



HAL
open science

Molecular cloning and characterization of a novel human variant of RIC-3, a putative chaperone of nicotinic acetylcholine receptors

Tamara Seredenina, Teresa Ferraro, Georg C Terstappen, Andrea Caricasole, Renza Roncarati

► To cite this version:

Tamara Seredenina, Teresa Ferraro, Georg C Terstappen, Andrea Caricasole, Renza Roncarati. Molecular cloning and characterization of a novel human variant of RIC-3, a putative chaperone of nicotinic acetylcholine receptors. *Bioscience Reports*, 2008, 28 (6), pp.299-306. 10.1042/BSR20080055 . hal-00479301

HAL Id: hal-00479301

<https://hal.science/hal-00479301>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Title: Molecular cloning and characterization of a novel human variant of RIC-3, a putative chaperone of nicotinic acetylcholine receptors.

Authors: Tamara Seredenina, Teresa Ferraro, Georg C. Terstappen, Andrea Caricasole, and Renza Roncarati.

Affiliation: Siena Biotech S.p.A. , Strada del Petriccio e Belriguardo 35, 53100 Siena, Italy

Short running title: Cloning of a novel human variant of RIC-3

Corresponding author: Renza Roncarati

Address:

Siena Biotech S.p.A.,
Strada del Petriccio e Belriguardo 35
53100, Siena

Italy

E-mail: roncarati@sienabiotech.com

Tel: +390577381332

Fax: +390577381303

Accepted Manuscript

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Abstract

Recent reports demonstrate that the RIC-3 protein is important for the maturation of nicotinic acetylcholine receptors. In the present work RIC-3e, a novel variant of RIC-3 is described. This variant carries a deletion of exons 4 and 5 resulting in a protein product lacking a conserved coiled-coil domain. Like RIC-3, the new variant is predominantly but not exclusively expressed in the brain. The analysis of expression of RIC-3 variants' mRNA and of $\alpha 7$ -nAChR mRNA in a set of human tissues shows a similar profile. The RIC-3e protein is functionally active and enables surface expression of mature $\alpha 7$ -nAChRs in cell lines otherwise not permissive for the expression of this receptor.

Keywords: alpha7-nAChR, alpha-bungarotoxin, chaperone, expression, RIC-3, splice variant

The nucleotide sequence reported here has been submitted to the GeneBank sequence data bank and is available under accession number AM422214

Accepted Manuscript

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels which are widely distributed in the central and peripheral nervous system. They are involved in a variety of physiological processes including cognition and development and their dysfunction has been associated with a variety of neurological disorders [1-5]. Hence, nicotinic receptors represent important and intensively studied targets for therapeutic intervention. The $\alpha 7$ -nAChR is of particular relevance for Alzheimer's disease and schizophrenia, but studies focusing on this receptor are challenging since it cannot be easily expressed in heterologous systems [6-9]. In fact, the complex process leading to functional expression of $\alpha 7$ -nAChR is likely to require the activity of multiple cell-specific assembly and trafficking proteins [10;11]. Recently it was shown that the protein RIC-3 (resistant to inhibitors of cholinesterase) is necessary for functional expression of $\alpha 7$ -nAChRs [12;13]. Human RIC-3 belongs to a conserved gene family [14] and is predicted to contain a cleavable signal peptide in vertebrate species, followed by a proline-rich domain, one transmembrane domain and a cytoplasmic component that consists of a conserved coiled-coil domain and a non conserved C-terminus [15]. Another experimental evidence suggests the absence of a signal peptide indicating that RIC-3 has two transmembrane domains [16]. The function of this protein is not yet clear; the prevailing hypothesis states that RIC-3 acts in the endoplasmic reticulum (ER) as a chaperone which regulates the expression of nicotinic and 5-HT₃ receptors by either promoting their maturation and functional expression or by retaining the receptors in the ER, depending on the composition and type of receptor [17;18]. The precise mechanism of action of this protein is not known, however experimental evidence supports an interaction of RIC-3 with unassembled nAChR subunits [18]. To date, the existence of four distinct transcripts of human RIC-3 has been demonstrated [14] but little is known about the function of these variants. Here, we report the identification, cloning and detailed pattern of expression of a new variant of human RIC-3, and demonstrate that this variant is able to promote the maturation and surface expression of $\alpha 7$ -nAChRs in cells that are otherwise not permissive for the expression of this receptor.

Materials and methods

Molecular cloning. PCR amplification was carried out using a proofreading enzyme (Phusion High-Fidelity DNA Polymerase, Fintzymes) following the manufacturer's instructions. 3% DMSO was added to the reactions. Primer sequences were as follows: 5'-CACCATGGCGTACTCCACAGT-3' (forward) and 5'-TCACTCTAAACCCTGGGGGT-3' (reverse). Reaction details were as follows: 98°C/1 min; 10 cycles of (30 sec at 98°C; 30 sec at 65°C, decreasing by 1°C every cycle; 30 sec at 72°C), followed by 30 cycles of (30 sec at 98°C; 30 sec at 55°C, 30 sec at 72°C), followed by 7 min at 72°C. cDNA from adult human hippocampus (see below) was used as template. PCR products were purified using Qiagen gel extraction kit, cloned into pCR-Blunt II-TOPO (Invitrogen) and subcloned into pcDNA3.1 Zeo(+). The inserted DNA was sequenced by standard protocols (MWG-Biotech). The alignment of the amino acid sequences was performed using MUSCLE 3.41 and visualized with GeneDoc 2.6.002.

Real-time quantitative PCR. cDNA was synthesized from 2 μ g of polyadenylated RNA (Clontech) employing Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primer (Proligo France SAS) according to the manufacturer's instructions. Real-time PCR was performed using an I-Cycler and the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. One μ l of cDNA was used as template. Primer concentration was 10 μ M. Reactions were carried out in triplicate. Reaction conditions were as follows: 5 min at 95°C; 45 cycles of (30 s at 95°C; 30 s at 55°C). As control, amplification without template was performed. The fluorescence threshold cycle (Ct) was determined using the iCycler iQ real-time PCR detection system software (BioRad). The relative starting quantities of each transcript were determined by interpolating the Ct values of the unknown samples to the standard curve. Real time PCR

experiments were repeated twice for each target sequence. Quantification of β -actin expression was carried out in parallel in order to normalize the levels of expression in each sample. Primer sequences were designed to different exons of the human RIC-3, RIC-3e and $\alpha 7$ -nAChRs genes. Primer sequences were as follows: 5'-CAGATCATTTGGGTTGGGAAA-3' (forward) and 5'-CACACGAGGTAACAGAATTATCTTCCT-3' (reverse) to amplify a product located within exon 6 of RIC-3; 5'-GGGAAAACAACACTGCAGAGGAT-3' (forward) and 5'-TCTTCAGGGTAACTAATTTTCCTG-3' (reverse) to amplify specifically the new variant RIC-3e; 5'-GCAAGAGGAGTGAAAGGTTCTATGA-3' (forward) and 5'-CATGGTCACTGTGAAGGTGACA-3' (reverse) was designed to amplify a product within human $\alpha 7$ -nAChRs; 5'-CCTGGCACCCAGCACAAT-3' (forward) and 5'-GCCGATCCACACGGAGTACT-3' (reverse) to amplify a fragment within human β -actin.

Cell culture and transfection. CHO-K1 cells were cultured in Ham's F12 medium with 10% FBS, HEK 293 cells were cultured in DMEM with 10% FBS. All media were supplemented with 2 mM Glutamax, 100 U/ml penicillin and 100 μ g/ml streptomycin. All media and supplements for cell culture were from Gibco, if not specified otherwise. For transient transfections 7×10^4 cells/cm² were plated one day before transfection on 13 mm coverslips treated with 10 μ g/ml poly-D-lysine. For FLIPR-Ca²⁺ measurements HEK 293 cells were plated in 96-well clear-bottom, black-well plates (Costar) at a density of 4×10^4 cells/well and cultured for 24 hours prior to transfection. All transfections were carried out using Lipofectamine-2000 (Invitrogen) following the instructions of manufacturer. 0.1 μ g of DNA per cm² was used for each transfection. The ratio DNA/Lipofectamine was 1/3 w/v. Transfection mix was prepared in OptiMEM. Incubation time with transfection mix was 4h. After the transfection, fresh medium was added and cells were incubated at 37°C. Assays were performed 24-48 h after transfection. All manipulations with cells were carried out in sterile conditions. Cells were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO₂/air.

α -Bgt fluorescent staining. For fluorescence microscopy, HEK 293 and CHO-K1 cells were cultured on PDL-coated glass coverslips at a density of 7×10^4 cells per cm². Labeling was performed with 0.5 μ g/ml of α -Bgt conjugated with Alexa FluorTM-555 (Molecular Probes); for 45 min at 4°C. After three washes with PBS cells were fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. Coverslips were subsequently mounted with Prolong[®] gold antifade reagent (Molecular Probes) on glass slides and analyzed with a Zeiss LSM510 confocal microscope equipped with Argon and He/Ne lasers.

Ca²⁺ flux measurements using a FLIPRTM system

FLIPR-Ca²⁺ experiments were performed as described previously [19]. In brief, 24 hours after transfection the medium was replaced with 100 μ l of Hank's Balanced Salt Solution-Hepes 20 mM, pH 7.4 (assay buffer) containing 4 μ M Fluo-4-AM, 0.02% pluronic acid and 5 mM probenecid. After 30 minutes of incubation at 37°C the labeling solution was removed and 200 μ l of assay buffer containing 2.5 mM probenecid and 10 μ M MLA where appropriate were added. Plates were then transferred to a Fluorometric Imaging Plate Reader system (FLIPR, Molecular Devices) with excitation and emission wavelengths set to 488 nm and 540 nm, respectively. Basal fluorescence was recorded for 30 seconds, followed by addition of 50 μ l of a 5x concentrated solution of nicotine or PNU-120596 and measurement of fluorescence for 10 minutes. Subsequently, 50 μ l of a 6x solution of nicotine were added and fluorescence was measured for 3 minutes. Measurements were made at 1 second intervals for 1 minute after compound addition and at 30 second intervals for the remaining time. The results were exported from the FLIPR raw data as MAX-MIN of fluorescence signal intensity in two intervals corresponding to the first and the second addition of compounds.

Results and discussion

Identification of a novel variant of human RIC-3

In order to clone RIC-3 transcripts, proofreading PCR was carried out using cDNA from adult human hippocampus as template. Primers employed in the reactions were designed based on the known human RIC-3 sequence (RefSeq NM_024557) and comprised initiation and stop codons. Four distinct products were obtained in the RT-PCR (Fig.1). The specificity of the primers employed was confirmed by performing the reactions with single primer controls; also, a negative control PCR lacking template cDNA did not produce any amplification (data not shown). The band of ca. 1100 bp (Fig. 1) corresponds to the major form of RIC-3 (AY326435 [14]), as confirmed by DNA sequencing. Four human RIC-3 variants (a, b, c and d) have been previously described [14]. RIC-3a is the major sequence of RIC-3; for simplicity we will refer to this splice variant as "RIC-3". Based on the apparent length, the two faster migrating products most likely correspond to the shorter variants of RIC-3 (GenBank accession numbers AK021670 and AL832601) [14]. The minor product of ca. 900 bp does not correspond to any of the previously identified RIC-3 variants. This putative human RIC-3 variant was cloned into the pCR-Blunt II-TOPO vector and sequenced. The sequence (accession number AM422214), demonstrates that the cloned fragment corresponds to human RIC-3. We named the novel variant "RIC-3e", following the nomenclature proposed by Halevi et al. [14]. The initiation and stop codons are the same as those reported for RIC-3 [14]. A comparison with the gene structure of the human RIC-3 locus shows that RIC-3e contains the exons 1, 2, 3 and 6. The deletion of 240 bp corresponds to the lack of exons 4 and 5, probably due to alternative splicing. The prediction of the amino acidic sequence and alignment with the known human RIC-3 variants confirmed that the novel cDNA carries a deletion of 240 bp and encodes for a RIC-3 protein of 288 amino acids which lacks the coiled-coil domain and a part of the C-terminal sequence (Fig. 2).

Expression profile of RIC-3 variants and $\alpha 7$ -nAChR in human tissues

Little is known about tissue specific distribution of RIC-3. Studies in *C. elegans* [12] show that RIC-3 is expressed in almost all neurons and the body wall muscles. *In situ* hybridization of mouse brain with a RIC-3 probe produces a low signal in most regions of the brain with higher levels in cerebellum and some hippocampal areas. Northern blot analysis demonstrates RIC-3 expression in human brain and in some peripheral tissues [14]. The detailed analysis of the distribution of RIC-3 expression is an important step towards the understanding of its biological function. Therefore, a series of quantitative PCR (qPCR) studies was carried out to determine the expression profile of RIC-3 and RIC-3e transcripts in human tissues. A panel of cDNAs synthesized from mRNA samples derived from different human tissues, including several distinct brain regions, was used. The first set of primers (see Materials and methods) was designed to amplify a product located within exon 6 of RIC-3. This set of primers recognizes all known transcripts except for the truncated form AY326436/AY358475 (see Fig. 2B). A second set of primers was designed to specifically amplify the new variant RIC-3e. Since exons 4 and 5 are missing in this variant, the reverse primer was designed across the junction of exon 3 with exon 6. As expected, a single product corresponding to RIC-3e was amplified from cDNAs from different tissues using this set of primers (data not shown). The results presented in Fig. 3 (A, B) indicate a tight correlation of the distribution and level of expression of the novel RIC-3e variant and the major RIC-3 isoform. The human RIC-3 gene is expressed predominantly in the central nervous system with the highest levels in cerebellum and pituitary gland. These data on mRNA expression levels in different areas of human brain are in general in accordance with recently published work which describes RIC-3 protein localisation in the rat brain [16]. In peripheral tissues high levels of RIC-3 are observed in pancreas and testis with much lower levels of expression in other tissues.

Since RIC-3e has a similar expression profile as compared to RIC-3, the functional significance of this new variant remains elusive. However despite the similarity of tissue distribution of the two

variants cell-type or cell compartment-specific expression can not be excluded. A detailed comparison of RIC-3 and RIC-3e proteins in tissues, at the single-cell level resolution will be required to verify this possibility.

RIC-3 was shown to be necessary for functional expression of $\alpha 7$ -nAChRs [13;14]. However, a detailed comparative expression analysis of both genes is not available. Therefore, qPCR of $\alpha 7$ -nAChR was performed on the same panel of human tissues in order to determine if RIC-3 and $\alpha 7$ -nAChR transcripts are coherently expressed (Fig. 3C). Figure 3C shows that $\alpha 7$ -nAChRs are widely expressed within the central nervous system. The highest levels of $\alpha 7$ -nAChR transcripts were found in the cerebral cortex, hippocampus and amygdala. This quantitative analysis correlates well with the distribution pattern of $\alpha 7$ -nAChR described by *in situ* hybridization and [125 I]-Bgt-binding studies [20;21]. Analysis of the abundance of $\alpha 7$ -nAChR transcripts in the peripheral tissues shows high level of expression in human testis. Interestingly, some recent publications suggest a function for $\alpha 7$ -nAChR in the reproductive system [22;23]. Comparison of the distribution pattern of RIC-3 and $\alpha 7$ -nAChR shows an overlapping profile of the two genes, which are both expressed at higher levels in the CNS than in the periphery. In general, all the tissues that express $\alpha 7$ -nAChR also express significant levels of RIC-3, consistent with the proposed function of RIC-3 as a chaperone of nicotinic receptors [12]. Nevertheless, high levels of expression of RIC-3 are also seen in some brain regions (corpus callosum, pituitary gland, cerebellum) which express relatively low levels of $\alpha 7$ -nAChR. This suggests that RIC-3 might possess other, as yet unknown functions not confined to the process of maturation of $\alpha 7$ -nAChR.

Analysis of functional activity of RIC-3e

RIC-3 was shown to promote the folding, assembly and membrane targeting of recombinant $\alpha 7$ -nAChR in different mammalian cell lines [13;18]. In particular, co-expression of RIC-3 and $\alpha 7$ -nAChR resulted in the formation of surface α -Bgt-binding receptors in host cells that are otherwise unable to form functional $\alpha 7$ -nAChRs, such as HEK 293, COS and tsA201 [13;17;18]. As the novel variant RIC-3e lacks the evolutionarily conserved coiled-coil domain and a part of the C-terminal region present in RIC-3 proteins, we determined the capacity of RIC-3e to mediate proper membrane expression of $\alpha 7$ -nAChRs in a cellular background which is non-permissive to functional $\alpha 7$ -nAChRs expression. To this end, HEK 293 cells were transfected with $\alpha 7$ -nAChR, either alone or in combination with RIC-3 or RIC-3e. In order to detect $\alpha 7$ -nAChR on the cell surface, live cells were incubated with fluorescently labeled α -Bgt. As expected, expression of human $\alpha 7$ -nAChR alone did not result in the formation of α -Bgt-binding receptors (Fig. 4A,B), On the contrary, co-expression of $\alpha 7$ -nAChR with either RIC-3 (Fig. 4C,D) or RIC-3e (Fig. 4E,F) resulted in α -Bgt binding to cell membranes, indicating that both isoforms are able to promote surface expression of mature $\alpha 7$ -nAChRs. Similar results were also observed using CHO-K1 cells (data not shown).

In order to demonstrate that co-expression of RIC-3e does not only lead to expression of $\alpha 7$ -nAChRs on the plasma membrane but also promotes formation of functionally active channels, we performed measurements of Ca^{2+} influx as this channel is known to possess a high conductivity for Ca^{2+} ions in the activated state. To this end HEK 293 cells were transiently transfected with constructs encoding human $\alpha 7$ -nAChR together with RIC-3 or RIC-3e and with each plasmid alone as control. Subsequently, cells were stimulated with nicotine in the absence or presence of the positive allosteric modulator PNU-120596 [24]. The latter was necessary in order to increase the open-time of the $\alpha 7$ -nAChR (which is known to desensitize very rapidly) such that the Ca^{2+} influx is sufficient to be measured with a FLIPR system. In fact, we had previously shown that - depending on the cell line used - potentiation of the activity of $\alpha 7$ -nAChR with PNU-120596 was necessary in order to enable functional measurements of Ca^{2+} flux with a FLIPR system [19].

As expected, treatment with nicotine alone did not elicit any measurable changes of the intensity of fluorescence (Fig. 5). Conversely, the addition of nicotine after treatment with PNU-120596 resulted in a significant increase of intracellular Ca^{2+} concentrations. Importantly, this effect was

only observed in the cells co-transfected with $\alpha 7$ -nAChR and RIC-3 or $\alpha 7$ -nAChR and RIC-3e. Moreover, the increase of fluorescence intensity was completely abolished when the cells were pretreated with methyllycaconitine (MLA), a selective antagonist of $\alpha 7$ -nAChR, thus confirming specificity of the obtained results. In all experiments, the magnitude of the functional response of co-transfected $\alpha 7$ -nAChR and RIC-3e was comparable to $\alpha 7$ -nAChR in combination with RIC-3. More detailed studies of the two splice variants cannot be made in this system as subtle differences may be masked by the strong potentiation induced by PNU-120596. Taken together, we can conclude that RIC-3e is a functional splice variant leading to expression of functional $\alpha 7$ -nAChRs on the plasma membrane. As compared to RIC-3, the variant RIC-3e carries a deletion of the coiled-coil domain and part of the C-terminal domain. Previously published deletion analyses of RIC-3 suggested that ablation of the coiled-coil domain does not influence the capability of RIC-3 to modulate the expression of nicotinic and 5-HT₃ receptors [17;25]. No changes were found in the chaperone activity of naturally occurring RIC-3 variants lacking the coiled-coil domain in *Drosophila melanogaster* [26]. Our results demonstrate that a variant, which does not contain the coiled-coil domain region (plus an adjacent C-terminal part) naturally exists, promotes $\alpha 7$ -nAChR surface expression and functional receptor activity. Coiled-coil domains are known to be important for protein-protein interactions in the organization of molecular scaffolds, in addition to other functions [27]. At least two other regions of RIC-3 have been predicted to provide protein-protein interactions [14] that may be sufficient to form the scaffold recruiting the complex necessary for maturation of $\alpha 7$ -nAChRs. The role of the missing segment may be to gather additional regulatory partners, to specify differential subunit composition of mature receptors, or be related to as yet undiscovered functions of RIC-3.

Conclusions

In conclusion, here we report the existence of a new human variant of RIC-3 which lacks the coiled-coil domain but is still capable to promote functional expression of $\alpha 7$ -nAChRs in mammalian cells. The detailed analysis of the expression patterns of RIC-3, RIC-3e and $\alpha 7$ -nAChRs has shown that both variants are very similarly distributed in human tissues, with a major overlap to $\alpha 7$ -nAChRs in the nervous system. Further studies regarding the biological function of RIC-3 and its variants are necessary to clarify the specific roles of the various isoforms and to identify new targets of this important regulator of neurotransmitter receptor expression.

Acknowledgments. We would like to thank Dr. Giuseppe Pollio for excellent support provided for cloning and molecular biology. We would also like to thank Dr. Hendrick Bothmann for helpful discussions regarding FLIPR experiments.

REFERENCES

- 1 Dajas-Bailador, F. and Wonnacott, S. (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol. Sci.*, **25**, 317-324.
- 2 Gotti, C., Riganti, L., Vailati, S. and Clementi, F. (2006) Brain neuronal nicotinic receptors as new targets for drug discovery. *Curr. Pharm. Des.*, **12**, 407-428.
- 3 Levin, E.D. and Rezvani, A.H. (2002) Nicotinic treatment for cognitive dysfunction. *Curr. Drug Targets. CNS. Neurol. Disord.*, **1**, 423-431.
- 4 Rusted, J.M., Newhouse, P.A. and Levin, E.D. (2000) Nicotinic treatment for degenerative neuropsychiatric disorders such as Alzheimer's disease and Parkinson's disease. *Behav. Brain Res.*, **113**, 121-129.
- 5 Martin, L.F., Kem, W.R. and Freedman, R. (2004) Alpha-7 nicotinic receptor agonists: potential new candidates for the treatment of schizophrenia. *Psychopharmacology (Berl)*, **174**, 54-64.
- 6 Cooper, S.T. and Millar, N.S. (1997) Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor alpha7 subunit. *J. Neurochem.*, **68**, 2140-2151.
- 7 Quik, M., Choremis, J., Komourian, J., Lukas, R.J. and Puchacz, E. (1996) Similarity between rat brain nicotinic alpha-bungarotoxin receptors and stably expressed alpha-bungarotoxin binding sites. *J. Neurochem.*, **67**, 145-154.
- 8 Rangwala, F., Drisdell, R.C., Rakhilin, S., Ko, E., Atluri, P., Harkins, A.B., Fox, A.P., Salman, S.S. and Green, W.N. (1997) Neuronal alpha-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors. *J. Neurosci.*, **17**, 8201-8212.
- 9 Sweileh, W., Wenberg, K., Xu, J., Forsayeth, J., Hardy, S. and Loring, R.H. (2000) Multistep expression and assembly of neuronal nicotinic receptors is both host-cell- and receptor-subtype-dependent. *Brain Res. Mol. Brain Res.*, **75**, 293-302.
- 10 Chen, D., Dang, H. and Patrick, J.W. (1998) Contributions of N-linked glycosylation to the expression of a functional alpha7-nicotinic receptor in *Xenopus* oocytes. *J. Neurochem.*, **70**, 349-357.
- 11 Blumenthal, E.M., Conroy, W.G., Romano, S.J., Kassner, P.D. and Berg, D.K. (1997) Detection of functional nicotinic receptors blocked by alpha-bungarotoxin on PC12 cells and dependence of their expression on post-translational events. *J. Neurosci.*, **17**, 6094-6104.
- 12 Halevi, S., McKay, J., Palfreyman, M., Yassin, L., Eshel, M., Jorgensen, E. and Treinin, M. (2002) The *C. elegans ric-3* gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J.*, **21**, 1012-1020.
- 13 Williams, M.E., Burton, B., Urrutia, A., Shcherbatko, A., Chavez-Noriega, L.E., Cohen, C.J. and Aiyar, J. (2005) Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. *J. Biol. Chem.*, **280**, 1257-1263.
- 14 Halevi, S., Yassin, L., Eshel, M., Sala, F., Sala, S., Criado, M. and Treinin, M. (2003) Conservation within the RIC-3 gene family. Effectors of mammalian nicotinic acetylcholine receptor expression. *J. Biol. Chem.*, **278**, 34411-34417.
- 15 Cheng, A., Bolland, K.A., Greenwood, S.M., Irving, A.J. and Connolly, C.N. (2007) Differential subcellular localization of RIC-3 isoforms and their role in determining 5-HT3 receptor composition. *J. Biol. Chem.*
- 16 Castelan, F., Castillo, M., Mulet, J., Sala, S., Sala, F., Dominguez del, T.E. and Criado, M. (2008) Molecular characterization and localization of the RIC-3 protein, an effector of nicotinic acetylcholine receptor expression. *J. Neurochem.*, **105**, 617-627.
- 17 Castillo, M., Mulet, J., Gutierrez, L.M., Ortiz, J.A., Castelan, F., Gerber, S., Sala, S., Sala, F. and Criado, M. (2005) Dual role of the RIC-3 protein in trafficking of serotonin and nicotinic acetylcholine receptors. *J. Biol. Chem.*, **280**, 27062-27068.
- 18 Lansdell, S.J., Gee, V.J., Harkness, P.C., Doward, A.I., Baker, E.R., Gibb, A.J. and Millar, N.S. (2005) RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. *Mol. Pharmacol.*, **68**, 1431-1438.
- 19 Roncarati, R., Seredenina, T., Jow, B., Jow, F., Papini, S., Kramer, A., Bothmann, H., Dunlop, J. and Terstappen, G.C. (2008) Functional properties of alpha7 nicotinic acetylcholine receptors co-expressed with RIC-3 in a stable recombinant CHO-K1 cell line. *Assay. Drug Dev. Technol.*, **6**, 181-193.
- 20 Seguela, P., Wadiche, J., Dineley-Miller, K., Dani, J.A. and Patrick, J.W. (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.*, **13**, 596-604.
- 21 Dominguez del, T.E., Juiz, J.M., Peng, X., Lindstrom, J. and Criado, M. (1994) Immunocytochemical localization of the alpha 7 subunit of the nicotinic acetylcholine receptor in the rat central nervous system. *J. Comp. Neurol.*, **349**, 325-342.
- 22 Bray, C., Son, J.H., Kumar, P. and Meizel, S. (2005) Mice deficient in CHRNA7, a subunit of the nicotinic acetylcholine receptor, produce sperm with impaired motility. *Biol. Reprod.*, **73**, 807-814.
- 23 Kumar, P. and Meizel, S. (2005) Nicotinic acetylcholine receptor subunits and associated proteins in human sperm. *J. Biol. Chem.*, **280**, 25928-25935.
- 24 Hurst, R.S., Hajos, M., Raggenbass, M., Wall, T.M., Higdon, N.R., Lawson, J.A., Rutherford-Root, K.L., Berkenpas, M.B., Hoffmann, W.E., Piotrowski, D.W., Groppi, V.E., Allaman, G., Ogier, R., Bertrand, S.,

Bertrand,D. and Arneric,S.P. (2005) A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J.Neurosci.*, **25**, 4396-4405.

25 Ben Ami,H.C., Yassin,L., Farah,H., Michaeli,A., Eshel,M. and Treinin,M. (2005) RIC-3 affects properties and quantity of nicotinic acetylcholine receptors via a mechanism that does not require the coiled-coil domains. *J.Biol.Chem.*, **280**, 28053-28060.

26 Lansdell,S.J., Collins,T., Yabe,A., Gee,V.J., Gibb,A.J. and Millar,N.S. (2008) Host-cell specific effects of the nicotinic acetylcholine receptor chaperone RIC-3 revealed by a comparison of human and *Drosophila* RIC-3 homologues. *J Neurochem.*, **105**, 1573-1581.

27 Rose,A. and Meier,I. (2004) Scaffolds, levers, rods and springs: diverse cellular functions of long coiled-coil proteins. *Cell Mol Life Sci.*, **61**, 1996-2009.

Accepted Manuscript

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Figure legends

Fig.1. Molecular cloning of a novel variant of RIC-3. Agarose gel electrophoresis of the products resulting from PCR amplification of human RIC-3 cDNAs obtained from hippocampus. Four amplification products were obtained. According to described sequences of human RIC-3, the upper band (ca. 1100 bp) corresponds to the major variant and two lower bands (ca. 550 and 700 bp) correspond to known RIC-3 variants (sequence accession numbers are given next to the band).

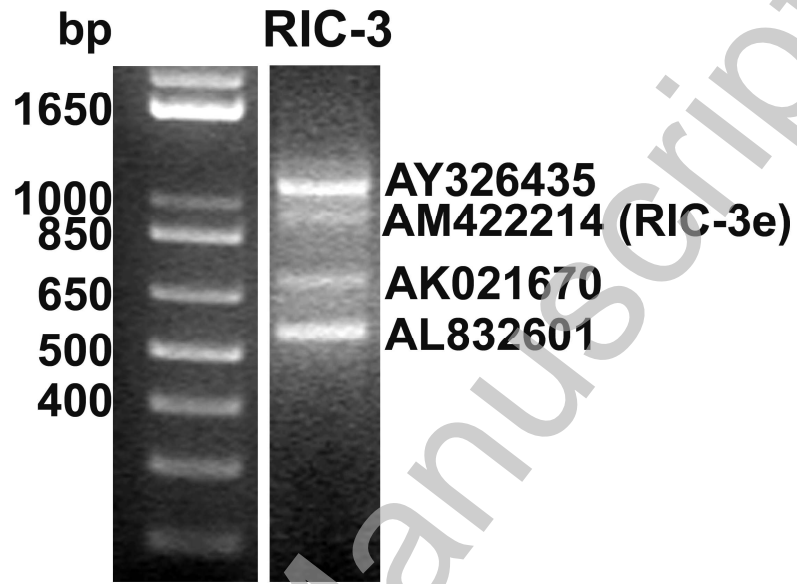
Fig.2. Amino acid sequence of RIC-3e and comparison to known variants. A. Alignment of the deduced amino acid sequences of RIC-3 (AY326435) and RIC-3e (AM 422214) was performed using MUSCLE 3.41 and visualized with GeneDoc 2.6.002. Black color indicates 100% sequence identity. RIC-3e carries a deletion of 240 bp and encodes a RIC-3 protein of 288 amino acids. B. Schematic representation of known human RIC-3 transcripts. The empty boxes E1-E6 correspond to RIC-3 exons. The predicted protein domains are represented with different colors: SP: signal peptide; Nterm: amino-terminal domain; TM: transmembrane domain; Cterm: carboxy-terminal domain; CCD: coiled-coil domain.

Fig.3. Quantitative real-time PCR expression analysis of RIC-3 variants and $\alpha 7$ -nAChR in a panel of human tissues. Results shown are the means and standard errors of three measurements per tissue. Absolute levels of expression were normalized to the expression levels of β -actin. A. Expression profile of RIC-3. B. Expression profile of RIC-3e. C. Expression profile of $\alpha 7$ -nAChR.

Fig.4. Bungarotoxin labeling of $\alpha 7$ -nAChR in HEK293 cells. HEK293 cells transfected with human $\alpha 7$ -nAChR (A, B), co-transfected with human $\alpha 7$ -nAChR and RIC-3 (C, D), or co-transfected with human $\alpha 7$ -nAChR and RIC-3e (E, F). Scale bar: 5 μ m.

Fig.5. Determination of functional $\alpha 7$ -nAChR activity using a FLIPR system. HEK 293 cells were transfected with $\alpha 7$ -nAChR, RIC-3, RIC-3e or combinations of $\alpha 7$ -nAChR and RIC-3 variants as indicated under the corresponding bars (NT, non transfected). Applied compounds are as indicated. MLA was added in the wash buffer and cells were pretreated for 10 min prior to addition of agonist and modulator. All compounds were used at the concentration of 10 μ M. Data obtained from one representative experiment (four replicates for each treatment) are presented as mean \pm SEM.

Figure 1



Accepted Manuscript

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Figure 2

A

```

*      20      *      40      *      60      *      80
AY326435 : MAYSTVQRVALASGLVLALSLLLKAFLSRGKRQEPPTPEGKLGFRFPMMHHHQAPSDGQTPGARFQRSHLAEAFAKAK :
AM422214 : MAYSTVQRVALASGLVLALSLLLKAFLSRGKRQEPPTPEGKLGFRFPMMHHHQAPSDGQTPGARFQRSHLAEAFAKAK :

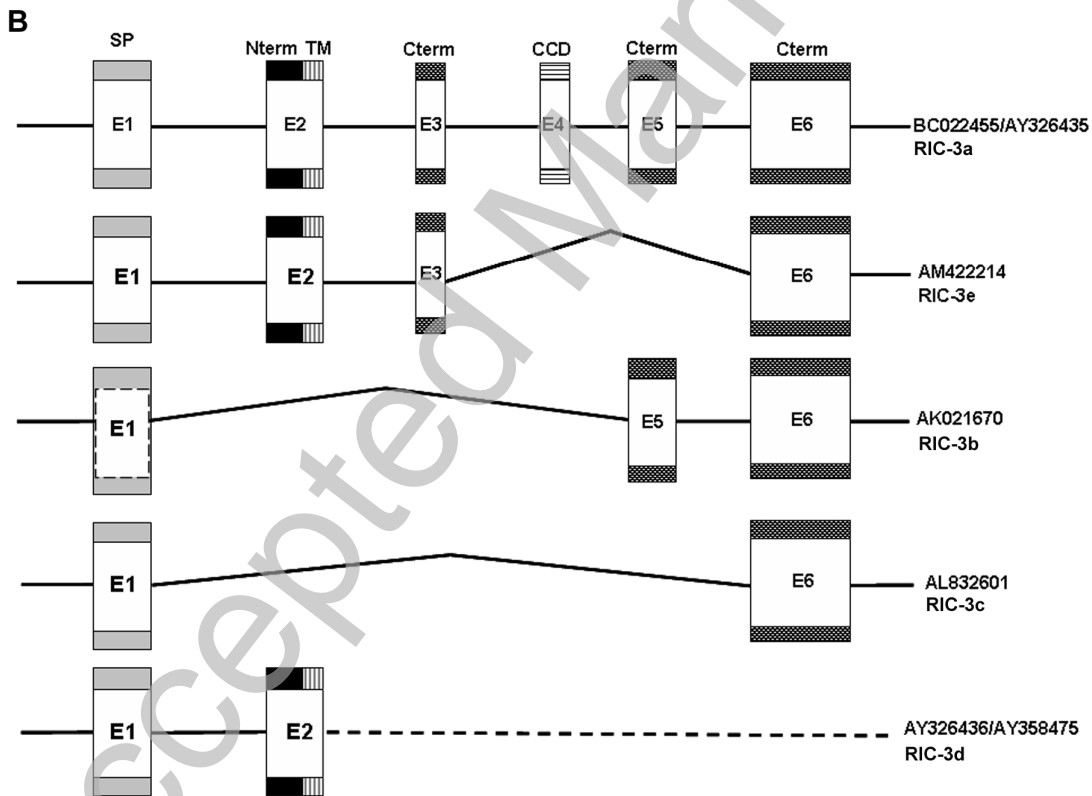
*      100     *      120     *      140     *      160
AY326435 : GSGGGAGGGGSGRGLMGQIIPYGFGLFLYILYILFKLSKGGKTTAEDGKCYTAMPGNTHRKITSFELAQLOEKLKTEAA :
AM422214 : GSGGGAGGGGSGRGLMGQIIPYGFGLFLYILYILFKLSKGGKTTAEDGKCYTAMPGNTHRKIS----- :

*      180     *      200     *      220     *      240
AY326435 : MEKLINRVGPNGESRAQTVTSDQEKRLHLQREITRVMKEGKFDIDRFSPKEAEEAPYMEDWEGYDEETYPIYDLSDCIK :
AM422214 : -----YDEETYPIYDLSDCIK :

*      260     *      280     *      300     *      320
AY326435 : RRQETILVDYPPDKELSAEELAEARMGMIEEESDHLGWESLPTDPRAQEDNSVTSCDPKPETCSCCFHEDEDPAVLAENA :
AM422214 : RRQETILVDYPPDKELSAEELAEARMGMIEEESDHLGWESLPTDPRAQEDNSVTSCDPKPETCSCCFHEDEDPAVLAENA :

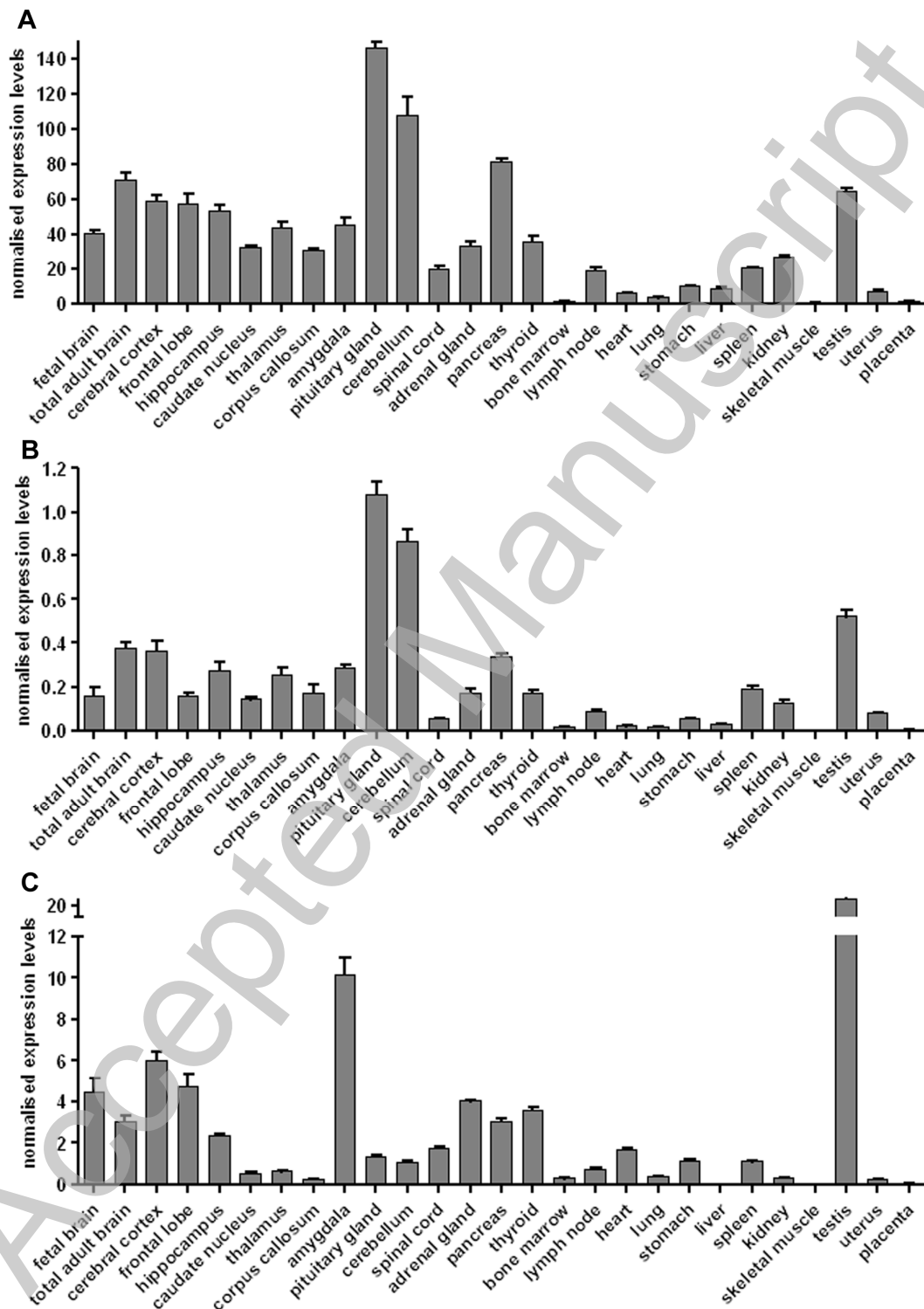
*      340     *      360
AY326435 : GFSADSYPEQEBTTKEEWSQDFKDEGLGISTDKAYTGSMRLKRNPOGLE : 369
AM422214 : GFSADSYPEQEBTTKEEWSQDFKDEGLGISTDKAYTGSMRLKRNPOGLE : 288

```



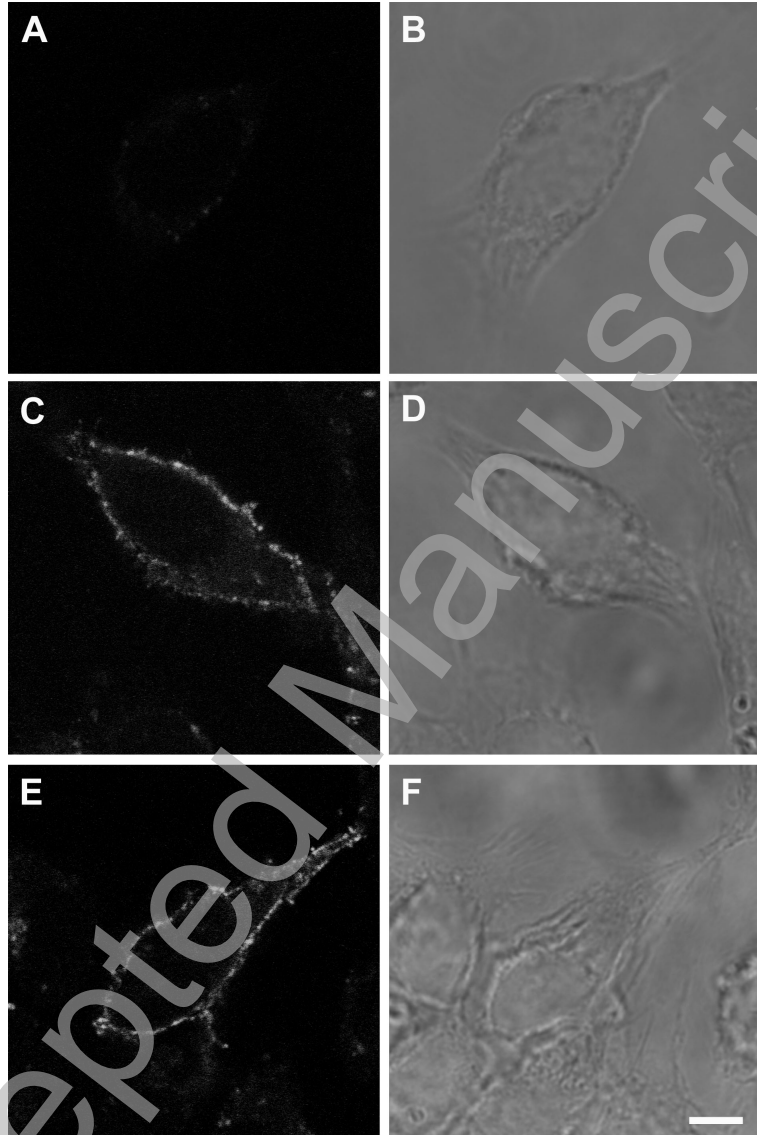
THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Figure 3



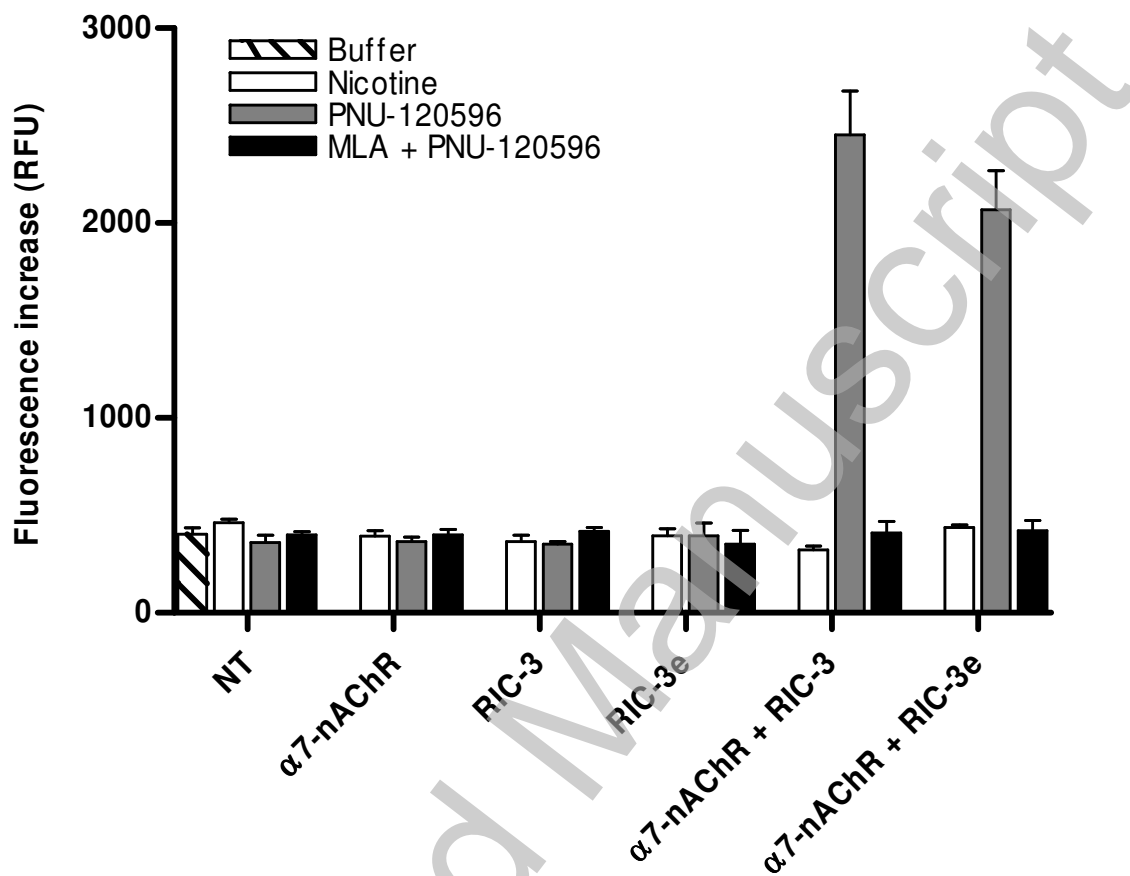
THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Figure 4



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Figure 5



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BSR20080055

Accepted Manuscript