*CNR, Neuroscience Institute-Milano, Biometra University of Milan, Milan, Italy*

9 [c] C.G. and R.M. equally contributed to the work.

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11 **Corresponding author**

12 Roberto Maggi, PhD
13 Dept. of Pharmacological and Biomolecular Sciences
14 Università degli Studi di Milano
15 Via G. Balzaretti, 9
16 20133 Milano (Italy)
17 Phone: +39 0250318233 Fax: +39 0250318204
18 email: roberto.maggi@unimi.it
19

20 **Abstract**

21 The activation of nicotinic cholinergic receptors (nAChR) inhibits the reproductive axis; however, it
22 is not clear whether nicotine may directly modulate the release of hypothalamic gonadotropin-
23 releasing hormone (GnRH). Experiments carried out in GT1-1 immortalized GnRH neurons reveal
24 the presence of a single class of high affinity $\alpha4\beta2$ and $\alpha7$ nAchR subtypes. The exposure of GT1-1
25 cells to nicotine does not modify the basal accumulation of GnRH. However, nicotine was found to
26 modify GnRH pulsatility in perifusion experiments and inhibits, the release of GnRH induced by
27 prostaglandin E_1 or by K^+-induced cell depolarization; these effects were reversed by D-
28 tubocurarine and α-bungarotoxin. In conclusion, the results reported here indicate that: functional
29 nAChRs are present on GT1-1 cells, the activation of the α-bungarotoxin-sensitive subclass ($\alpha7$)
30 produces an inhibitory effect on the release of GnRH and that the direct action of nicotine on
31 GnRH neurons may be involved in reducing fertility of smokers.

32

33 **Keywords**

34 GnRH, nicotine, prostaglandin E1, neurons, reproduction

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41 **1. Introduction**

42 Cigarette smoking may have adverse effects on fertility. It has been shown that the activation of
43 nicotinic acetylcholine receptors (nAChR) inhibits the activity of the hypothalamus-pituitary-
44 gonadal axis at several levels. For example, it has been reported that nicotine inhibits
45 steroidogenesis in Leydig cells as well as sperm motility and oocyte maturation (Condorelli et al.,
46 2013; Gocze et al., 1996; Yamamoto et al., 1998; Zenzes, 2000).
47 *In vivo* and *in vitro* activation of acetylcholine receptors leads to either stimulatory or inhibitory
48 effects on GnRH secretion (Kalra and Kalra, 1983; Kawai et al., 2013; Koren et al., 1992; Richardson
49 et al., 1982), leaving the nature of action of cholinergic input on GnRH neurons unresolved.
50 It has also been shown that the specific activation of nAChR affects the hypothalamic-pituitary-
51 gonadal axis. In particular, nicotine induces a decrease of the release of luteinizing hormone
52 possibly acting at the pituitary level (Blake et al., 1972; Fiorindo and Martini, 1975; Kanematsu and
53 Sawyer, 1973; Motta et al., 1973; Zemkova et al., 2013). However, it is not clear whether nicotine
54 can modulate the release of the hypothalamic gonadotropin releasing hormone (GnRH).
55 It has been shown that nicotine administration may inhibit the activity of the gonadotropin-
56 releasing hormone (GnRH) pulse generator in ovariectomized rats (Sano et al., 1999). However, it
57 has been postulated that nicotine action could be mediated by a potentiation of the inhibitory
58 tone exerted by opioid peptides on GnRH/gonadotropin release (Hodson et al., 1997); conversely,
59 experimental evidence might exclude this hypothesis (Sano et al., 1999). Kimura and coworkers
60 (Kimura et al., 2004) have further reported that, in the cultured embryonic olfactory placode,
61 nicotine inhibits GnRH secretion through a release of GABA and the consequent activation of
62 GABA-A receptor system.

63 It cannot be excluded that the nicotinic cholinergic system might also act directly on GnRH
64 secreting neurons, as acetylcholine induces a rapid, but transient, stimulation of GnRH release in
65 perifused hypothalamic and immortalized GnRH neurons (Krsmanovic et al., 1998).
66 Considering that the affinity of nicotine to nAChR ranges from pM to nM values and that after
67 inhalation of a single puff of cigarette smoke, the nicotine concentration in human arterial plasma
68 rise to a peak of about 50-100 ng/ml (0.3-0.6 M) in about 20 sec (Crandall et al., 1989), an effect
69 of nicotine on GnRH release should be carefully evaluated.
70
71 Neuronal nAChRs are a heterogeneous family of acetylcholine (Ach)-gated channels with a
72 pentameric structure resembling that of muscle AchRs. Mammalian nAChRs can be subdivided
73 into two main classes: homomeric or heteromeric α-bungarotoxin (αBgtx)–sensitive receptors
74 consisting of α7, α9, α9-α10, α7β2 subunits, and αBgtx-insensitive heteromeric receptors
75 consisting of α2–α6 and β2–β4 subunits (reviewed in (Millar and Gotti, 2009; Zoli et al., 2015)).
76 The two classes of receptors are characterized by distinct pharmacological profiles wherein: the
77 αBgtx-insensitive heteromeric receptors are bound by nicotine agonists with a very high affinity
78 but not by the antagonist αBgtx, whereas the αBgtx–sensitive receptors are bound by agonists
79 with lower affinity but with high αBgtx affinity (Dutton and Craik, 2001). All of the nAChR subtypes,
80 but in particular the α7 subtype, show pronounced permeability for Ca^{2+} relative to Na^{+}; and many
81 of the biological functions identified for nAChRs have been associated with receptor-mediated
82 changes in intracellular Ca^{2+} concentration which modulate the release of several
83 neurotransmitters. Data from distribution of nAChR in mouse brain indicate that 42 and 7 are
84 present in the hypothalamic region (see (Millar and Gotti, 2009) for a review) while α9 is
85 expressed predominantly in hair cells of the cochlea.
86 In the present work we evaluated the presence of nicotinic receptors and the nature of a direct
87 effect of nicotine on GnRH secretion in a neuronal isolated system using cell lines of immortalized
88 hypothalamic GnRH neurons, an *in vitro* model widely used to study the mechanisms that control
89 GnRH release in controlled conditions (Glidewell-Kenney et al., 2013; Gore and Roberts, 1997;
90 Krsmanovic et al., 1998; Maggi et al., 1995; Maggi et al., 1995; Maggi et al., 2000; Mellon et al.,
91 1990; Pal et al., 2007; Pimpinelli et al., 1999).

92 **2. Materials and Methods**

93 *2.1 Chemicals*

94 Nicotine tartrate and prostaglandin E_1 (PGE_1) were from Sigma Chemicals (St.Louis, MO),
95 nonradioactive epibatidine α-bungarotoxin (αBgtx) and D-tubocurarine (D-Tub) were from
96 Tocris Bioscience (Bristol, UK). When not specified, other reagents were from Sigma Chemicals
97 (St.Louis, MO).

98

99 *2.2 Cell cultures*

100 GT1-1 cells, generously provided by Dr. R.I. Weiner (San Francisco, CA) through Dr. B. Marchetti
101 (Catania, Italy) and human embryonic kidney cells (HEK)293 were routinely grown in monolayer at
102 37 °C in a humidified CO2 incubator in Dulbecco's Minimum Essential Medium (DMEM) containing
103 1 mM sodium pyruvate, 100 mg/ml streptomycin, 100 U/ml penicillin and 10 mg/l of phenol red
104 (Biochrom KG, Berlin, Germany) and supplemented with 10% fetal calf serum (FCS, Gibco, Grand
105 Island, NY). The medium was replaced at 2-day intervals. Subconfluent cells were routinely
106 harvested by trypsinization and seeded in 57 cm^2 dishes (1 x 10^6 cells) for propagation. For all the
107 experiments, GT1-1 cells within 6 passages were used.

108 *Receptor binding*

109 Subconfluent GT1-1 or HEK293 cells were detached from the subconfluent culture using
110 phosphate buffer saline (PBS) containing 1 mM PMFS, washed twice by centrifugation,
111 resuspended in 50 mM TrisHCl pH 7.4 containing 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 5 mg/ml
112 mixture of the protease inhibitors leupeptin, bestatin, pepstatin A, aprotinin and 1 mM PMFS and
113 homogenized to obtain a crude membrane preparation.

114 All the incubations were performed in a buffer containing 50 mM Tris-HCl pH 7, 150 mM NaCl, 5
115 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 2 mg/ml BSA.

116 The binding of ^{3}H-Epibatidine (Epi, epibatidine specific activity: 66 Ci/mmol was purchased from
117 Perkin Elmer (Waltham, MA, USA), and ^{125}I-α-bungarotoxin (αBgtx, specific activity: 122.8
118 Ci/mmol was purchased from Perkin Elmer (Waltham, MA, USA) were performed by a
119 homogeneous saturation binding assay according to a mixed protocol combining both saturation
120 of labeled ligand (the first 7-8 concentrations of respectively 0.005-5 nM and 0.01-20 nM) and
121 displacement curves (the last 3-4 concentrations of 50-1000 nM for both ligands) with unlabeled
122 ligand (Rovati et al., 1989). By effectively combining both saturation and competition protocols in
123 a single curve, high ligand concentrations can be reached without using excessive amounts of
124 labeled ligand (the competition part of the curve), while retaining adequate radioactivity in the
125 lower concentration range (the saturation part of the curve).

126 Non-specific binding (averaging 5-10% of total binding for Epi and 25-30% of total binding for
127 αBgtx was determined in the presence of 2 µM unlabeled ligands.

128 Saturation experiments were performed by incubating aliquots of GT1-1 crude membrane
129 preparations with ligands overnight at 20°C. At the end of the incubation, the samples were
130 filtered through a 24-channel Brandel cell harvester on GFC filters presoaked in buffer +1% BSA,
131 five times with ice-cold buffer, and the bound radioactivity then determined by means of liquid
132 scintillation spectrometer (Packard 1600 CA, Packard, Milano, Italy) with 60% of efficiency.

133

134 *2.3 RT-PCR assay*

135 For expression studies, cells were washed with cold PBS, and collected with TRIzol (Invitrogen) and
136 total RNA was extracted following the manufacturer's protocol. Mouse hypothalami, collected
137 from adult animals, were homogenized in TRIzol and RNA extracted following the manufacturer's
138 protocol. One microgram of total RNA was subjected to cDNA synthesis with Superscript II reverse
139 transcriptase (Invitrogen), using random hexamers according to standard procedures. PCRs were

140 performed using Taq PCR Core Kit (Qiagen) and the following oligonucleotides; (α4) forward 5'-
141 CAATGTACACCACCGCTCAC-3' and reverse 5'-TGGTCTGACACTGGAAGCTG-3', (α7) forward 5'-
142 GCACCTCATGCATGGTACAC-3' and reverse 5'-ATCCAGAGTGGGCAATGAC-3', (α9) forward 5'-
143 CCTTGCGTCCTCATATCGTT-3' and reverse 5'-CCCTGGAAGTTTGCCATAAA-3', (β2) forward 5'-
144 TGGCTGTGTTCAGGGGTTTT-3' and reverse 5'-CCTCAATCTTGCATGCGCTC-3'.
145 The mouse *Gapdh* gene was analyzed as housekeeping gene with the following PCR primers:
146 forward 5'-GGCCCCTCTGGAAAGCTGTGG-3' and reverse 5'-TCTTGCTCAGTGTCCTTGCTGGG-3'.
147 Amplification products were separated by 1% agarose gel electrophoresis and detected by
148 ethidium bromide fluorescence on a UV transilluminator (Bio-Rad).
149
150 *2.4 GnRH release form perifused GT1-1 cells*
151 Perifusion experiments were performed as already described (Magni et al., 1999). In brief, GT1–1
152 cells were grown on Cytodex-3 beads (Pharmacia Biotech, Uppsala, Sweden). After 3–4 days, cells
153 were loaded into temperature-controlled glass syringes, the final cell-matrix volume was adjusted
154 to 0.15 ml. Chambers were perifused at a flow rate of 10 ml/h with Locke's, gassed with 95% O2–
155 5% CO2 at 37 C. After a 2-h equilibration period, samples were collected every 90 sec and stored
156 at -20 C until radioimmunoassayed for GnRH. Cells were perifused for the first hour with Locke's
157 medium, and then with medium containing nicotine (500 μM) for 30 min. GnRH pulses were
158 identified and their parameters were determined by a computer algorithm cluster analysis
159 (Veldhuis and Johnson, 1986). The occurrence and the duration of pulses are shown above each
160 plot.
161
162 *2.5 GnRH accumulation in GT1-1 cell culture medium*
163 GT1-1 neurons were plated in 24-well plates (0.5 x 10^6 cells/cm^2) and used after five days of
164 culture. Art the day of the experiment, cells were washed with 1 ml of DMEM (prewarmed at 37°
165 C) and, when not otherwise specified, incubated for 30 min in DMEM containing the substances to
166 be tested. At the end of the incubation period, the medium was collected, centrifuged for 5 min at
167 12.000 rpm and the supernatant stored at -70 C until the GnRH RIA (Maggi et al., 1995). The cells
168 remaining in the culture wells were collected in 0.2 M NaOH, and assayed for protein content
169 using a microassay with human serum albumin as a standard. No variations of total protein/well
170 were detected in all the experimental groups (data not shown).
171 For the experiments performed in the presence of depolarizing extracellular concentrations of K^+
172 ions, DMEM was substituted by Locke's medium containing 5.6 mM or 56 mM K^+ (Pimpinelli et al.,
173 2003).
174
175 *2.6 GnRH radioimmunoassay*
176 The concentration of GnRH in the media and in the fractions collected during the perifusion as well
177 as static experiments was determined by RIA using a commercial antibody (Cod. L-8391, Sigma
178 Chemicals, St Louis, MO) and iodinated GnRH (Amersham, Milano, Italy). The GnRH standard was
179 from NovaBiochem (Laufelfingen, Switzerland) (Maggi et al., 1995; Pimpinelli et al., 1999). All
180 samples were run in duplicate; the detection limit was 3.9 pg/ml. The inter- and intra-assay
181 coefficients of variation were 9.4% and 6.6%, respectively. Each experiment was repeated at least
182 three times.
183
184 *2.7 cAMP assay*
185 For cAMP experiments, GT1-1 cells were plated in 24-wells plates (0.5 x 10^6 cells/cm^2) and used
186 after three days of culture (Pimpinelli et al., 1999). All the samples were assayed for protein
187 content using a microassay with human serum albumin as a standard. Intracellular cAMP

188 accumulation was measured over a 15-minutes incubation period with 1 µM of prostaglandin E1
189 (PGE_1) (Sigma, St. Louis, MO), as activators of adenylyl cyclase, after a 10 minutes preincubation
190 with 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma Chemicals, St. Louis, MO). A commercial
191 available binding-protein assay kit (Amersham, Milano, Italy) was used to evaluate cAMP levels in
192 ethanol extracted cells according to manufacturers' instructions.
193
194 *2.8 Statistical analysis*
195 Receptor binding experiments were optimized with the program DESIGN (Rovati et al., 1990) and
196 the results further analyzed by the program LIGAND (Munson and Rodbard, 1980). The statistic
197 software PRISM was used to analyze the dose-response curves, by a four parameter non-linear
198 regression, and the other results, by ANOVA and adequate post-hoc tests (Dunnett's or
199 Bonferroni's test).
200
201

202 **3. Results**

203 *3.1 Detection of nAChR in GT1-1 cells*
204 A series of experiments were performed to investigate, using different approaches, the presence
205 of nAChRs in GT1-1 cells.
206 Receptor binding assays on GT1-1 membrane preparations were carried out using labeled Epi, an
207 azabicycloheptane alkaloid exerting potent nicotinic agonist action for heteromeric receptors, and
208 αBgtx that binds with high affinity the homomeric nAChR. Epi binds to several heteromeric nAChR
209 subtypes with a Kd in the pM range whereas its binding to the α7 homomeric subtype is in the nM
210 range and competitive with that of αBgtx.
211 We then analyzed the saturation binding of Epi on separate preparations of GT1-1 cell
212 homogenates in the presence of 2 μM unlabeled αBgtx to avoid possible binding to homomeric
213 receptors. In these conditions, a specific and saturable Epi binding with a Kd of 13 pM and a Bmax
214 (mean + SE) of 6.6 + 3.5 fmol/mg of protein was observed (Fig. 1A).
215 Saturation binding experiments performed with αBgtx, also revealed a single high affinity site with
216 a Kd of 2.8 nM and a Bmax (mean + SE) of 16.6 + 3.5 fmol/mg of protein (Fig. 1B).
217 As a control the binding of both Epi and αBgtx was tested on membrane preparations from
218 HEK293 cells, that does not express nAchR (Chavez-Noriega et al., 2000; Craig et al., 2004). The
219 results, shown in Fig. 1 A and B clearly indicate a negligible, mainly nonspecific, binding of the two
220 ligands impossible to resolve for parameter estimation by LIGAND program.
221 These results indicate the presence of independent high affinity binding sites for both Epi and
222 αBgtx on GT1-1 cell membranes, and suggest the presence of at least two distinct populations of
223 nAChR subtypes.
224 Based on data indicating the presence of α4β2 and α7 nAChR subunits in mouse hypothalamus
225 and transcriptomic analysis (Affymetrics mouse 2.0, data not shown) of GT1 cell extracts revealing
226 the presence of signals for α7, α4 and β2 nicotine receptor subunits, we analyzed the presence of
227 specific transcripts for these nicotine receptor subunits by RT-PCR. The transcript analysis, carried
228 out on total RNA extracted from GT1-1 cells, showed the presence of transcripts for α7, α4 and β2
229 subunits of nAChR (Fig. 1C). The transcript for α9 subunit, analyzed as a control, was not
230 detectable in GT1-1 cell extract (data not shown).
231
232 *3.2 Effects of the activation of nAChR by nicotine on basal GnRH release*
233 The effect of receptor activation on the accumulation of GnRH released from GT1-1 cells was
234 evaluated. Graded concentrations of nicotine (1-500 μM) and/or nicotine antagonists (αBgtx and
235 D-Tub) were added to GT1-1 cells maintained in basal culture conditions and GnRH accumulation
236 in the culture medium over a 30 min interval was evaluated by radioimmunoassay. Nicotine or its
237 antagonists did not significantly affect basal GnRH release from GT1-1 cells (Fig. 2A). Similarly,
238 exposure to the nAChR antagonists αBgtx and D-Tub did not significantly affect GnRH release.
239
240 *3.3 Effects of the activation of nAChR by nicotine on pulsatile GnRH release*
241 To explore whether the possible action of nicotine receptor activation on GnRH release could have
242 modified the dynamic, rather than the total amount, of GnRH release, a series of perifusion
243 experiments were performed.
244 GT1-1 cells were cultured as described in Materials and Method and perifused for 30 minutes with
245 a 500 μM solution of nicotine. This concentration was selected considering the results obtained in
246 the experiment on GnRH accumulation and the consolidated notion that nAch requires high
247 concentrations of agonist to couple binding to channel opening and, conversely, low
248 concentrations of agonist may induce a rapid desensitization of receptors followed by an increase

in their number (Quick and Lester, 2002); this phenomenon may produce biphasic responses that complicate biological interpretation. The medium was collected at different time intervals during the infusion and subjected to radioimmunoassay.

As expected, we found that the basal release of GnRH by GT1–1 cells is intrinsically pulsatile (Fig. 2B) with a mean pulse frequency of 2.58 ± 0.54 pulses/hour (mean ± SD), as detected by cluster analysis on 6 independent experiments (Magni et al., 1999). After a 30-60 min of preconditioning in basal conditions, nicotine (at 500 μM concentration) was added to the perifusion medium. The cells were exposed to nicotine for 30 min followed by a 30-40 min of washing The results show a change of the secretory peaks characterized by a significantly increased pulse frequency (5.13 ± 1.03 pulse/hour; $p<0.05$), associated with a reduced pulse duration during nicotine exposure and for few minutes after the withdrawal of treatment withdrawal, followed by a rapid return to a basal frequency (3.25 ± 0.35 pulse/hour)(Fig. 2B). The baseline secretory activity of GT1–1 cells, as well as their response to nicotine were different among perifusion experiments due to intrinsic features of this system; however, the effect of nicotine was qualitatively similar when measured over independent experiments. However, in agreement with static experiments, the amount of GnRH released, calculated as the area under the curve, was not significantly different before and during nicotine exposure (data not shown).

3.4 Effects of the activation of nAChR by nicotine on PGE_1 stimulated GnRH release and cAMP accumulation

Since the release of hypothalamic GnRH is regulated by several stimulatory inputs impinging on GnRH neurons (Hrabovszky and Liposits, 2013), we explored the possibility that the activation of nAChRs could exert an inhibitory effect on stimulated GnRH release.
The release of GnRH was induced by exposure of GT1-1 cells to PGE_1 as a GnRH secretagogue (Maggi et al., 1995; Pimpinelli et al., 1999) and treated with nicotine and the two nAChR antagonists □□T□□ and αBgtx.
Under these conditions, nicotine shows a significant and dose-dependent inhibition (IC_{50} 214.0 ± 26.6 μM) of GnRH accumulation induced by exposure of the cells to PGE_1 (1 mM) (Fig. 3A). The inhibitory effect of nicotine is completely reversed by the presence of either of the nicotinic general nAChR antagonist D-Tub or antagonist of homomeric nAChR αBgtx, which, when given alone, do not significantly affect GnRH release in either basal or stimulated conditions (Fig. 3B and 3C).
Since in GT1-1 cells the secretagogue effect of PGE_1 is mediated by intracellular accumulation of cAMP (Pimpinelli et al., 1999), we analyzed whether the effect of nicotine was mediated by modifications of the formation of this intracellular second messenger. The results (Figure 4) indicate that nicotine exposure does not affect the PGE_1-induced of cAMP accumulation in GT1-1 cells suggesting that a different intracellular pathway is involved.

3.5 Effects of nicotine on high K^+-induced GnRH release

The effect of nicotine under GnRH stimulation by direct GT1-1 cell depolarization was subsequently investigated. During direct cell depolarization, induced by the exposure to high extracellular K^+ concentration (56 mM) (Pimpinelli et al., 2003), nicotine still inhibits the release of GnRH in a dose-dependent manner (IC_{50} 121.7 ± 18.8 μM) (Fig. 5A); an effect that is reversed by the presence of either of the nicotine antagonists (Fig. 5B and 5C).

295 **4. Discussion**

296 At least two classes of functional nAChR receptors are expressed in GT1-1 immortalized GnRH
297 neurons. One class of such receptors is bound by Epi, a potent cholinergic agonist (Houghtling et
298 al., 1995), with high affinity (pM range) and specificity. Epi is known to interact with high affinity
299 with many heteromeric nAch receptors, and in particular with α4β2 (Gotti et al., 1997), the most
300 represented form in mouse brain and hypothalamus (Flores et al., 1992; Millar and Gotti, 2009).
301 However, it may also bind homomeric α7 nAch receptors, although with an affinity that is four
302 orders of magnitude lower (Sullivan et al., 1994).
303 Labeled αBgtx was also found to bind, with nM affinity, to a single class of sites on GT1-1 cell
304 membranes. αBgtx selectively interacts with muscle nicotinic receptors (α1) and homomeric α7,
305 expressed in different neural tissues, suggesting the presence of these receptors (McGehee and
306 Role, 1995).
307 The presence of nAChR in GT1-1 cells was then confirmed by RT-PCR experiments that revealed
308 the presence of transcripts specific for α7 , α4 and β2 nAChR subunits.
309 Considering the limitations of the *in vitro* cell model, these findings are in agreement with the
310 reported main distribution of α7 and α4β2 nAChR in several hypothalamic structures and in the
311 median eminence (Clarke et al., 1985; Dominguez del Toro et al., 1994; Flores et al., 1992; Michels
312 et al., 1986; Millar and Gotti, 2009), the brain region where the axons of GnRH-secreting neurons
313 make contact with the hypothalamo-pituitary portal vessels.
314 An anatomical relationship of cholinergic neuronal pathways and gonadotropin-releasing hormone
315 neurons of the preoptic area has been indicated by the detection of cholinergic axons in
316 apposition to gonadotropin-releasing hormone immunoreactive cell bodies and dendrites,
317 providing direct neuromorphological evidence for the involvement of the cholinergic system in the
318 regulation of gonadotropin-releasing hormone neurons (Turi et al., 2008). Similarly, we have
319 previously demonstrated the presence of delta opiod receptors both on GT1-1 cells and in
320 hypothalamic GnRH terminals (Maggi et al., 1995; Pimpinelli et al., 2006).
321 The data reported here indicate that the activation of nAChRs present in GT1-1 cells by nicotine
322 does not modify the constitutive basal GnRH release, although increased pulse frequency and
323 decreased pulse duration of GnRH secretion was observed on exposure to the alkaloid.
324 Using hypothalamic primary cell cultures and GT1-7 clone, Krsmanovic and coworkers (Krsmanovic
325 et al., 1998) found a dual effect of Ach on GnRH neurons; while activation of M2 muscarinic
326 receptors reduced basal GnRH release, the activation of M1 receptors resulted in a rapid and
327 transient increase in GnRH neurosecretion. In addition, these authors reported that the treatment
328 of GT1-7 neurons with nicotine (at 10 μM concentration) caused a transient increase in GnRH
329 pulsatility, even if no further characterization of the nicotine receptor involved or its specific
330 activation were provided.
331 On the other hand, the decrease of the pulse frequency reported here agrees with the observation
332 that 'in vivo' nicotine may inhibit the activity of the GnRH pulse generator (Sano et al., 1999) by
333 suppressing the neuronal multiunit activity (MUA) at the level of the median eminence. However,
334 this study was carried out in ovariectomized animals, where the release of GnRH is highly
335 stimulated, due to the lack of gonadal steroid-mediated negative feedback and the activation of
336 neurostimulatory inputs. In fact, neurons in culture lack multiple neuronal afferents that impinge
337 on hypothalamic GnRH neurons (Gore and Roberts, 1997; Pimpinelli et al., 1999). Accordingly, a
338 potent dose-dependent inhibitory effect of nicotine was observed during the stimulation of GnRH
339 release by PGE_1 or to high extracellular K^+ concentration, an experimental condition more similar
340 to that of native hypothalamic GnRH neurons (Pimpinelli et al., 1999; Pimpinelli et al., 2003).

341 Both these two conditions are powerful stimuli to increase cytosolic Ca^{2+} levels (Krsmanovic et al.,
342 1996; Martinez de la Escalera et al., 1995; Stojilkovic et al., 1994) that activates the release
343 machinery of GnRH, even though they act with a different mechanism. In fact, while exposure to
344 high K^+ induces a direct depolarization of the cell (Mellon et al., 1991; Pimpinelli et al., 2003), PGE_1
345 acts through the formation of cAMP (Pimpinelli et al., 1999). We have similarly reported that
346 opioid peptides were unable to modify the basal secretion of GnRH from the GT1-1 cells but they
347 exerted an inhibitory effect under stimulation of GnRH release with prostaglandins (PGE_1 and
348 PGE_2) (Maggi et al., 1995).
349 This observation, indicating a significant inhibitory effect of nicotine on GnRH release, further
350 affirms that GT1 cells resemble hypothalamic GnRH-secreting neurons only when they are
351 properly stimulated (Gore and Roberts, 1997).
352 The inhibitory effect of nicotine, both on cells stimulated with K^+ or with PGE_1, was efficiently
353 blocked by D-Tub confirming the interaction of nicotine with nAChR. Significantly, αBgtx also
354 completely blocks the effect of nicotine, indicating the involvement of αBgtx-sensitive nAChR
355 present in GT1-1 neurons (α7) in the control of secretion of GnRH.
356 Moreover, our investigation on the possible mechanism underlying these effects demonstrates
357 that the inhibition of PGE_1 -induced secretion of GnRH by nicotine is not mediated by a change in
358 cAMP accumulation, suggesting a possible action downstream to the activation of this intracellular
359 pathway.
360 It has been described that the protein kinase A, protein kinase G and protein kinase C pathways
361 are all functionally coupled to regulation of GnRH secretion by GT1 cells; in particular, the pulsatile
362 secretion of GnRH is coupled to the entry of extracellular Ca^{2+} via L-type Ca^{2+} channels (Martinez
363 de la Escalera et al., 1995; Zheng et al., 1997). Therefore cell depolarization triggers the Ca^{2+} entry
364 response with consequent exocytosis of GnRH. The importance of cytoplasmic concentration of
365 Ca^{2+} in GnRH release has been established both in native GnRH (Drouva et al., 1981; Ojeda et al.,
366 1988) and GT1 neurons (Krsmanovic et al., 1992).
367 Prostaglandins were found to induces a membrane depolarization in native mouse GnRH neurons
368 by the involvement of a non-selective cation current that require the cAMP/protein kinase A (PKA)
369 pathway activation (Clasadonte et al., 2011; Coleman et al., 1994; Ojeda and Negro-Vilar, 1985;
370 Roland and Moenter, 2011; Sang et al., 2005; Zhang et al., 2008). Accordingly, prostaglandin
371 induces GnRH release in GT1 cells by promotion of cAMP formation and calcium mobilization
372 (Ojeda and Negro-Vilar, 1985; Ojeda et al., 1985; Rage et al., 1997)
373 Direct membrane depolarization induced by high extracellular concentration of K^+ (from 7.5 to 60
374 mM) also induces a dose-dependent increase of intracellular Ca^{2+} levels in GT1 neurons, with a
375 consequent GnRH release, that involves voltage dependent N- and L-type Ca^{2+} channels (Javors et
376 al., 1995; Krsmanovic et al., 1992; Stojilkovic et al., 1994). The action of K^+ on GnRH release is not
377 affected by a pretreatment with TTX excluding the involvement of sodium channels in such effect
378 (Mellon et al., 1991).
379 Collectively, several studies confirm that depolarization of the plasma membrane and influx of Ca^{2+}
380 through L-type, and possibly N-type, calcium channels are associated functionally with the
381 stimulated release of GnRH and may be shared by PGE_1 and K^+ action. Therefore, the observation
382 that nicotine inhibits the PGE_1-induced GnRH release from GT1-1 cells without affecting the cAMP
383 formation as well as blocks the release of GnRH promoted by exposure to high extracellular
384 concentration of K^+, lead to hypothesize that the activation of nAchRs might affect membrane
385 depolarization. According to their pharmacological profile, the activation nAChRs may change ion
386 permeability of the GT1-1 cell membrane, making the cells insensitive to external stimuli
387 irrespective of the intracellular pathway activated. A postsynaptic inhibitory action of nAChRs in
388 central neurons is supported by a study carried out on rat brain slices, in which the application of

389 nicotine was found to induce a marked neuronal hyperpolarization (Wong and Gallagher, 1989);
390 however, the analysis of membrane ions currents under nicotine exposure by targeted
391 electrophysiological experiments will help to clarify such hypothesis.
392 It has been proposed that the inhibitory action of nicotine observed *in vivo* is not due to a direct
393 effect of the cholinergic agonist on GnRH neurons. The initial hypothesis suggesting the
394 involvement of the opioid system (Pomerleau, 1998) was excluded by the observed insensitivity of
395 nicotine effect to the opioid antagonist naloxone (Sano et al., 1999); more recently, it has been
396 proposed that nicotine may stimulate the release of GABA which may inhibits GnRH release
397 through GABA-A receptor (Kimura et al., 2004).
398 It should be underlined that the results from experiments in organotypic cultures or *in vivo* may be
399 altered by the interference of other neuronal inputs to GnRH neurons possibly activated or
400 inhibited by modification of the cholinergic tone, possibly by pharmacological doses of nicotine
401 that might not reflect the physiological role of the activation of nAChRs.
402 GT1-1 cells may release GABA (Ahnert-Hilger et al., 1998) and the activation of GABA-A receptors
403 induces an initial stimulatory action on GnRH release followed by an inhibitory phase possibly
404 mediated by GABA-B receptors (Martinez de la Escalera et al., 1994). However, in contrast with
405 the present results, in this case the inhibitory effect is mediated by a decrease of intracellular
406 cAMP levels (Martin et al., 2007).
407 Results from GT1-1 cells seem to confirm the hypothesis that the effects of nicotine on the
408 reproductive system "in vivo" can be largely mediated by a direct inhibitory action on GnRH
409 neurons, mediated by αBgtx-sensitive receptors (α7 nAChR); this result does not exclude that 'in
410 vivo' an additional indirect action of nicotine might be mediated by the activation of opioidergic or
411 GABA interneurons.
412 Our results apparently contrast with a previous study (Krsmanovic et al., 1998) suggesting that the
413 activation of nicotinic receptors in perifused immortalized GnRH neurons causes a prompt
414 transient increase in basal GnRH release followed by a return to basal levels.
415 The discrepancy might be due to the different experimental procedures adopted in the two
416 studies. Krsmanovic and coworkers (Krsmanovic et al., 1998) used immortalized GnRH neurons
417 exposed to a lower concentration of nicotine (10 μM), than in the present perifusion study (500
418 μM), and the effect of nicotine was not tested in conditions of stimulated GnRH release.
419 Moreover, although the nAChR involved in the observed phenomena was not characterized, it is
420 possible to speculate that exposure of the cells to low concentrations of nicotine might have
421 induced a receptor desensitization-upregulation and biphasic response (as stated in the Results
422 section) (Quick and Lester, 2002).
423 The results of our study indicate that the GnRH release-inhibitory potency of nicotine (IC_{50} 100-200
424 μM) is congruent with the potency found in activating channel current in neuronal cells (Arneric et
425 al., 1994) and its affinity to rat brain α7 nAChR (Rueter et al., 2006).
426 Evidence of a direct inhibitory effect of nicotine in central neurons, provided by studies in which
427 nicotine application reduced Purkinje cells discharge (de la Garza et al., 1989) or induced
428 membrane hyperpolarization mediated by an increase in potassium conductance (Wong and
429 Gallagher, 1989), are consistent with the results of the current study.
430 It is interesting to note that a greater number of nicotinic receptors are present in the brain of
431 smokers (Benwell et al., 1988) since chronic nicotine induces a receptor upregulation with a partial
432 receptor desensitization (Govind et al., 2009); this is true for α4/β2 nAChRs, the main high-affinity
433 nicotine binding sites present in the brain; however, α7 nAChR were found not to be inactivated
434 after up-regulation induced by chronic nicotine exposure (Kawai and Berg, 2001) making smokers
435 more prone to the inhibitory effects of nicotine on GnRH release.

In conclusion, the results presented in this study demonstrate for the first time the presence of at least two classes of nAChRs on immortalized GnRH neurons (GT1-1 cell line) and that the activation of the αBgtx-sensitive subclass (possibly α7) produces an inhibitory effect on the release of GnRH.

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Conflict of interest

The authors declare no competing or financial interests.

Author contributions

E.M., F.P., C.G and R.M. conceived the project.
E.M., F.P. and R.M. performed all the experiments on living cells
C.G. performed binding experiments.
V.A. performed RT-PCR experiments
C.G., V.A., C.R. and R.M. assisted with data interpretation and manuscript preparation.

Legends to figures

Figure 1 Detection of nicotinic acetylcholine receptors in GT1-1 cells.

Homologous saturation curves of the binding of (A) ^{3}H-epibatidine (^{3}H-Epi) and (B) ^{125}I-a-bungarotoxin (^{125}I-αBgtx) on GT1-1 (circle) and HEK392 (triangle) cell membrane preparations Binding isotherms have been analyzed by LIGAND program. (C) RT-PCR amplified transcripts for α4, α7 and β2 nicotine receptor subunits in GT1-1 cell total RNA. Normal adult mouse brain total RNA was used as internal control. Mouse *Gapdh* was used as housekeeping gene.

Figure 2 Effect of the exposure to nicotine on the basal release of GnRH from GT1-1 cells.

(A) Basal accumulation of immunoreactive GnRH in culture medium of GT1-1 cells after a 30 min static incubation in control (C) or in presence of increasing concentration of nicotine and cholinergic antagonists αBgtx (2.5 mM) and D-Tub (250 mM). Values are expressed as mean ± SEM (n=8). (B) Representative graphs (of four separate experiments) of perifused GT1-1 cells in absence and in presence of a 500 μM concentration of nicotine; the segmented bar on top of the profile of GnRH release indicates the position and duration of secretory pulses identified by cluster analysis.

Figure 3 Effect of the exposure to nicotine on the release of GnRH induced by PGE_1 .

(A) Representative dose response curve of the inhibitory action of nicotine on PGE_1-induced GnRH release. The effect of nicotine is abolished in the presence of the antagonists (B) D-Tub and (C) αBgtx. Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B); * $p<0.05$ vs 0.

Figure 4 Nicotine does not modify the PGE_1-induced cAMP accumulation in GT1-1 cells.

The exposure of GT1-1 cells to nicotine or the antagonist D-Tub does not affect the intracellular accumulation of cAMP induced by PGE_1.
Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B).

483
484 **Figure 5 Effect of the exposure to nicotine on the release of GnRH induced by 56 mM K^+- .**
485 (A) Representative dose response curve of the inhibitory action of nicotine on 56 mM K^+-induced
486 GnRH release. The effect of nicotine is abolished in the presence of the antagonists (B) D-Tub and
487 (C) αBgtx. Values are expressed as mean ± SEM (n=8); § $p<0.05$ vs basal release levels (B); * $p<0.05$
488 vs 0.
489
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491 **References**
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Highlights

- Immortalized GnRH neurons (GT1-1) express acetylcholine nicotinic receptors
- Nicotine has not effect on basal accumulation of GnRH
- Nicotine affects the pulsatility of GnRH release from GT1-1 cells
- Nicotine exerts an inhibitory action on stimulated GnRH release
- The effect of nicotine does not affect intracellular cAMP levels

Figure1

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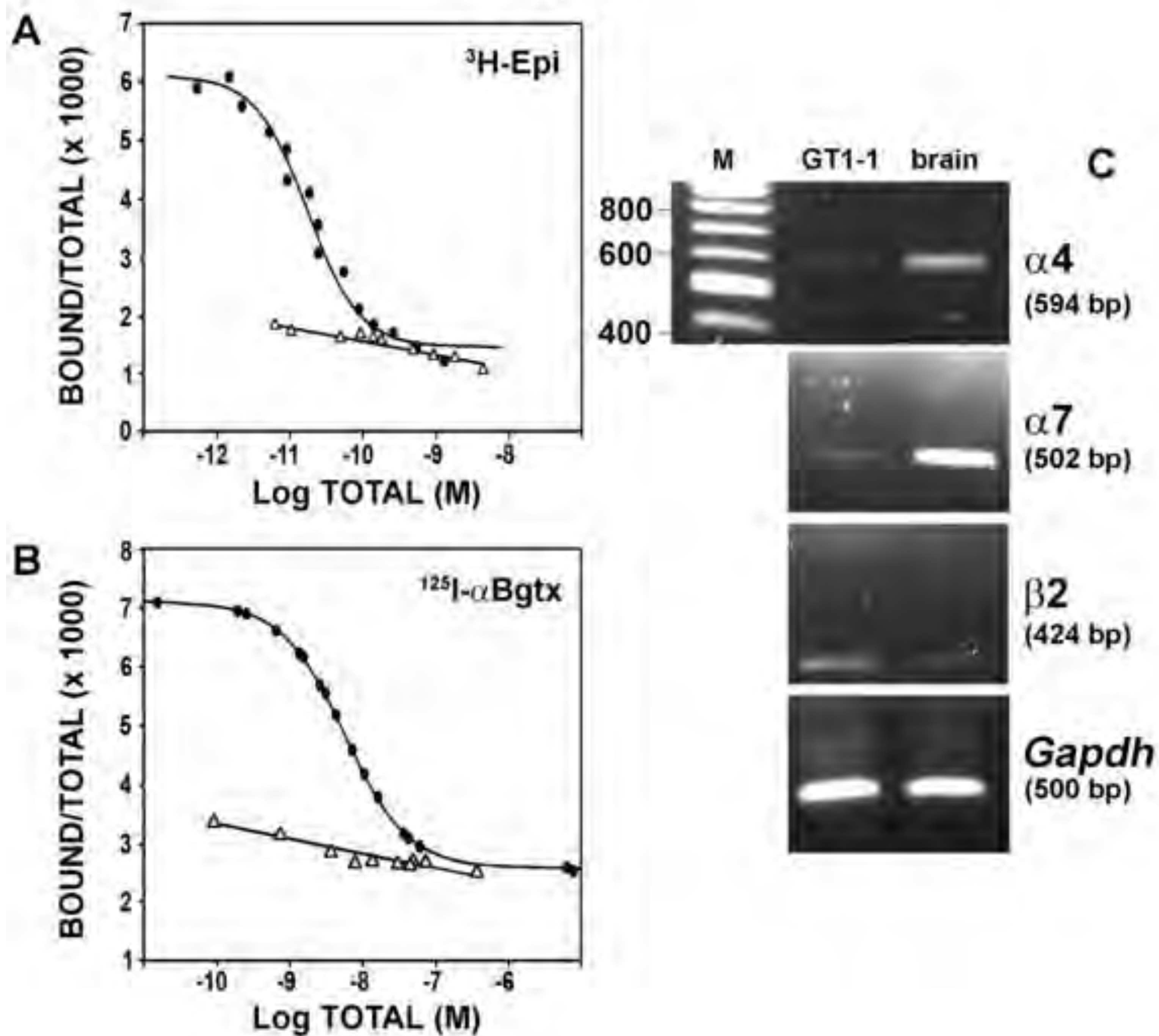

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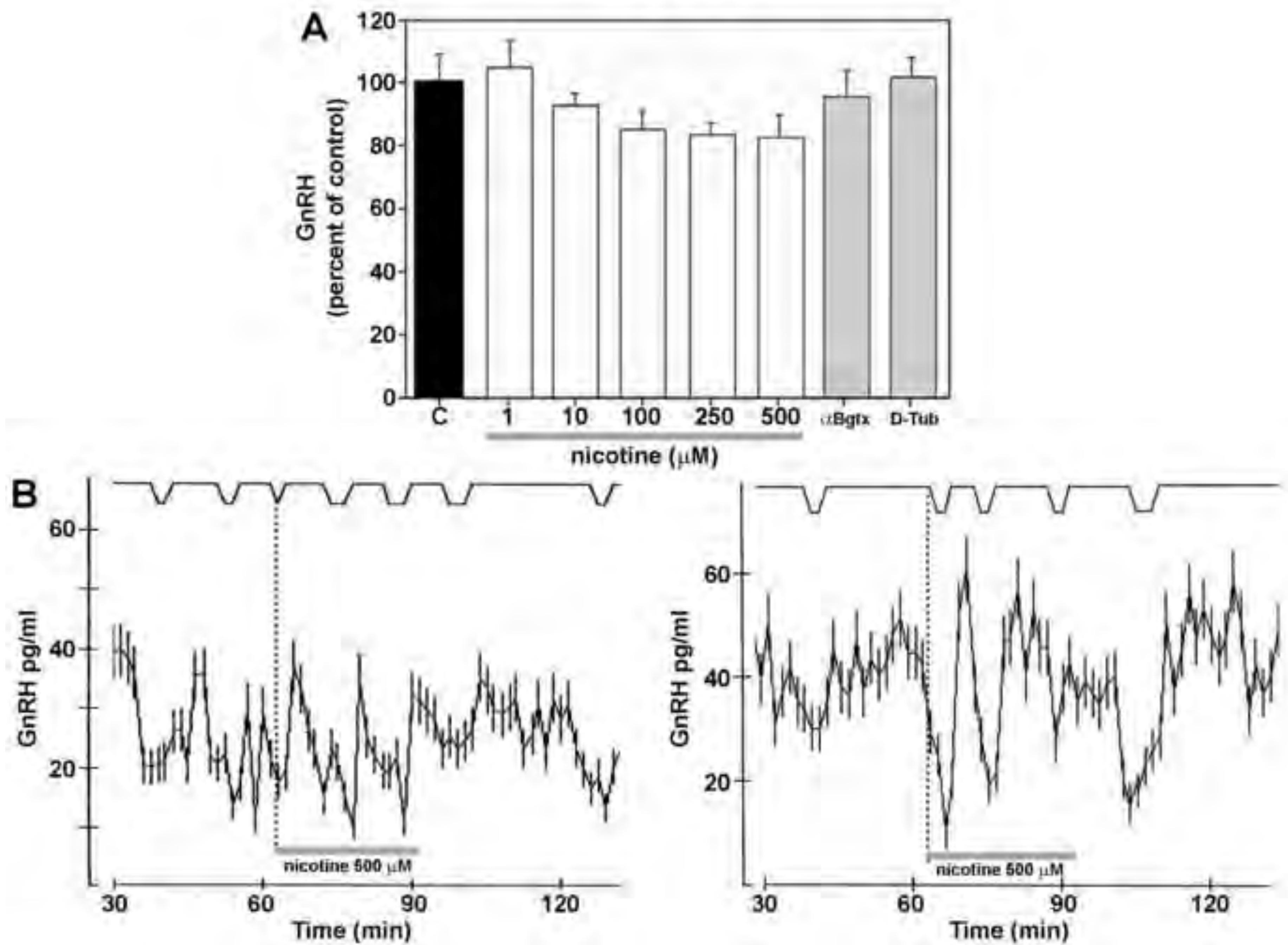

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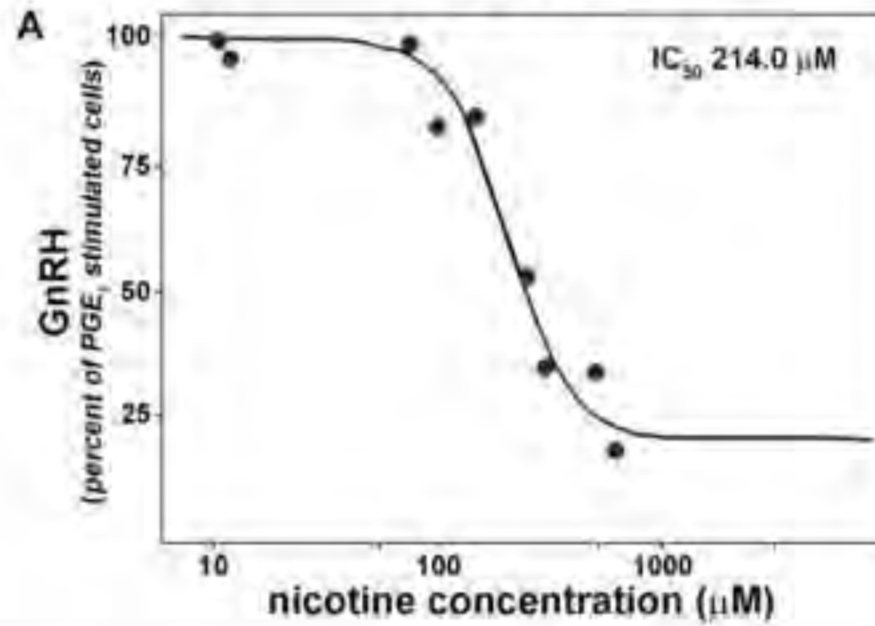
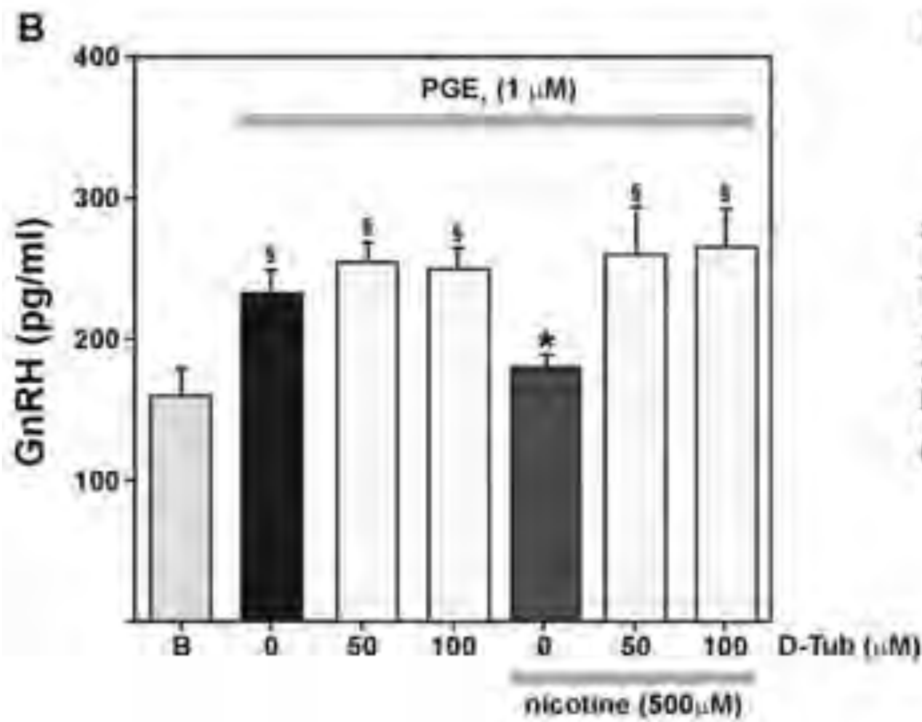
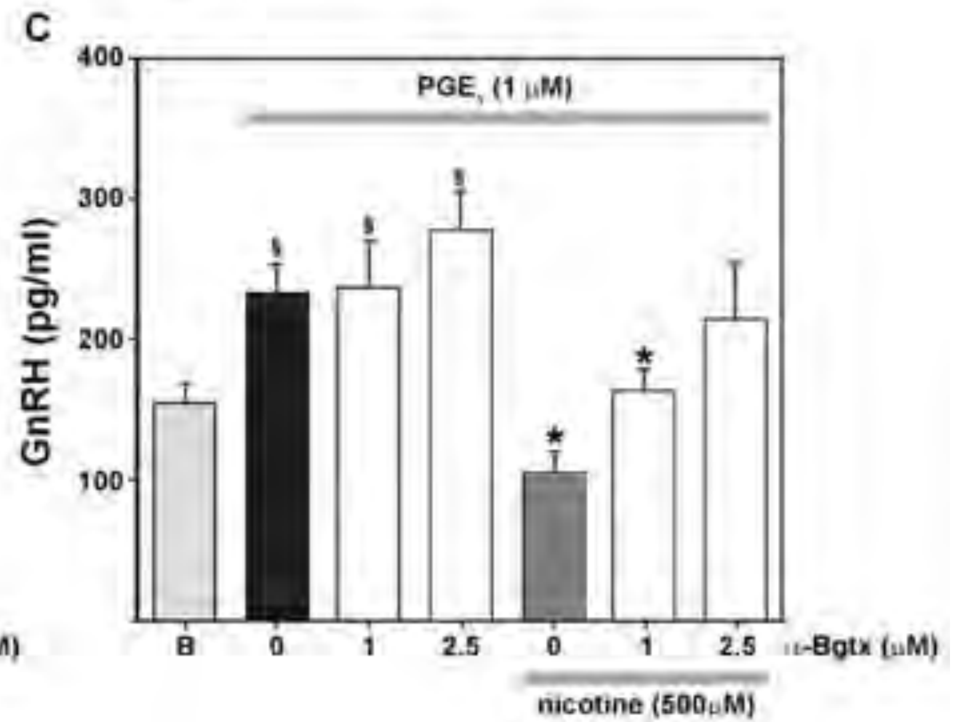
Figure 3







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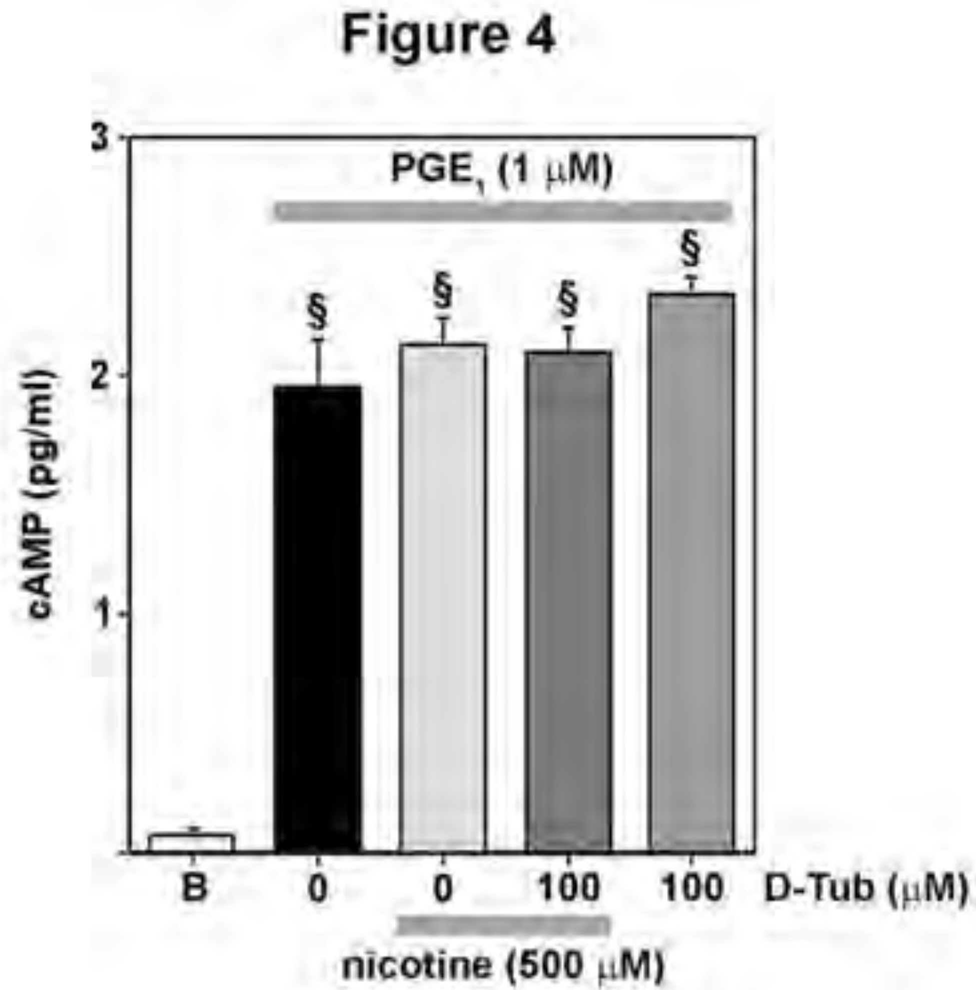

Figure5

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