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CHOLINE AND NICOTINE INCREASE GLIOBLASTOMA CELL PROLIFERATION BY BINDING AND ACTIVATING α7- AND α9- CONTAINING NICOTINIC RECEPTORS

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Running title

α–Bungarotoxin-sensitive nicotinic receptors increase proliferation of glioblastoma cells

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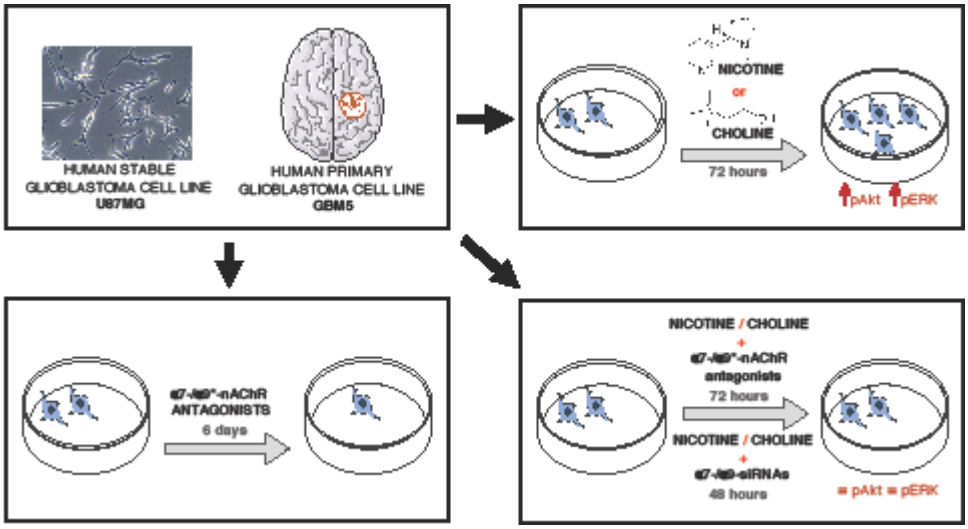
Graphical abstract



Highlights

- Choline compounds are highly increased in glioblastoma cells
- Choline is also an agonist of α7- and α9-containing nicotinic receptors
- Both choline and nicotine promote glioblastoma cell proliferation and survival.
- These effects are blocked by specific nicotinic antagonists or α7- and/or α9 siRNAs.
- Nicotinic antagonists also decrease basal glioblastoma cell proliferation

Abstract

Glioblastomas (GBMs), the most frequent and aggressive human primary brain tumours, have altered cell metabolism, and one of the strongest indicators of malignancy is an increase in choline compounds. Choline is also a selective agonist of some neuronal nicotinic acetylcholine receptor (nAChR) subtypes.

As little is known concerning the expression of nAChR in glioblastoma cells, we analysed in U87MG human grade-IV astrocytoma cell line and GBM5 temozolomide-resistant glioblastoma cells selected from a cancer stem cell-enriched culture, molecularly, pharmacologically and functionally which nAChR subtypes are expressed and,whether choline and nicotine can affect GBM cell proliferation.

We found that U87MG and GBM5 cells express similar nAChR subtypes, and choline and nicotine increase their proliferation rate and activate the anti-apoptotic AKT and pro-proliferative ERK pathways. These effects are blocked by the presence of non-cell-permeable peptide antagonists selective for α7- and α9-containing nicotinic receptors. siRNA-mediated silencing of α7 or α9 subunit expression also selectively prevents the effects of nicotine and choline on GBM cell proliferation.

Our findings indicate that nicotine and choline activate the signalling pathways involved in the proliferation of GBM cells, and that these effects are mediated by α7 and α9-containing nAChRs. This suggests that these nicotinic receptors may contribute to the aggressive behaviour of this tumor

and may indicate new therapeutic strategies against high-grade human brain tumours.

Key words: Neuronal nicotinic receptors; glioblastoma cell lines; proliferation; gene silencing; AKT1 and ERK1/2.

1.Introduction

Gliomas account for 81% of all malignant brain tumours, and almost half of them are highly aggressive glioblastomas (GBMs) [1]. GBMs originate from brain neural cells and have high levels of proliferation, migration and invasion. Potential initiating cells include astrocytes, glial precursor cells and neural stem cells, but the underlying molecular and pathophysiological mechanisms causing the malignant transformation of precursor cells are still poorly understood. There is currently no viable treatment other than surgical resection, radiotherapy and chemotherapy, and median overall survival is only 15 months from the time of diagnosis [2].

There is evidence that the risk of developing various types of cancer is greater among smokers than non-smokers [3], but the role of smoking in the etiology of malignant gliomas remains controversial [4-6]. Tobacco smoke contains at least 4000 compounds, including nicotine and a number of carcinogenic compounds such as exogenous N-nitroso compounds, which one study has shown to be potent neurocarcinogens that can induce glioma formation in rats [7]. Nicotine, the most addictive component of tobacco smoke, is not a direct carcinogen but it enhances cell proliferation, migration, invasion and angiogenesis in multiple cancers [3]. The primary mechanism by which nicotine acts as a promoter is by binding and activating neuronal acetylcholine nicotinic receptors (nAChRs) [8] a very heterogenous class of receptors that consist of various combinations of α2–α10 and β2–β4 subunits, and generate heteromeric or homomeric pentameric ligand-gated ion channels [9].

In non-neuronal cells, signalling cascades downstream of nAChR activation may involve both ionic and non-ionic mechanisms and, depending on the type of cell and the expressed nAChR subtypes, the binding of endogenous ACh or choline, or exogenous nicotine ligands can induce conformational changes in nAChRs and/or their associated proteins that can activate different intracellular signalling pathways and regulate gene expression [10]. All nAChR subtypes are permeable to Ca^{2+} ions, but the most permeable are those containing the α7 or α9 subunits [11].

The main glial cell types in mammalian brain are astrocytes, microglia, oligodendrocytes, and chondroitin sulfate proteoglycan NG2-expressing (NG2) cells, but astrocytes are the most predominant. The presence of nAChRs in astrocytes was first described in 1988 [12], and it was later shown that cultured hippocampal astrocytes may express α3, α4, α7, β3 or β4 subunits (reviewed in

[13]. Astrocytes and NG2 cells in the CA1 of hippocampal slices express functional α7-containing nAChRs whose activation increases intracellular calcium levels [14, 15].

Cell metabolism is altered in gliomas, and one of the most robust indicators of malignancy is an increase in the choline compounds involved in the membrane phospholipid metabolism that accompanies the cell cycle [16]: anabolic phosphocholine, catabolic glycerophosphocholine, and free choline. In *vivo* and *ex vivo* magnetic resonance studies (MRS) have shown that phosphocholine is the predominant choline compound in high-grade gliomas (particularly GBMs), whereas glycerophosphocholine is predominant in low-grade gliomas and normal [17]. The higher phosphocholine levels in GBMs have been attributed to the greater expression and/or activity of choline kinase [17], which is enhanced by hypoxia.

Choline is also a selective agonist of native α7-containing nAChRs [18] and heterologously expressed α9-containing neuronal nAChRs [19]. It has recently been shown that choline and phosphocholine are ligands of the α9 and α9/α10-containing nAChRs present in monocytes, and that they can efficiently inhibit the ATP-dependent release of interleukin-1β from human and murine monocytes by means of a mechanism involving the nAChRs containing these subunits [20, 21]. Moreover, choline elicits ionotropic current responses in homomeric α9 nACRs, whereas phosphocholine does not trigger ionotropic responses in either homomeric α9 or heteromeric α9/α10 nAChRs [20].

Apart from three recent studies showing that nicotine enhances the proliferation, migration, radio-resistance and invasion of malignant human glioma cells [22], that functional nAChRs and muscarinic acetylcholine receptors (mAChRs) are present in GBM cells [23] and in GBM cancer stem cells [24], very little is known about the role of nAChRs in glioma cells.

The aim of this study was to investigate the expression of nAChRs in widely used human grade-IV astrocytoma U87MG cells and the GBM cell line GBM5 [25] selected from patient-derived GBM cancer stem cell (CSC)-enriched cultures resistant to temozolomide (TMZ) treatment [25] in order to determine whether GBM cells are responsive to choline and nicotine, and whether selective nAChR subtype antagonists can interfere with their signalling.

2. Experimental procedures

2.1 Materials

Nicotine (-)-nicotine hydrogen tartrate salt), choline (choline chloride) and MLA (methyllycaconitine citrate salt) were purchased from Sigma-Aldrich; the αBgtx (α-Bungarotoxin) was purchased from Tocris Bioscience (Biotechne).

The α-conotoxin ArIB [V11L; V16D] (AR) and RGIA4 an analog of α-conotoxin RgIA, were synthesized as previously described [26, 27].

2.2 Cell culture

The human U87MG and GBM5 GBM cells were kindly provided by Dr. Antonio Daga (IRCSS – San Martino Hospital, Genoa, Italy).The U87MG cells came from ATCC®, and TMZ-resistant GBM5 cells were selected from CSC-enriched culture [25].

The U87MG cells were grown in a high glucose DMEM medium (Gibco®, Life Technologies) supplemented with 10% fetal bovine serum (Carlo Erba Reagents) of South American origin, 2 mM L-glutamine (100x 200 mM solution, Euroclone), 100 U/ml penicillin G, and 100 µg/ml streptomycin (100x solution, Euroclone).

The GBM5 cells were grown in a 1:1 solution of DMEM/F-12 (Gibco®, Life Technologies) and Neurobasal™ (Gibco®, Life Technologies), supplemented with 1% B-27™ Supplement minus vitamin A (50x solution, Gibco®, Life Technologies), 20 ng/mL human EGF (Gibco®, Life Technologies), 2 mM L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin. The plastic surfaces were pre-treated with a 1:250 solution of Matrigel® Matrix GFR (Growth Factor Reduced, Corning) and DMEM/F-12 or Neurobasal™ for at least 30 minutes at 37°C. The cells were maintained in a 5% CO_2 environment at 37°C.

The cells used in the experiments are between passaga 2 and 12.

2.3 Antibody against the human α7 nAChR subunit

To detect the α7 subunit of nAChRs, we used affinity-purified, subunit-specific, polyclonal antibody against an intracytoplasmic loop (peptide sequence: ACSPTHDEHLLHGGQPPEGDPDL) of the human α7 nAChR subunit produced in rabbit as previously described [28] at concentration of 1 µg/ml. In order to exclude any cross reactivity between nAChR subunits, the anti-α7 antibodies were tested as previously described [28].

2.4 Total RNA extraction and reverse transcription

Total RNAs were extracted using the *RNeasy Mini kit* and accompanying *QIAshredder* (Qiagen), according to the manufacturer's instructions. Briefly, a maximum of 9 x 10^6 cells, to detect the basal levels of transcripts or cells harvested from multiwell plates 48 hours after transfection with siRNAs (to assess the CHRNA7 or CHRNA9 gene knock down), were collected by centrifugation and lysed with 600 µL or 350 µL of buffer RLT, containing β-mercaptoethanol (10 µL mL-1 RLT buffer), respectively. The lysate was homogenized by means of QIAshredder column centrifugation for 2 min at maximum speed. To avoid DNA contamination, samples were on-column incubated with DNAse I for 15 min and RNA was eluted with 50 µL of RNase-free water. The amount of eluted total RNA was determined by spectrophotometer at 260 nm and its purity was evaluated using the 260/280 ratio; 0.5 - 1 µg per sample was reverse transcribed using the GoScriptTM Reverse Transcriptase (Promega), according to information provided by the company.

2.4.1 Quantitative real-time PCR (q-PCR)

Gene expression analyses were performed by qPCR assay using the ABI Prism Thermocycler QuantStudio 5. The target sequences were amplified from 50 ng of cDNA in the presence of TaqMan® Gene Expression Master Mix (Life Technologies, Inc.).

The TaqMan® primer and probe assays used were human *CHRNA2* (ID #Hs00181237_m1), *CHRNA3* (ID #Hs01088199_m1), *CHRNA4* (ID #Hs00181247_m1), *CHRNA5* (ID #Hs00181248_m1), *CHRNA6* (ID #Hs00610233_m1), *CHRNA7* (ID #Hs01063373_m1), *CHRFAM7A* (ID #Hs04189909_m1), *CHRNA9* (ID #Hs00214034_m1), *CHRNA10* (ID #Hs00220710_m1), *CHRNB2* (ID #Hs00181267_m1), *CHRNB3* (ID #Hs00181269_m1), *CHRNB4* (ID #Hs00609520_m1). *GAPDH* (ID #Hs99999905_m1) was used as endogenous control, as described in the figure legends. The expression level of each subunit in the U87MG and GBM5 cell lines was determined by five independent experiment, run in triplicate. The "Quant Studio DA2 software (Thermo Fisher)" was used to create a project that calculated the mean value of the five replicates, according to the $2^{-\Delta CT}$ method Gene expression analyses after siRNAs transfection has been conducted on two (U87MG) or three (GBM5) independent samples as an exploratory experiment to choose the most efficent siRNA for silencing α7 and α9 expression. In this case the $2^{-\Delta\Delta CT}$ method was applied for comparison with the calibrator (scrambled siRNAs) for each experiment (set to a value of 1).

2.5 Proliferation assay

On the day before treatment with the designated chemical (or chemicals) 1.8-20 x 10^3 U87MG or GBM5 cells were seeded in 24-well multiwells.

The plastic was pretreated with Matrigel® Matrix before seeding the GBM5 cells. The nicotinic antagonists MLA (500 nM), αBgtx (1 µM), AR (1 µM) or RGIA4 (1 µM) were pre-incubated 30 minutes before adding nicotine or choline. Where not specified in the figures, nicotine was used at 50 nM (U87MG cells) or 100 nM (GBM5 cells) and choline was used at 1 µM (U87MG cells) or 50 µM (GBM5 cells). The treatments were 72 hours or or six-day long with change of nicotine or choline and the antagonists every 24 hours (or 72 hours).

Only for siRNA-transfected cells the treatment with nicotine or choline lasted 48 hours.

After 72 hours or six days (48 hours in the case of siRNA-transfected cells), the cells were trypsinised, resuspended in 600 µL of phosphate-buffered saline (PBS), and counted manually at least three times using a Bürker chamber.

Each 24-well plate in the proliferation assays was equally randomised to one of seven experimental conditions (one basal and six different concentrations of nicotine or choline) in duplicate or triplicate. Three separate fields were counted for each well, and the independent values generated by each well under the same experimental condition were averaged. One plate represented a single experiment under each experimental condition, and the number of separate experiments is shown in the figure

(the number in the lower part of the bar).The independent values (n) generated by the separate experiments (including outliers (n) were used for the statistical analysis.

A 24-well plate was also used to assess the inhibitory effects of the antagonists or subtype-specific toxins on cell proliferation under six experimental conditions: basal, nicotinic ligands (50 nM or 100 nM nicotine, and 1 or 50 µM choline), and nicotinic ligands in the presence of antagonists (500 nM MLA or 1 µM αBgtx) or toxins (1 µM RGIA4 or 1 µM toxin AR). Three separate fields were counted for each well, and the average of the counts of 2-3 wells per condition/per plate was determined as the data for one experiment.

2.6 AKT1 and ERK1/2 pathways activation

On the day before treatment 7-9 x 10^4 GBM5 cells were seeded in 24-well multiwells pretreated with Matrigel® Matrix. Two days before treatment, 7-9 x 10^4 U87MG cells were seeded in 24-well multiwells. The day after, U87MG cells were overnight deprived from serum before treatment.

The cells were then treated with the designed chemicals from 5 minutes to one hour, lysed with 80-90 µL of Laemmi sample buffer and subjected to SDS-PAGE. The nicotinic antagonists MLA (500 nM), αBgtx (1 µM), AR (1 µM) or RGIA4 (1 µM) were pre-incubated 30 minutes before adding nicotine or choline. Nicotine was used at 50 nM (U87MG cells) or 100 nM (GBM5 cells) and choline was used at 5 mM on both cell lines.

The Western blot analyses of AKT and ERK pathway activation were also made using 24-well plates, each of which was used to evaluate one antagonist or one toxin. Each plate always had one or two wells under basal condition, one well with the control antagonist or toxin, one well stimulated for five, ten, 30 or 60 min with the indicated concentration of nicotine or choline, and an equal number of wells pre-incubated with the antagonists or toxins for 30 min and subsequently stimulated for five, ten, 30 or 60 min with the indicated concentration of nicotine or choline.

After quantifying band intensity (expressed as the pAKT/AKT or pERK/ERK ratio), the basal values or the mean values of the two wells under basal condition were considered as to 1 and used to normalise the values of each blot under each condition. The experimental value for each plate under each experimental condition is the mean of the quantification of two gels loaded with samples from the same well treated in the same way and normalised to the basal value (or the mean of two basal values) within the corresponding blot. The numbers in the figures thus represent independent experiments performed using independent plates.

2.7 Western blotting and signal intensity quantification of bands

Cells in the multiwell plates were scraped after addition of Laemmli sample buffer. Samples were separated by means of SDS-polyacrylamide gel electrophoresis using 9% acrylamide, and electrophoretically transferred to nitrocellulose membranes with 0.45 µm pores (GE Healthcare

Amersham).

The blots were blocked overnight in 4% non-fat milk (Hipp 2) in Tris-buffered saline (TBS), washed in a buffer containing 4% non-fat milk and 0.3% Tween 20® (Merck) in TBS, incubated for 2 hours at room temperature with the primary antibodies and then with the appropriate secondary antibodies for 1 hour at room temperature keeping the blots in the dark.

The commercial primary antibodies were purchased from BD Transduction Laboratories (anti-human AKT1 mouse IgG1, ID #610860), Invitrogen (anti-human pSer473-AKT1 rabbit IgG, Thermo Fisher Scientific ID #44-621G), Cell Signaling Technology (anti-human ERK1/2 rabbit IgG1 ID #9102 and pThr202/Tyr204-ERK1/2 mouse IgG1 ID #9106) and Merck (monoclonal mouse anti-actin Abs n# A4700) used in accordance with the manufacturer's instructions.The secondary antibodies employed were from Li-Cor:goat anti-rabbit IRDye800RD ID #926-3221 and goat anti-mouse IRDye680RD ID #926-68070.

After ten washes with TBS with decreasing concentrations of Tween® 20 (starting from 0.3% to 0%), the membranes were dried overnight in the dark at room temperature. The immunoreactive signal was measured using an Odissey CLx Near-Infrared Imaging System (Li-Cor) and quantified through Image Studio software version 2.1.10.

2.8 siRNA transfection

The siRNAs used to knock down endogenous α7 and α9 nAChR subunits were obtained from Dharmacon™ (Horizon Discovery). For each subunit, two different siRNAs were used, together with a non-targeting pool of scrambled siRNAs as a control of transfection.

On the day before transfection 3.5-4 x 10^5 U87MG and GBM5 cells were seeded in 6-well multiwells (the plastic was pretreated with Matrigel® Matrix before seeding the GBM5 cells). One well was transfected with 75 pmol of siRNAs or scrambled pool diluted with 250 µL of Opti-MEM (Gibco®, Life Technologies) using the Lipofectamine™ 3000 Reagent (Invitrogen™, Life Technologies) in accordance with the manufacturer's instructions. A total of 4 µL of transfection reagent per well was used for the U87MG and 5 µL for the GBM5 cells. The medium was replaced with fresh medium without antibiotics just before transfection.

The cells for qPCR analysis were harvested after 48 hours from transfection and immediately frozen in liquid nitrogen.

In the case of α7 nAChR subunit knocking down screening by means of Western blotting, the cells were co-transfected with the corresponding siRNA or scrambled pool and a human α7-pCDNA3.1 expression vector by adding 5 µL of P3000™ Enhancer Reagent per well in accordance with the manufacturer's intructions. After 24 hours, culture medium was replaced with complete fresh medium. After 48 hours, the cells were scraped and harvested with 200 µL of Laemmli sample buffer per well, and loaded onto gels as described below.

In the case of the proliferation and AKT/ERK pathways activation experiments, the cells were

trypsinised after 4-5 hours, counted and re-seeded in 24-well multiwells using complete medium as described below.

The design of the experiments investigating the effects of siRNA on AKT and ERK pathway activation was essentially the same as that used for the antagonists or toxins: the differences were that the basal condition was determined using scrambled siRNAs, and the five, ten, 30 or 60 min stimulation with choline or nicotine was performed on U87MG or GBM5 cells transfected with scrambled or α7- or α9-specific siRNAs.

2.9 Statistical analysis

The experiments were performed at least five times, and each sample in each experiment was at least duplicated; the qPCR experiments were performed five times for each cell line, and each sample was triplicated. The results are expressed as mean values ± SEM.

The parametric data were analysed using one-way ANOVA and, if there was a significant between-group difference, Bonferroni's *post hoc*. The non-parametric data were analysed using a Kruskall-Wallis test followed by Dunn's *post hoc* test. The accepted level of significance was p <0.05. All of the statistical analyses were made using GraphPad Prism software, version 6.0.

3. Results

3.1 Relative qPCR analyses of GBM nicotinic receptors

The gene expression profile of the nAChR subunits was studied in the GBM cell lines U87MG and GBM5 by means of qPCR analysis. No *CHRNA2* transcript was detected, and *CHRNA4* and *CHRNB4* were expressed at very low levels in both cell lines (Figure 1, panels A and B). *CHRNA3* expression was higher in the U87MG cell line, whereas *CHRNB2* and *CHRNB3* transcripts were more expressed in GBM5 cells (Figure 1, panels A and B). *CHRFAM7A*, *CHRNA7* and *CHRNA10* mRNAs were present at low but comparable levels in both cell lines. The most highly expressed subunits in both U87MG and GBM5 cells were *CHRNA5* and *CHRNA9* (Figure 1, panels A and B).

Comparison of mRNA expression in U85MG and GBM5 cells with that in a publicly available DNA microarray (accession number GSE16805) obtained from long-term GBM cultures derived from patients at different tumour stages showed that they were very similar, with the *CHRNA5,* and *CHRNA9* genes being the most highly expressed.

3.2 Nicotine increases U87MG and GBM5 cell proliferation

The effect of nicotine on the growth of U87MG and GBM5 cells was investigated by incubating the cells with increasing concentrations of nicotine (from 10 nM to 100 μM) for 72 hours. As shown in Figures 2A and 2D, very low nicotine concentrations (from 10 to 100 nM) increased the proliferation of both cell lines. As the qPCR studies had indicated that the most highly expressed ligand-binding subunit is that coding for the α9 subunit, and the α7 subunit is expressed in both cell lines (albeit at a lower level), the effect of cell incubation with αBgtx or MLA, which are known antagonists of α7- and α9-containing nAChRs, was tested in order to identify the receptor subtypes involved in nicotine-induced proliferation.

As shown in Figures 2B and 2E, 500 nM MLA or 1 μM αBgtx blocked the U87MG cell proliferation induced by 72h exposure to 50 nM nicotine, and the GBM5 cell proliferation induced by 100 nM nicotine. When given alone, the MLA has a slight reducing effect on the basal proliferation, that however was not statistically significative.

As αBgtx and MLA act on both α7- and α9-containing receptors, the same experiments were repeated using RGIA4 (a selective antagonist of α9-containing receptors) [27], and AR (a selective antagonist of α7-containing receptors) [26], in order to distinguish the subtypes involved.

One micromolar of RGIA4 or AR completely abolished the U87MG cell proliferation induced by 72h exposure to 50 nM nicotine (Figure 2C) and the GBM5 cell proliferation induced by 100 nM nicotine (Figure 2F). When administered alone at a concentration of 1 μM RGIA4 and AR slightly (but not significantly) decreased the basal proliferation (Figures 2C and 2F) .

3.2.1 Choline increases U87MG and GBM5 cell proliferation

Choline is a precursor of ACh synthesis and a product of ACh hydrolysis that elicits responses in native α7 and oocyte-expressed α9 nAChRs [18, 19].

We tested the possible effect of choline on GBM cell proliferation, and found that it increased the proliferation of the U87MG and GBM5 cell lines, with the maximum effects being observed at doses of 1 μM (U87MG, Figure 2G) and 50 μM (GBM5, Figure 2J). These doses were therefore used to test whether the non-selective α7 and α9 antagonists MLA (500 nM) and αBgtx (1 μM), the α7-selective antagonist AR (1 μM) or the α9-selective antagonist RGIA4 (1 μM) blocked choline-induced proliferation. The effect of choline on U87MG (Figures 2H, 2I) was blocked by αBgtx, MLA, RGIA4, and AR and significantly decreased by the same toxins in GBM5 cells (Figures 2K,2L).

3.2.2 α7 and α9 antagonists decrease the basal proliferation of GBM cells

When given to U87MG and GBM5 cells for 72 hours, MLA and RGIA4 toxins slightly decreased basal proliferation of GBM cells, so we decided to investigate over a longer time (6 days) the effect of all the four antagonists on basal U87MG and GBM5 cell proliferation. We found that after 6 days incubation with the non-selective antagonists MLA and αBgtx both U87MG and GBM5 cells had a reduced basal proliferation (Figures 3A and C). Moreover, a dose–response experiment using the

α7- and α9- selective antagonists AR and RGIA4 showed that both antagonists significantly reduced U87MG and GBM5 cell proliferation not only at 1 μM but also at the lower concentrations investigated, either alone (with the exception of AR 250 nM in U87MG cells) and in combination (Figures 3B and D).

3.3 Nicotine and choline increase the phosphorylation of AKT and ERK in GBM cells

As nicotine and choline increased U87MG cell proliferation, we investigated the signalling pathways potentially involved in order to improve our understanding of the underlying mechanisms by testing the activation of AKT1 (which promotes cell growth and survival) and ERK1/2 (which is associated with cell proliferation).

After being preincubated for 30 min with or without one of the four antagonists, the cells were treated with nicotine 50 nM for 5-60 minutes, lysed and collected, and assessed by means of immunoblotting. Nicotine induced a significant increase in the levels of phosphorylated AKT1 (Ser473) and ERK1/2 (Thr202/Tyr204) (Figures 4A,B,C,D and 4E,F,G,H). This activation was time dependent: nicotine-stimulated phosphorylation peaked after 5-10 min and returned to baseline levels after 30–60 min.

As shown in Figure 4, MLA (4A and E), αBgtx (4B and F), AR (4C and G) and RGIA4 (4D and H) all prevented nicotine-induced AKT1 and ERK1/2 phosphorylation.

The same protocol was used to test the effect of 5 mM choline on AKT1 and ERK1/2 phosphorylation (Figure 5). Choline also increased AKT1 and ERK1/2 phosphorylation in a time-dependent manner, and these effects were reduced by all of the α7 and α9 selective and non-selective antagonists.

We also examined the effects of nicotine and choline on AKT1 and ERK1/2 activation in GBM5 cells (Supplementary Figures S1 and S2), and found that the extent and time-course of nicotine- and choline-induced AKT1 activation was very similar to that observed in U87MG cells, but the peak level of choline-induced ERK1/2 phosphorylation was much greater (Figures S1 E,F,G,H). Once again, all of the four α7 and α9 selective and non-selective antagonists blocked or strongly reduced the effects of nicotine and choline on the GBM5 cell line.

3.4 Silencing α7 or α9 subunits decreases GBM cell proliferation and signalling

In preliminary experiments, we tested two siRNAs that we have previously used to silence *CHRNA7* and *CHRNA9* expression in U87MG and GBM5 cell lines [28]. The qPCR analysis shown in Supplementary Figure 3 revealed that, in comparison with scrambled siRNAs, α7siRNAI and α7siRNAII knocked down the *CHRNA7* gene transcript by more than 65% (U87MG cells: 70% by α7siRNAI and 84% by α7siRNAII; GBM5 cells: 80% by both α7siRNAI and by α7siRNAII (Figures S3A and S3E), and α9-siRNAI and α9-siRNAII knocked down the α9 subunit by respectively 41% and 66% in U87MG cells (Figure S3B) and by 70% in GBM5 cells (Figure S3F). As a control, we tested the specificity of each siRNA in the U87MG cell line, as shown in Figure S3D.

3.4.1 Effects of siRNA-mediated knock down of α7-containing nAChRs

The level of native α7 protein in U87MG and GBM cells is very low, and cannot be determined using traditional binding studies or immunoblotting. Consequently, in order to investigate the effect of α7siRNAs on α7 protein expression, we co-transfected a human α7 expression vector with a pool of scrambled siRNAs, α7siRNAI or α7siRNAII in U87MG and GBM5 cells for 48 hours, and measured the level of knock down by means of Western blotting with an anti-α7 antibody. As shown in Supplementary Figures S3C and S3G, in comparison with the scrambled siRNAs, both α7siRNAI and α7siRNAII significantly knocked down the transfected α7 protein in U87MG and GBM5 cells. Assuming that the signal of scrambled siRNAs were 100%, the α7 signal remaining in the U87MG cells was 67.5±2.5% after α7siRNAI, and 26.5±1.5% after α7siRNAII, and α7 signal remaining in the GBM5 cells was 31.5±3.5% after α7siRNAI and 5.5±2.5% after α7siRNAII.

In order to analyse the effect of scrambled siRNAs and α7siRNAII on the nicotine-induced stimulation of cell proliferation, the cells were first transfected with scrambled siRNAs or α7siRNAII and, on the following day, were treated with 50 nM nicotine for 48h. As expected, nicotine increased the proliferation of the scrambled-transfected U87MG (Figure 6A) and GBM5 cells (Figure S4A), but not that of the cells transfected with α7siRNAII. There was no difference in the level of nicotine-induced proliferation between the untransfected and scrambled-transfected cells (data not shown).

The effects of the pool of scrambled siRNAs and α7siRNAII on the choline-induced stimulation of cell proliferation were similar: choline increased the proliferation of the scrambled-transfected U87MG (Figure 6G) and GBM5 cells (Figure S4G), but not that of the cells transfected with α7siRNAII.

Silencing the α7 subunit with α7siRNAII reduced the nicotine-induced activation of AKT1 signalling after 5, 10 and 30 min, and the nicotine-induced activation of ERK1/2 pathway after 5 and 10 min in U87MG cells (Figures 6B and 6C). In GBM5 cells, the nicotine-induced activation of AKT1 and ERK1/2 signalling cascades was delayed, but the silencing of α7 reduced these nicotine-mediated effects (Figures S4B and C). Silencing the of α7 subunit with α7siRNAII did not significantly reduce the choline-induced activation of AKT1 signalling (Figure 6H) or the choline-induced phosphorylation of ERK1/2 (Figure 6I) in U87MG cells, nor did it reduce the choline induced phosphorylation of AKT1 (Figure S4H) and ERK1/2 (Figure S4I) in GBM5 cells.

3.4.2 Effects of the siRNA-mediated knock down of α9-containing nAChRs

As shown in Figure 1, qPCR indicated that the α9 subunit is more highly expressed than the α7 subunit in both U87MG and GBM5 cells. Silencing the α9 subunit in U87MG cells by means of α9-siRNAI blocked nicotine-induced cell proliferation (Figure 6D) and greatly reduced the nicotine-induced activation of the AKT1 (Figure 6E) and ERK1/2 signalling pathways (Figure 6F). Scrambled-transfected GBM5 cells continued to show nicotine-increased cell proliferation and AKT1 and ERK1/2 pathway activation, but not those transfected with α9-siRNAI (Figure S4D).

As shown in Figures 6G and S4G transfection of U87MG and GBM5 cells with scrambled siRNAs did not affect the ability of 1 μM (U87MG) or 50 μM choline (GBM5) to increase cell proliferation, but transfection with α9siRNAI completely abolished the choline-induced proliferation of both cell types, and reduced the effect of choline on AKT1 (Figures 6J and S4J) and ERK1/2 phosphorylation (Figures 6K and S4K).

4. Discussion and conclusions

The findings of this study show that, when stimulated by the nicotinic ligands nicotine and choline, nAChRs containing the α7 or α9 subunits increase the proliferation of GBM cells. The acute activation of α7- or α9-containing receptors by nicotine and choline on GBM cells activates the AKT anti-apoptotic pathway and the ERK proliferation pathway. These effects are due to interactions between the ligands and surface α7- and/or α9-containing receptors because the presence of non-cell-permeable peptide antagonists selective for α7- and/or α9-containing receptor blocks both ligand-induced and long-term basal proliferation, as well as the increases in pAKT and pERK. Silencing the expression of the α7 or α9 subunit by means of siRNAs also selectively strongly reduced the effects of nicotine and choline on GBM cells**.**

Although cigarette smoking is a known risk factor for numerous cancers, its association with gliomas has not been systematically studied in population-based case-control studies, cohort studies or meta-analyses. However, one case-control study of a Chinese population [4] has shown that smoking is associated with gliomas, particularly in subjects aged ≥50 years*.* An experimental study by Kahill et al. (2013)[22] has found that nicotine stimulates the malignant behaviour of glioma cells by activating epidermal growth factor receptors and protein kinase B, thus affecting cell proliferation, migration, signalling and radiation sensitivity. It has also been shown that functional nAChRs and mAChRs are expressed on GBM cells, and play a role in promoting tumour invasiveness by increasing the activity of metalloprotease-9 [23].

In order to investigate the effect of nicotine and choline on glioma cells and identify the nicotinic receptor(s) involved, we preliminarily analysed the nAChR subunits on publically available DNA microarray (accession number GSE16805) obtained from long term GBM cultures derived from patients at different tumour stages, and found that the most frequently expressed nAChR subunit mRNAs were *CHRNA5* and *CHRNA9* and at lower level *CHRNA7 and CHRNA10.* The expression of nicotinic subunits on GBM cells was expected, as it is known that nAChRs are expressed on glial cells reviewed in [13], but very little is known about their signalling mechanisms under physiological or pathological conditions.

The α7 subunit is ubiquitously expressed in brain and peripheral neurons; the α5 subunit is only expressed in restricted brain areas and in autonomic ganglia; and the α9 and α10 subunits are not expressed by neurons but highly expressed by the cochlear and vestibular hair cells of adult or

embryonic rat brain [29, 30]. Many non-neuronal and tumour cells express α5 and α7 mRNAs (reviewed in [9, 31, 32], and the α9 subunit has been found in nasal epithelium, the *pars tuberalis* of the pituitary gland [29] bone marrow, bronchial epithelia, keratinocytes, immune cells [33], human breast [34], lung tumours [28], and GBMs [23,24].

Previous studies have shown that, when expressed alone, the α10 subunit does not form functional channels but, when co-expressed with α9, it forms a subtype whose functional and pharmacological profile is very similar to that of the homomeric α9 receptor [30]. Receptors containing the α9 subunit show unusually fast and extensive agonist-mediated desensitisation, a distinct current-voltage relationship, and a biphasic response to changes in extra-cellular Ca^{2+} ions. They also have a particular pharmacology, and their ionotropic effects are blocked by classical nicotinic agonists (nicotine, epibatidine, cytisine), and a muscarinic agonist (muscarine) or antagonist (atropine) [19].

After making q-PCR analyses of a number of GBM cell lines, we selected U87MG and GBM5 cells because, they express the α5, α7, α9 and α10 subunits, and their expression of α10 mRNA is relatively low like primary cultures of GBM cells. Furthermore, U87MG is a continuous GBM cell line and GBM5 was obtained from GBM cancer stem cell (CSC)-enriched cultures resistant to temozolomide treatment [25].

However, we were unable to confirm the protein expression of these subunits in GBM cells using Western blotting because of the low protein expression and the lack of subunit-specific antibodies against α9. For these reasons, we used subtype-specific antagonists to identify the role of these receptors in the proliferation and signalling of GBM cells .

We used a wide range of nicotine concentrations for the proliferation assay, and found that concentrations in the range of those detected in the serum of smokers (10^{-8}–10^{-7} M) increased the proliferation of U87MG and GBM5 cells, which was blocked by both αBgtx and MLA (acting on both α7 and α9 receptors), as well as by the subtype-specific toxins AR (α7-selective) and RGIA4 (α9-selective).

We also found that choline increased proliferation of GBM cells in a dose-dependent manner, and that the dose inducing proliferation is in the low μM range, which is similar to the concentration present in the brains of humans and experimental animals and that is increased in GBMs [35].

Both U87MG and GBM5 cells express a very high level of α5 subunit mRNA, but as no α5- selective antagonists are available, we could not pharmacologically study the possible involvement of α5-containing receptors in choline and nicotine-induced proliferation of GBM cells.

Nicotine and choline stimulation of the nAChRs in U87MG and GBM5 cells triggered a number of protein kinase signalling cascades, thus simultaneously altering gene expression and inducing cellular changes. Both increased p-ERK/ERK and p-AKT/AKT ratios after a very short time of exposure (5-30 min), and the α7/α9 toxins (MLA and αBgtx), the α9-selective peptide RGIA4, and the α7-selective peptide AR all strongly decreased nicotine-induced ERK and AKT pathways

activation. Notably, choline was much more active in stimulating the ERK pathway in GBM5 cells than in U87MG cells.

In general, blockade of either α7 or α9 nAChRs with selective antagonists or siRNAs was able to substantially abolish choline or nicotine-induced pro-proliferation and decreases phosphorylation effects in GBM cells, indicating that both subunits are necessary for those effects of nicotinic agonists. This may have several explanations. One possibility is that there are two populations of nAChRs, α7 or α9 containing nAChRs, that have complementary effects and are both necessary to achieve a sufficient level of target cell activation. A second possibility is that heteropentameric α7α9 containing nAChRs are expressed in GBM cells and are principally responsible for the effects of choline or nicotine observed here.

The nicotine and choline concentrations that increased cell proliferation were much lower than those necessary to activate α7 receptors in electrophysiological experiments [31]. Together with the fact that nicotine acts as an antagonist on the ionotropic effects of α9 receptors, this suggests that, although nAChRs are ion channels mediating the influx of Na^+ and Ca^{2+} and the efflux of K^+, the ligand occupancy in U87MG and GBM5 cells leads to a ligand-associated conformational state that may alter nAChR scaffolds for interactions with other intracellular signalling molecules or cytoskeletal elements that elicits non-ionic, metabotropic signalling events that regulate the phosphorylation and dephosphorylation of the target proteins mediating some of the effects of nicotine [36]. Recent proteomic studies have also shown that α7 protein binds $G\alpha$ and $G\beta\gamma$ proteins through the intracellular M3–M4 loop, and that this enables downstream signalling, but it is still unknown how extracellular ligand binding is coupled to this important putative G-protein binding epitope [37, 38].

Metabotropic signalling after ligand binding is a property of α7 receptors in many non-neuronal cell types: for example, cholinergic signalling plays an important role in regulating cytokine release from immune cells, although no ACh-dependent currents have been recorded despite the fact that the α7 receptors are on the outer membrane [33, 39]. In addition, different metabotropic functions of α7 and α9 nAChRs have been described in monocytic cells including the inhibition of ATP-induced ion currents, inflammasome activation, and interleukin-1β (IL-1β) release [20, 21]. Moreover, silent α7 agonists (i.e. compounds with very weak partial agonist activity that convert α7 receptors to a long-lived desensitised state) are active in modulating nAChR activity in immune cells and in animal models of immune diseases [40].

As a further means of demonstrating the role of nAChRs in GBM cells, we interfered with the RNA of the α7 and α9 subunits, and found that decreasing or abolishing the expression of these subunits paralleled a decrease in nicotine-induced proliferation and intracellular signalling, thus replicating the results obtained using the α7- and α9-specific antagonists.

Similarly, α9 subunit silencing prevents choline-induced GBM cell proliferation and intracellular signalling. Conversely, α7 knockdown only prevents choline-induced proliferation.

Choline is an essential nutrient for all cells because it participates in the synthesis of the phospholipid components of cell membranes as a methyl-group donor in methionine metabolism, and in the synthesis of the neurotransmitter ACh. Our findings that αBgtx-sensitive nAChRs are present in GBM cells and that added choline binds to them to activate proliferation and the AKT and ERK pathways support the idea that choline stimulates GBM growth and proliferation in an autocrine or paracrine manner. As choline is essential for the synthesis of ACh, we cannot say whether the increase in GBM cell proliferation after 72 hours exposure to choline is due to the direct action of choline itself or to that of ACh; however, the blocking effect of the α7/α9 nicotinic antagonists clearly indicates the mediation of nAChRs. The possibility that ACh is the signalling molecule acting on nAChRs is suggested by the findings of Thompson and Sontheimer (2019) [23], who evaluated the expression of various components of ACh synthesis and catabolism in a large GBM cohort and found that the expression of the mRNA of many choline transporters, choline acetytransferase (the enzyme responsible for acetylcholine synthesis) and vescicular acetylcholine transporters are significantly increased in GBM tissues in comparison with their non-tumour counterparts. In line with these findings, we determined the presence of both choline and ACh in the supernatant of U87MG cells (data not shown). Their levels did not show any increase with the increased time in culture.

Thompson and Sontheimer (2019)[23] also examined the expression of nAChRs and mAChRs in GBM data sets, GBM cell lines, and patient-derived xenograft lines and (in agreement with our own data) found a high expression level of α9 mRNA in GBM cells. Using calcium time-lapse studies, they also showed that the activation of both mAChRs and nAChRs leads to an increase in intracellular Ca^{2+}. The time-course of nicotine and ACh activation on the homomeric α7 receptor is very fast in the order of milliseconds [41], whereas they found that both nicotine and an α7-specific compound activate Ca^{2+} influx with time course of hundreds of seconds. However this could be due to release of calcium from intracellular stores. In fact although 100 μM of nicotine is highly desensitising, it has long been known [42] that activated α7 receptors in hippocampal astrocytes generate calcium transients mainly as a result of calcium-induced calcium release (CICR), and that this calcium signalling response persists beyond the expected time course of channel activation. Thus, the capability of α7 to operate as a metabotropic/ionotropic receptor prolongs the transient electrical response, involving intracellular calcium influx and membrane depolarization, in a more prolonged and sustained response involving multiple intracellular events.

In conclusion, our findings indicate that nicotine and choline activate the signalling pathways involved in the proliferation of GBM cells, and that these effects are mediated by α7/ α9-containing nAChRs. This suggests that these receptors are potential targets for new therapeutic strategies and, for this reason, we are currently evaluating the possible anti-GBM effects of new nicotinic compounds [43]. A better understanding of the role played by nAChRs in the pathogenesis of glioblastoma will help in the development of new treatments targeting this very aggressive tumour.

Conflit of interest

Dr Michael Mcintosh declares that Conotoxin peptides, including those referenced in this paper, have been patented by the University of Utah where he is listed as an inventor.

All the other authors declare that do not have conflict of interest , with no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.

Declaration of competing interest

Conotoxin peptides, including those referenced in this paper have been patented by the University of Utah with Dr Michael McIntosh listed as an inventor.

All the other authors declare that do not have conflict of interest , with no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted.

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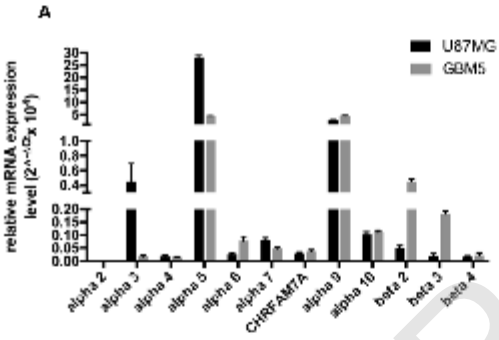
Figure 1. Relative expression of nAChR subunits in U87MG and GBM5 cell lines

A) Results of the qPCR analysis of nAChR gene expression in human U87MG (black bars) and GBM5 cells (grey bars). The data relate to relative expression ± SEM (normalised to endogenous *GAPDH* expression, in accordance with the $2^{-\Delta Ct}$ method) calculated in five independent experiments performed in triplicate. B) Expression of nAChR subunits in U87MG and GBM5 cell lines calculated as $2^{-\Delta Ct} * 10^4$. The most highly expressed subunits are in bold.



B

nAChR	U87MG	GBM5
CHRNA2	nd	nd
CHRNA3	0.451 ± 0.131	0.018 ± 0.002
CHRNA4	0.017 ± 0.003	0.015 ± 0.003
CHRNA5	**28.13 ± 0.435**	**4.378 ± 0.079**
CHRNA6	0.025 ± 0.003	0.078 ± 0.009
CHRNA7	0.080 ± 0.005	0.047 ± 0.003
CHRFAM7A	0.030 ± 0.002	0.037 ± 0.003
CHRNA9	**2.688 ± 0.057**	**4.450 ± 0.150**
CHRNA10	0.103 ± 0.005	0.111 ± 0.003
CHRNB2	0.052 ± 0.005	0.452 ± 0.016
CHRNB3	0.021 ± 0.004	0.181 ± 0.007
CHRNB4	0.016 ± 0.002	0.022 ± 0.005

Figure 2. Effects of nicotine, choline and nicotinic antagonist exposure on U87MG and GBM5 cell proliferation. Exposure to the indicated concentrations of nicotine for 72 hours significantly increased U87MG (A, n=10-12) and GBM5 cell proliferation (D, n=6,10). Treatment with 1 μM αBgTx or 500 nM MLA in the respective presence of 50 nM or 100 nM nicotine for 72 hours inhibited

nicotine-induced U87MG (B, n=11-14) and GBM5 cell proliferation (E, n=13-16). Exposure to 1 µM of the α9 subtype-selective peptide RGIA4 or 1 µM of the α7 subtype-selective peptide AR for 72 hours prevented the nicotine-induced proliferation of U87MG (C, n=10) and GBM5 cells (F, n=10-12).

Exposure to the indicated concentrations of choline for 72 hours significantly increased U87MG (G, n=10) and GBM5 cell proliferation (J, n=16). Treatment with 1 µM αBgTx or 500 nM MLA in the respective presence of 1 µM or 50 µM choline for 72 hours, inhibited the choline-induced proliferation of U87MG (H,n=11) and GBM5 cells (K,n=14-15) respectively. The α9 or α7 subtype-specific toxins RGIA4 and AR blocked the choline-induced proliferation of U87MG (I,n=13) and GBM5 cells (L,n=16).

The proliferation analysis was made using one-way ANOVA followed by a *post hoc* Bonferroni test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* untreated or nicotine/choline-treated cells), and the results are expressed as the mean values ± SEM in each group

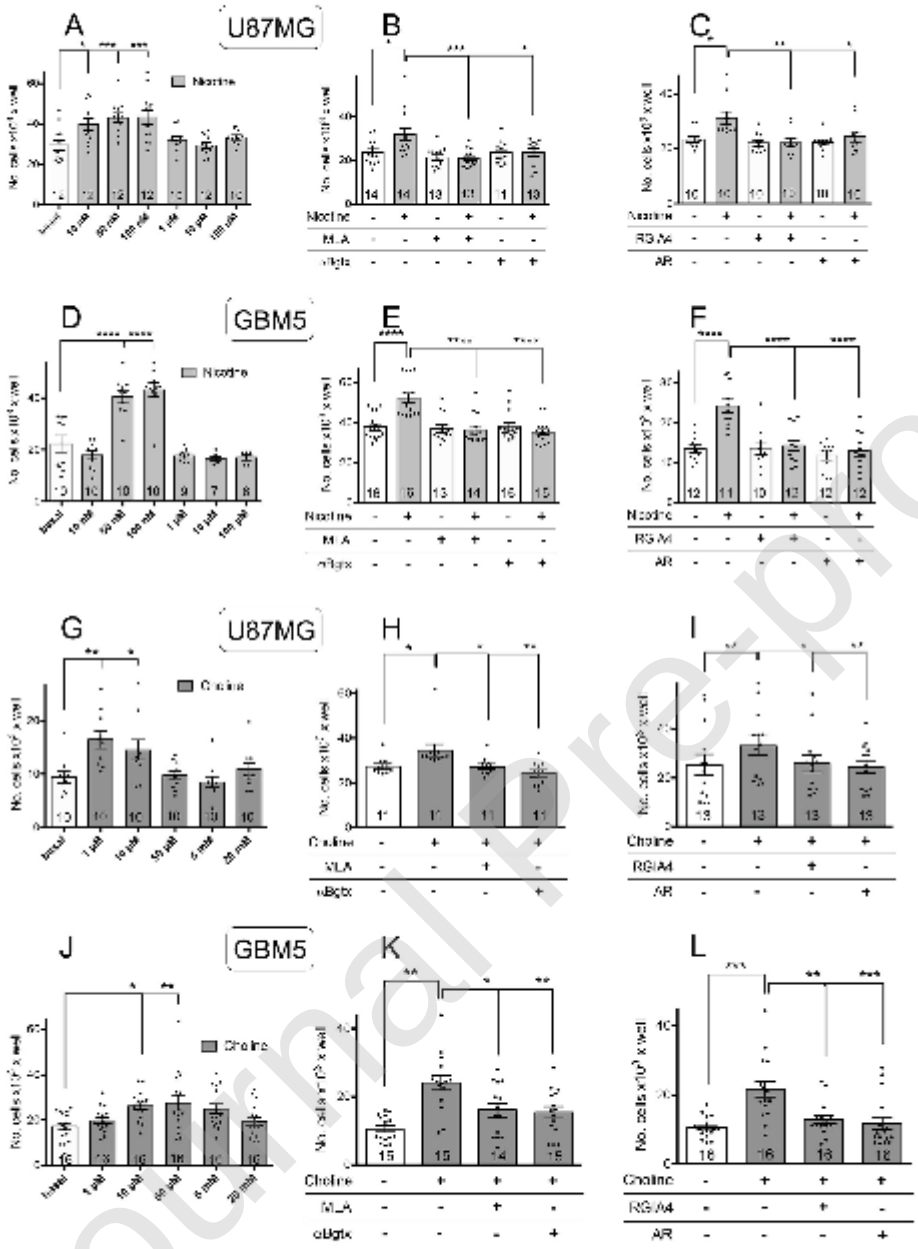


Figure 3. Effects of chronic (six-day) nicotinic antagonist exposure on U87MG and GBM5 cell proliferation.

Six-day exposure to 1 μM αBgTx or 500 nM MLA significantly reduced U87MG (A, n=15) and GBM5 cell proliferation (C, n=17). Treatment with the α9 subtype-selective peptide RGIA4 and the α7

subtype-selective peptide AR, alone or together, also blocked U87MG cell proliferation at all of the tested concentrations (100 nM, 250 nM and 1 µM) (B, n=11). When used together, AR and RGIA4 inhibited the proliferation of GBM5 cells (D,n=10-13) at all of the tested concentrations, whereas RGIA4 alone was only effective at the concentration of 250 nM and 1 µM and AR alone was only effective at the concentration of 1 µM. The analysis was made using one-way ANOVA followed by a *post hoc* Bonferroni test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* untreated cells), and the results obtained from the indicated number of experiments are expressed as the mean values ± SEM in each group.

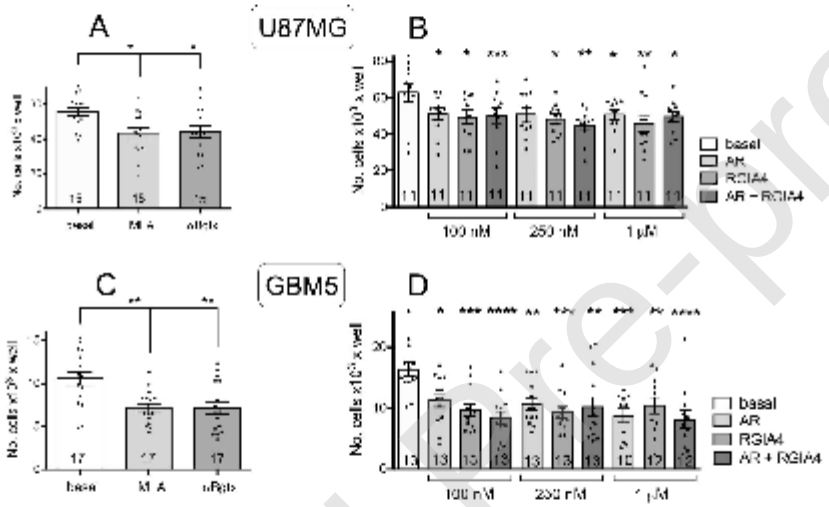


Figure 4. Effects of nicotine and nicotinic antagonist exposure on U87MG signalling.

The cells were pre-incubated or not with 500 nM MLA (A, n=6,8), 1 µM αBgTx (B, n=6,8), 1 µM AR (C, n=6,7) or 1 µM RGIA4 (D, n=7,9) for 30 minutes, and then treated for the indicated times with 50 nM nicotine in the presence or absence of each antagonist. The Western blot results are

expressed as the increase in the pAKT/AKT ratio (A,B,C,D) with the untreated cells (basal) considered as 1. The same protocol was used to analyse the effects of the same concentrations of MLA (E,n=5,6), αBgTx (F, n=5,7), AR (G, n=5,8) and RGIA4 (H, n=6,7) on the pERK/ERK ratio. The graphs show the mean values ± SEM of each group obtained from the indicated number of experiments. A representative blot at the corresponding times is shown below the graph. The Western blotting data were analysed using the Kruskal-Wallis test followed by Dunn's test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* untreated cells and °P<0.05; °°P<0.01; °°°P<0.001; °°°°P<0.0001 *vs* nicotine-treated cells).

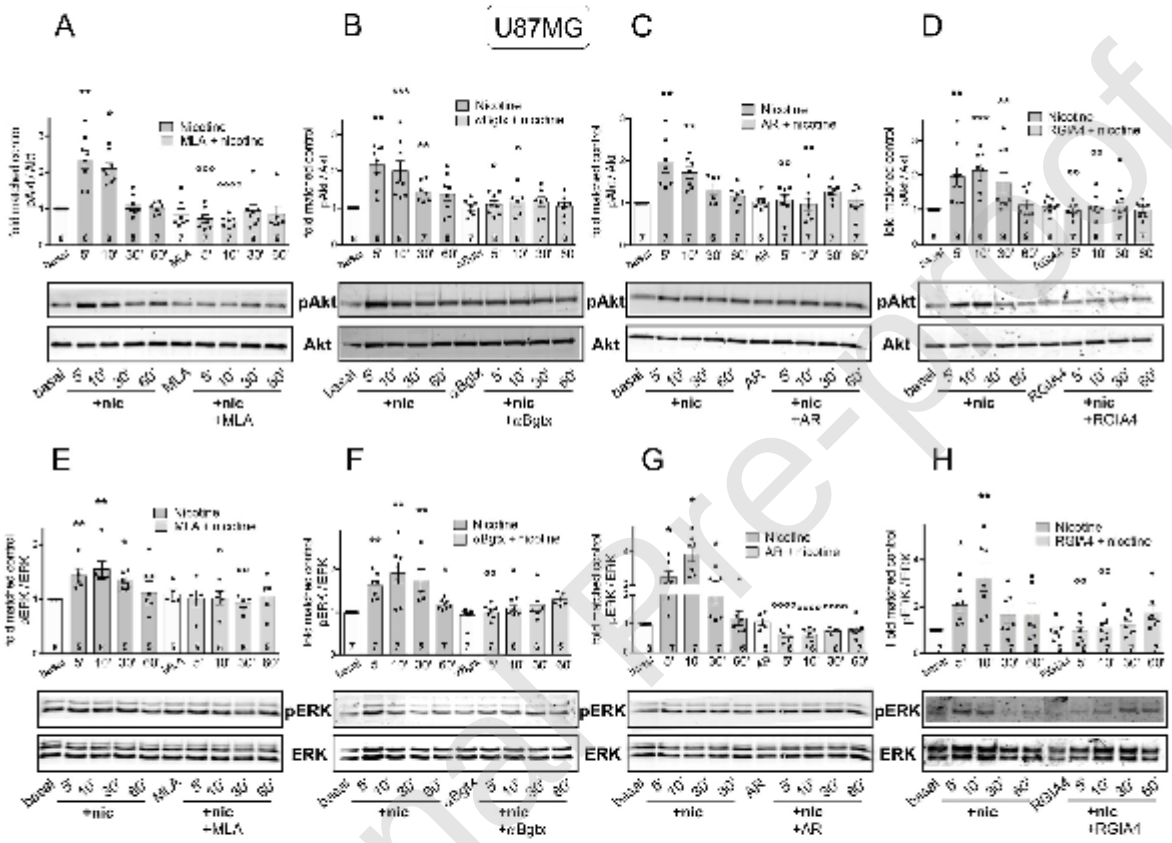


Figure 5. Effects of choline and nicotinic antagonist exposure on U87MG signalling.

The cells were pre-incubated or not with 500 nM MLA (A, n=7,9), 1 µM αBgTx (B, n=7,9), 1 µM AR (C, n=7,9) or 1 µM RGIA4 (D, n=6,7) for 30 minutes, and then treated for the indicated times with 5 mM choline in the presence or absence of each antagonist. The Western blot results are expressed

as the increase in the pAKT/AKT ratio (A,B,C,D) with the untreated cells (basal) considered as 1. The same protocol was used to analyse the effects of the same concentrations of MLA (E,n=6,8), αBgTx (F, n=6,8), AR (G, n=7,8) and RGIA4 (H,n=7,9) on the pERK/ERK ratio.

The graphs show the mean values ± SEM of each group obtained from the indicated separated experiments. A representative blot at the corresponding times is shown below the graph. The Western blotting data were analysed using the Kruskal-Wallis test followed by Dunn's test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* untreated cells and °P<0.05; °°P<0.01; °°°P<0.001; °°°°P<0.0001 *vs* choline-treated cells).

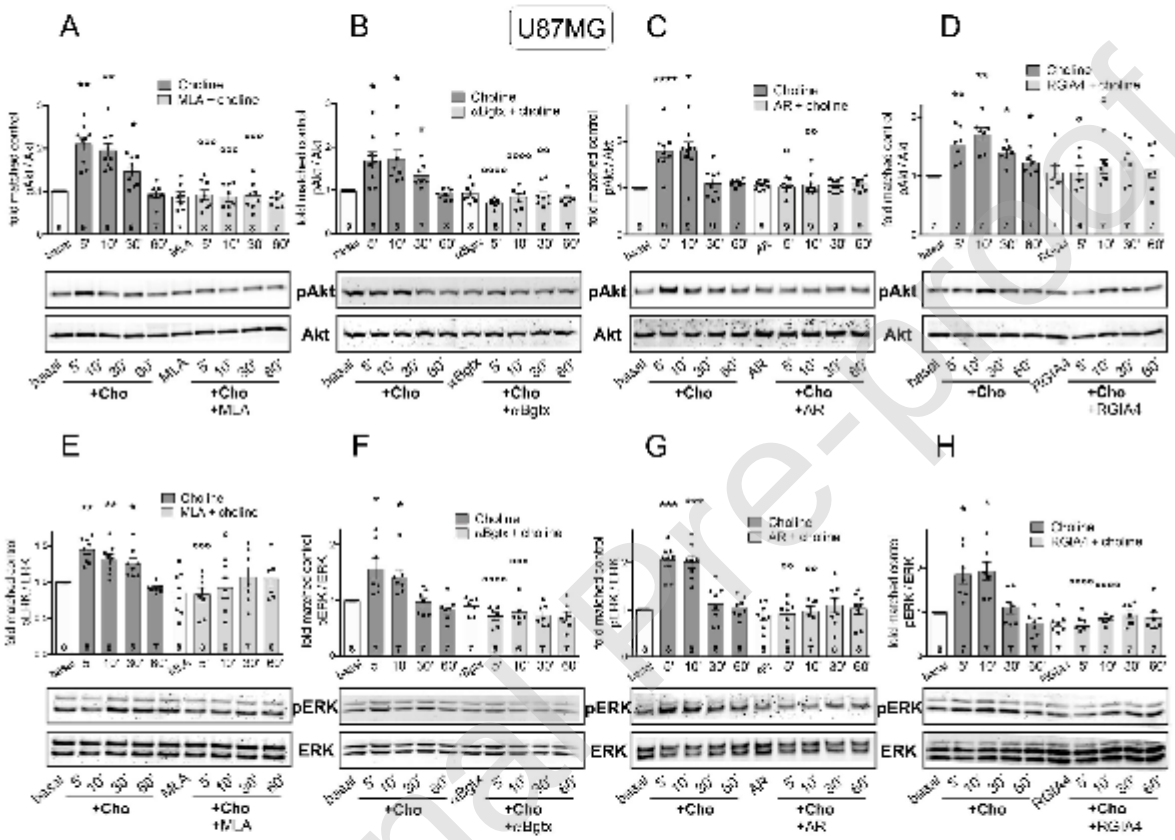


Figure 6. siRNA knock down of the α7 or α9 subunit reduces nicotine-induced proliferation and the activation of AKT signalling in the U87MG cell line.

(A) Effect of knocking down the α7 subunit on nicotine-induced cell proliferation. U87MG cells transfected with α7siRNAII or scrambled siRNAs were treated for 48 hours with 50 nM nicotine and

then counted. The data (n=9,11) were analysed using one-way ANOVA followed by a *post hoc* Bonferroni test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* cells transfected with scrambled siRNAs), and the results of the indicated experiments are expressed as the mean values ± SEM for each group.

(B) Western-blot analysis and a representative panel of AKT pathway activation in U87MG cells transfected with α7siRNAII or scrambled siRNAs, and treated with 50 nM nicotine for the indicated times. The Western blot data (n=9) are expressed as the increase in the pAKT/AKT ratio, with the untreated cells transfected with scrambled siRNAs considered as 1.

(C) Western-blot analysis and a representative panel of ERK pathway activation in U87MG cells transfected with α7siRNAI or scrambled siRNAs, and treated with 50 nM nicotine for the indicated times. The Western blot data (n=8,9) are expressed as the increase in the pERK/ERK ratio, with the untreated cells transfected with scrambled siRNAs considered as 1.

(D) Effect of knocking down the α9 subunit on nicotine-induced cell proliferation. U87MG cells transfected with α9siRNAI or scrambled siRNAs were treated for 48 hours with 50 nM nicotine and then counted (n=13).

(E) Western-blot analysis and a representative panel of AKT pathway activation in U87MG cells transfected with α9siRNAI or scrambled siRNAs, and treated with 50 nM nicotine for the indicated times (n=7).

(F) Western-blot analysis and a representative panel of ERK pathway activation in U87MG cells transfected with α9siRNAI or scrambled siRNAs, and treated with 50 nM nicotine for the indicated times. (n=5,6).

(G) Effect of knocking down the α7 or α9 subunit on choline-induced cell proliferation. U87MG cells transfected with α7siRNAII, α9siRNAI or scrambled siRNAs were treated for 48 hours with 1 μM choline and then counted. The data (n=13) were analysed using one-way ANOVA followed by a *post hoc* Bonferroni test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* cells transfected with scrambled siRNAs) and the results of the indicated experiments are expressed as the mean values ± SEM for each group.

(H) and (I) Western blot analysis and a representative panel of AKT (H,n=9) or ERK pathway activation (I,n=5,6) in U87MG cells transfected with α7siRNAII or scrambled siRNAs, and treated with 5 mM choline for the indicated times.

(J) and (K) Western blot analysis and a representative panel of AKT (J,n=7,9) or ERK pathway activation (K,n=6,8) in U87MG cells transfected with α9siRNAI or scrambled siRNAs, and treated with 5 mM choline for the indicated times.

The graphs show the mean values ± SEM obtained from the indicated experiments for each group. A representative blot at the corresponding times is shown below the graph.

The Western blot results are expressed as pAKT/AKT and pERK/ERK ratios, with the untreated cells transfected with scrambled siRNAs considered as 1. The Western blot analyses of AKT and

ERK pathway activation were made using the Kruskal-Wallis test followed by Dunn's test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* untreated scrambled-transfected cells and °P<0.05; °°P<0.01; °°°P<0.001; °°°°P<0.0001 *vs* scrambled-transfected cells treated with nicotine or choline at each time). The results of the indicated experiments are expressed as the mean values ± SEM for each group.

The analyses of the effect of knocking down the α7 and α9 subunit on cell proliferation were made using one-way ANOVA followed by a *post hoc* Bonferroni test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 *vs* cells transfected with scrambled siRNAs), and the results of the indicated experiments are expressed as the mean values ± SEM for each group.