THE CHOLINERGIC ANTAGONIST GYMNODIMINE IMPROVES Aβ AND TAU NEUROPATHOLOGY IN AN *IN VITRO* MODEL OF ALZHEIMER DISEASE

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Running title: Effect of gymnodimine in an in vitro model of AD

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Keywords: Gymnodimine, Alzheimer disease, tau protein, beta-amyloid, acetylcholine.

ABSTRACT

Gymnodimine (GYM) is a marine phycotoxin with a macrocyclic imine structure, isolated from extracts of the dinoflagellate *Karenia selliformis* known to act as a cholinergic antagonist with subtype selectivity. However, no data on the chronic effects of this compound has been reported so far. In this work, we evaluated the effect of long term exposure of cortical neurons to gymnodimine in the progress of Alzheimer disease (AD) pathology *in vitro*. Treatment of cortical neurons with 50 nM gymnodimine decreased the intracellular amyloid beta (Aβ) accumulation and the levels of the hyperphosphorylated isoforms of tau protein recognized by AT8 and AT100 antibodies. These results are suggested to be mediated by the increase in the inactive isoform of the glycogen synthase kinase-3 (phospho GSK-3 Ser9), the decrease in the levels of the active isoform of the ERK1/2 kinase and the increase in acetylcholine (Ach) synthesis elicited by long term exposure of cortical neurons to the toxin. Moreover, gymnodimine decreased glutamate-induced neurotoxicity *in vitro*. Altogether these results indicate that the marine phycotoxin gymnodimine may constitute a valuable tool for the development of drugs to treat neurodegenerative diseases.

INTRODUCTION

Gymnodimine is a marine phycotoxin first described in 1994 and initially thought to be produced by the dinoflagellate *Gymnodinium,* although later the dinoflagellate *Karenia Selliformis* was identified as the real producer *o*f the toxin [1]. This marine toxin has a structure with an imine cyclic ring and is included in the imine cyclic group of toxins with low toxicity after oral administration [2]. Since its toxicity is higher after intracerebral than after intraperitoneal injection, its main pharmacology target appears to be the central nervous system (CNS) [3]. Recently, it has been demonstrated that gymnodimine binds to both muscular and neuronal nicotinic acetylcholine receptors (nAChRs) with high affinity [3]. In addition, GYM also binds to α7nAChRs expressed in Xenopus oocytes in the nanomolar range, inhibiting the Ach-induced currents in this system [4].

Alzheimer´s disease is one of the most important neurodegenerative diseases, which nowadays affects more than 36 million people through the world (Alzheimer disease International association 2009) and currently it is estimated that 50-80% of all the cases of dementia are caused by AD. This cognitive disorder is characterized by neural loss, synaptic alterations, neuroinflamation and accumulation of Aβ and phosphorylated tau [5, 6]. In the last years a link between nAChRs and the development of AD has been demonstrated [7]. Thus, loss of nicotinic receptors, reduction in Ach levels and increase in acetylcholine esterase (AchE) activity combined with decreased choline acetyl transferase (ChAT) levels has been revealed in Alzheimer´s patients brains [8, 9]. These observations led to the development of nAchRs agonists and antagonists as drug targets for the development of AD therapies [10]. In fact, the agonist nicotine protected neurons against amyloid-beta toxicity [11] but later it was revealed that this compound increased tau pathology [12]. Currently, the α7nAchRs antagonist methyllycaconitine (MLA)

showed a neuroprotective effect in cortical neurons suggesting that nicotinic receptor agonism is not the main responsible of the beneficial effect of cholinergic therapies in AD but rather the neuroprotective effect of cholinergic agents arises from receptor inhibition/desensitization suggesting that α7nAchRs antagonists may constitute useful therapies in Alzheimer´s disease [13].

The recent description of the interaction of gymnodimine with cholinergic transmission [4] prompted us to evaluate the effect of this toxin on AD pathology *in vitro*. In order to do this, primary cortical neurons derived from the 3xTg-AD mice, which present a temporal profile of amyloid-beta and tau overexpression [14], were used and the long-term effect of the toxin on receptor expression and Aβ and tau pathologies was evaluated in this cellular model. Moreover, so far no reports on the effect of long term exposure to gymnodimine have been released. In this work, we studied the effects of GYM in tau and Aβ pathologies, several AchRs expression, Ach release and neuroprotection.

MATERIAL AND METHODS

Primary cortical neurons

Two colonies of homozygous 3xTg-AD mice and wild type non-transgenic (NonTg) mice were established at the animal facilities of the University of Santiago de Compostela, Spain, where animals were used to obtain primary cultures of cortical neurons. All protocols were approved by the University of Santiago de Compostela Institutional animal care and use committee.

Primary cortical neurons were obtained from embryonic day 15-17 NonTg and homozygous 3xTg mice as previously described [14]. Briefly, cerebral cortex were removed and dissociated by mild trypsinization, followed by mechanical titration in a

DNAse solution (0.005% w/v) containing a soybean trypsin inhibitor (0.05% w/v) at 37ºC. The cells were suspended in Dulbecco`s Modified Eagle´s medium (DMEM) supplemented with p-amino benzoate, insulin, penicillin and 10% foetal calf serum. The cell suspension was seeded in 12 or 96 multiwell plates precoated with poly-D-lysine and incubated for 7-10 days *in vitro* (div) in a humidified 5% CO_2/95% air atmosphere at 37ºC. Cytosine arabinoside 20 µM was added before 48h in culture to prevent glial proliferation. Cortical neurons from NonTg and 3xTg-AD mice were prepared and processed simultaneously.

Chemicals and solutions

Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Foetal calf serum was obtained from Gibco (Glasgow, UK) and DMEM was from Biochrom (Berlin, Germany). Fura 2-acetoxymethyl ester (Fura 2-AM) was from Molecular Probes (Leiden, The Netherlands). All other chemicals were reagent grade and purchased from Sigma-Aldrich (Madrid, Spain). Gymnodimine was purchased from the National Research Council of Canada (NRC-CNRC, Canada).

Determination of cellular viability

Cell viability was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test, as previously described [15]. This test, which measures mitochondrial function, was used to assess cell viability as it has been shown that in neuronal cells there is a good correlation between a drug-induced decrease in mitochondrial activity and its cytotoxicity [16]. The assay was performed in cultures grown in 96 well plates and exposed to different concentrations of gymnodimine added to the culture medium. Cultures were maintained in the presence of the toxin at 37ºC in humidified 5% CO_2/95% air atmosphere for 120 hours. Sodium azide, at a concentration of 1 M, was used as cellular death control. After the exposure time cells

were rinsed and incubated for 60 min with a solution of MTT (500 µg/ml) dissolved in Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 5.6 Glucose and 10 HEPES, pH 7.4 adjusted with Tris. After washing off excess MTT, the cells were disaggregated with 5% sodium dodecyl sulfate and the absorbance of the colored formazan salt was measured at 590 nM in a spectrophotometer plate reader.

Western blotting

Cultured neurons pretreated with the biotoxin through 3 to 7 div were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mMNa_3VO_4, 1 mg/ml pepstatin A and 1 mg/ml benzamidine. The total protein concentration was determined by the Bradford assay. Samples of cell lysates with 20 µg of total protein were resolved in gel loading buffer (50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 20% glycerol, 0.05% bromophenol blue, pH 6.8) by SDS-PAGE and transferred onto PVDF membranes (Millipore). The Snap i.d protein detection system was used for blocking and antibody incubation as previously described [14]. The primary antibodies were applied at the concentrations shown in Table 1.

The immunoreactive bands were detected using the supersignal West pico chemiluminiscent substrate (Pierce) and the Diversity 4 gel documentation and analysis system (Syngene, Cambridge, UK). Chemiluminiscence was measured with the Diversity GeneSnap software (Syngene). β-actin was used as control for lane loading.

ELISA

Extracellular amyloid-beta in the culture medium was measured with the Colorimetric BetaMark$_{TM}$ x-42 ELISA kit (SIGNET) following manufacturer's protocol. The culture medium was collected at the same days in vitro from NonTg and 3xTg-AD cultures

after the treatment with the compound from 3 to 7 div. The optical density was measured at 620 nm in a Syngene multiwell plate reader.

Ach levels and AchE activity determination in cellular lysates were meassured with an Amplex® Red Acetylcholine/Acetylcholinesterase assay kit (Invitrogen) following manufacturer's protocol.

Cell labelling and determination of the cytosolic calcium concentration $[Ca^{2+}]_c$

Cultured cortical neurons from NonTg and 3xTg-AD mice treated with gymnodimine from 3 to 7 div were loaded with the Ca^{2+} sensitive fluorescent dye Fura-2AM, 2.5 μM for 10 min at 37ºC. Then, cells were washed 3 times with cold buffer. The coverslips were inserted into a thermostated chamber at 37ºC (Life Science Resources, Royston, Herts, UK) and viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x-immersion UV-Fluor objective). The $[Ca^{2+}]_c$ images were obtained from the images collected by double excitation fluorescence with a Life Science Resources equipment. The light source was a 175 W xenon lamp. The calibration of the fluorescence was made by the Grynkiewicz method [17]. For the calcium experiments the extracellular medium was Locke's buffer, pH 7.4 adjusted with Tris. All experiments were carried out in duplicate.

Statistical analysis

All data are expressed as means ± SEM of three or more experiments (each performed in duplicate). Statistical comparison was by non-paired Student's t-test. p values < 0.05 were considered statistically significant.

RESULTS

In order to evaluate the effect of gymnodimine over the main cellular alterations found in AD, cortical neurons were incubated with the toxin between the third and the seventh day in culture. At seven days *in vitro* the AD cellular model shows increased expression

of the principal neuropathological hallmarks of the disease, consisting on a marked increase in the levels of intracellular Aβ and phosphorylated tau isoforms [14]. All experiments and toxin-treatments were performed simultaneously in 3xTg-AD cultures and control NonTg neurons. Control cultures were incubated with the same concentration of toxin dissolvent, in this case methanol-trifluoroacetic acid (TFA). A final concentration of less than 0.05% of methanol-TFA was used in each treatment.

Prolonged exposure of cortical neurons to gymnodimine prevents glutamate-induced neuronal death

In order to assess the effect of prolonged exposure of cortical neurons to gymnodimine, we first evaluated the effect of the toxin on cellular viability by the MTT assay. As shown in Table 2, none of the gymnodimine concentrations employed decreased cellular viability in the NonTg neurons. Therefore, a concentration of 50 nM GYM was chosen to evaluate the potential therapeutic effects of the toxin in the 3xTg-AD cellular model.

Nowadays, it is clear that glutamate-induced neurotoxicity is related to some neurodegenerative diseases, including Alzheimer´s disease [18-20]. Moreover, amyloid-beta accumulation increases the vulnerability of neurons to glutamate toxicity [11]. In addition, nicotine and AchE inhibitors can protect neurons against glutamate-induced toxicity through nAChRs but not through muscarinic acetylcholine receptors (mAChRs) [21]. Therefore, the effect of prolonged exposure of cortical neurons to 50 nM gymnodimine over glutamate-induced neurotoxicity was analyzed. In order to do this, 3xTg-AD and NonTg cortical neurons were preincubated with 50 nM GYM in the culture medium during 24 hours, later 100 μM glutamate was added to the medium and cells were maintained in culture for an additional period of 48 hours in the simultaneous presence of the toxin and glutamate. After this treatment, the cell viability was evaluated by the MTT test. As shown in Figure 1A, glutamate decreased neuron

viability by about 20% both in NonTg and 3xTg –AD neurons while pretreatment of cortical neurons with 50 nM GYM abolished glutamate-induced neurotoxicity in control and 3xTg-AD neurons. Since it is widely accepted that glutamate-induced neurotoxicity involves alterations in cytosolic calcium homeostasis [22, 23], we evaluated whether exposure of cortical neurons to GYM modified the glutamate-induced calcium increase. For these experiments, neurons were treated with the toxin from 3 to 7 days in culture and after this treatment, the cytosolic calcium increase evoked by extracellular application of 50 μM glutamate was evaluated in GYM-treated and non-treated 3xTg-AD neurons. As shown in Figure 1B bath application of glutamate caused a rapid rise in the cytosolic calcium concentration in non-treated 3xTg-AD neurons which was not altered by pretreatment of the neurons with gymnodimine.

Effect of long term exposure of cortical neurons to gymnodimine on cholinergic neurotransmission

Since gymnodimine targets nicotinic receptors with high affinity [3] and cholinergic transmission is severely affected in AD [24-27], we evaluated the effect of the compound on cholinergic transmission. Moreover, the first drugs developed for AD were AchE inhibitors which increase brain Ach levels [28] but nowadays there are no reports on the effects of gymnodimine on intracellular Ach levels. Therefore, we first analyzed the effect of long term treatment of cortical neurons with 50 nM gymnodimine on the expression level of the choline acetyl transferase enzyme which is involved in the synthesis of the neurotransmitter acetylcholine. Figure 2A shows representative Western blot bands for the expression of ChAT in control, 3xTg-AD and GYM-treated 3xTg-AD neurons. As shown in Figure 2B, quantitative measurement of the ChAT Western blot band intensities indicated no differences in ChAT levels between NonTg and 3xTg-AD neurons, however, these levels were significantly increased by 46.7 ±

6.0% in 3xTg-AD cortical neurons grown in the presence of 50 nM gymnodimine versus non-treated 3xTg-AD neurons ($p = 0.007$), therefore suggesting an increase in Ach levels after toxin treatment. In order to confirm this, the levels of Ach in cells lysates were evaluated after exposure of cortical neurons to 50 nM gymnodimine. As shown in Figure 2C, Ach levels were about 23.8 ± 0.04% lower in 3xTg-AD versus control cell lysates ($p < 0.01$; $n = 3$) and treatment of 3xTg-AD neurons with 50 nM GYM abolished the decrease in Ach levels in transgenic neurons. In contrast, exposure of cortical neurons to 50 nM GYM did no modify the activity of the AchE enzyme as illustrated in Figure 2D. Therefore, the data reported here indicates that gymnodimine may be a useful tool to increase brain Ach levels, most likely due to the stimulation in Ach synthesis.

In view of the previous results we next analyzed the effect of the toxin on the expression levels of the main acetylcholine receptors involved in AD, namely α7nAChRs and M1 and M2 mAChRs. Muscarinic AchRs are well-known transmembrane receptors widely expressed in the nervous system and among them, the M1 and M2 subtypes are the most studied in AD [29, 30]. Although M1 and M2 mAChRs subtypes have been targeted for the development of drugs therapies against AD, M1 levels are unaffected in AD brains [31-33], pointing to a reduced functionality and not to a reduced expression level of these receptors in AD. M2 mAChRs are another target for AD drug development, since its inhibition results in increased Ach levels [34] and this increase is more physiological that the obtained with AchE inhibitors [35]. Figure 3A shows representative Western blot bands for the M1 mAChRs immunoreactivity in control and 3xTg-AD neurons maintained either in the absence or in presence of 50 nM GYM from 3 to 7 days in culture. As previously reported for the *in vivo* mice model [36] and in AD brains [31, 32] quantitative analyses of the M1 band intensities showed no differences in M1

receptor expression between control and 3xTg-AD cultured cortical neurons (Figure 3B). Moreover, treatment of 3xTg-AD neurons with the toxin did not alter the expression levels of M1 mAChRs. Figure 3C shows representative Western blot bands for M2 mAChRs immunoreactivity in non-treated control and 3xTg-AD neurons and 3xTg-AD neurons exposed to GYM from 3 to 7 days in culture. As shown in Figure 3D, quantitative analysis of M2 Western band intensities revealed no differences in M2 receptor expression between control and 3xTg-AD neurons, while exposure of 3xTg-AD cortical neurons to 50 nM GYM decreased M2 mAChRs levels by 34.4 ± 8.7% versus non treated 3xTg-AD neurons ($p = 0.03$, $n = 3$).

Due to the fact that nicotine showed neuroprotective effect against Aβ cytotoxicity [11, 37] and both nicotine and nAChRs antagonists such as MLA and α-bungarotoxin reduced *in vitro* Aβ42 levels [35], and also these receptors are the targets of gymnodimine [4], we next evaluated the effect of the toxin on α7nAChRs expression. Figure 3E shows representative Western blot bands for α7nAChRs immunoreactivity in control and 3xTg-AD neurons. As shown in Figure 3F quantitative analysis of α7 band intensities showed a 12.9 ± 5.6% decrease in the levels of expression of this receptor between non-treated control and 3xTg-AD neurons at 7 days in culture ($n = 4$; $p = 0.03$), while no significative differences in α7 band intensities were observed between 3xTg neurons cultured either in the absence or in the presence of 50 nM gymnodimine.

Gymnodimine decreases intracellular amyloid-beta levels in 3xTg-AD cortical neurons

Regulation of the cholinergic system modulates Aβ-secretion [11, 26] and several nAChRs regulators have been found to decrease Aβ levels [35]. Therefore, we next studied the effect of gymnodimine treatment on amyloid-beta pathology. In order to do this, cortical neurons were lysed and processed for immunocytochemical analysis by

Western blot using the 6E10 specific antibody which reacts with the abnormally processed isoforms and precursors forms of the Aβ peptide. Figure 4A shows representative Western blot bands indicating Aβ immunoreactivity in control, NonTg, and 3xTg-AD neurons exposed to gymnodimine from 3 to 7 days in culture. As shown in Figure 4B quantitative analysis of Aβ band intensities revealed a marked increase in the intracellular levels of Aβ in 3xTg-AD neurons versus control neurons ($p = 0.01$; $n = 3$), while exposure of 3xTg-AD neurons to 50 nM GYM from 3 to 7 days in culture reduced Aβ expression by 20.9 ± 0.6 % versus non treated 3xTg-AD cortical neurons ($p < 0.001$; $n = 3$). In addition, extracellular Aβ in the culture medium was also increased in 3xTg-AD neurons versus control neurons, as shown in Figure 4C. However, exposure of 3xTg-AD cortical neurons to gymnodimine did not modify extracellular Aβ levels.

Exposure of 3xTg-AD neurons to gymnodimine decreases tau hyperphosphorylation

One of the main pathological hallmarks in AD is the hyperphosphorylation of tau. Therefore, we analyzed the effect of gymnodimine on tau hyperphosphorylation using different antibodies against phosphorylated tau in non-treated 3xTg-AD neurons and in 3xTg-AD neurons cultured in the presence of 50 nM gymnodimine from 3 to 7 days *in vitro*. First, the effect of GYM on tau phosphorylation was evaluated with the AT8 antibody which recognizes a tau epitope phosphorylated at serine 202 and threonine 205 [38]. Figure 5A shows representative Western blot bands of phospho tau expression in non-treated control, 3xTg-AD neurons and in 3xTg-AD neurons cultured in the presence of the toxin. Quantification of the Western blot band intensities for AT8 immunoreactivity indicates that band intensity is increased in 3xTg-AD versus NonTg neurons, while AT8 band intensity is decreased by 34.4 ± 11.9% ($p = 0.03$; $n=4$) in GYM-treated 3xTg-AD cortical neurons versus non treated 3xTg-AD neurons as shown

in Figure 5B. Among the phosphorylation-specific anti-tau antibodies, the AT100 antibody which recognizes phosphorylated tau at serine 212 and threonine 214 is one of the most used of these antibodies [39]. Therefore, the effect of GYM on tau phosphorylation *in vitro* was also evaluated with the AT100 antibody. Figure 5C shows representative Western blot bands for AT100 immunoreactivity in non-treated control, 3xTg-AD neurons and 3xTg-AD neurons cultured in the presence of 50 nM GYM from 3 to 7 days *in vitro*. As shown in Figure 5D, quantification of AT100 band intensity yielded a significative increase in 3xTg-AD neurons at 7 days in culture versus NonTg neurons. Moreover, exposure of 3xTg-AD neurons to GYM decreased AT100 band intensity by 37.3 ± 3.3 % ($p < 0.001$; $n = 3$). Next, the mechanism by which the toxin decreases tau hyperphosphorylation was evaluated. In this regard, it is now widely accepted that the abnormal activation of several kinase pathways including glycogen-synthase kinase-3β (GSK-3β), cyclin-dependent kinase-5 (CDK5), mitogen activated protein kinases (MAPK) and the c-Jun N-terminal kinase (JNK) is involved in the neurodegenerative progression of AD. Therefore, in view of the positive effects of gymnodimine over tau phosphorylation the effect of the toxin on GSK-3β expression was analyzed. GSK-3β is a serine/threonine kinase that phosphorylates several substrates involved in cellular signaling and survival that has been implicated in the abnormal phosphorylation of tau in AD [40, 41] and it is also activated by amyloid-beta [42]. In order to evaluate the effect of gymnodimine on GSK-3β expression two different antibodies were used: the antibody that recognizes the GSK-3β enzyme phosphorylated in Ser 9, which is the inactive or inhibited isoform of the kinase, and the total GSK-3β antibody, which recognizes both the active and inactive form of the enzyme. Figure 6A shows representative Western blot bands indicating phospho GSK-3β and total GSK-3β levels in non-treated control, 3xTg-AD and 3xTg-AD neurons

cultured in the presence of the toxin from 3 to 7 days in culture. As shown in Figure 6B, quantitative analysis of the Western blot band intensities yielded a significant increase of about 73.7 ± 32.9 % ($p = 0.04$; n=3) in the levels of the inactive isoform of GSK-3β in 3xTg neurons grown in the presence of the toxin while no significant differences were observed in the levels of total GSK-3β between control, 3xTg-AD and 3xTg-AD neurons cultured in the presence of gymnodimine. Moreover, no differences in the levels of the inactive isoform of the enzyme were found between control and 3xTg -AD neurons. Therefore, these results indicate that exposure of cortical cultures to low gymnodimine concentrations decreases the levels of the active isoform of GSK-3β and this effect may account for the decrease in tau phosphorylation observed after toxin treatment. Additionally, since Aβ can increase ERK phosphorylation primarily through interaction with α7 nicotinic receptors [43, 44] we evaluated the effect of gymnodimine treatment on phosphorylated ERK1/2 levels. Figure 7A shows representative Western blot bands for the levels of phospho ERK in non-treated control, 3xTg-AD neurons and in 3xTg-AD neurons cultured in the presence of 50 nM gymnodimine from 3 to 7 days in culture. Quantitative analysis of the Western blot band intensities for the phospho ERK levels is shown in Figure 7B and yielded a significant decrease of 39.5 ± 4.9% in phosphorylated ERK levels in 3xTg-AD neurons grown in the presence of the toxin versus non-treated 3xTg-AD cultures (n=4; $p < 0.01$). No differences were observed in the kinase levels between NonTg and 3xTg-AD neurons Therefore, these results indicate that prolonged gymnodimine treatment decreases the levels of two of the main kinases involved in AD, thus explaining the beneficial *in vitro* effect of the toxin on Aβ and tau pathology.

DISCUSSION

The aim of this study was to analyze *in vitro* the long term effect of the marine phycotoxin gymnodimine over the main pathological hallmarks of Alzheimer disease. The results presented here indicate that the presence of gymnodimine in the extracellular culture medium does not affect the cellular viability at 120 hours even at a final concentration of 100 nM gymnodimine in the culture medium. The *in vitro* results for the absence of toxicity of gymnodimine described in the present work are in agreement with previous *in vivo* data indicating a very low toxicity of the compound when it is administered with food [2]. Moreover, the toxin showed a neuroprotective effect against glutamate-induced neurotoxicity in cortical neurons. Although the exact mechanism of this neuroprotective effect was not identified, chronic treatment of cortical neurons with the toxin did not modify the glutamate-induced calcium response. Since nAchRs are the main target of gymnodimine and the toxin is an antagonist of these receptors [2, 3, 45], we explored whether the interaction between GYM and AchRs could have a relationship with the beneficial effect of the toxin against glutamate-induced neurotoxicity. Previous reports have shown that α7nAchR can protect against Aβ and glutamate induced neurotoxicity [7]. However, long-term exposure of cortical neurons to GYM did not affect the steady-state levels of neither the α7nAChRs or the expression of the M1 mAChRs. Interestingly, exposure of cortical neurons to gymnodimine also increased the intracellular levels of acetylcholine as well as the ChAT expression without affecting AchE levels. Therefore, the increase in intracellular Ach levels after gymnodimine treatment is most likely due to the increase in Ach synthesis by upregulation of the ChAT enzyme *in vitro*. To our knowledge, there are no previous reports on the effect of gymnodimine on intracellular acetylcholine levels. Moreover, although the toxin is thought to block mainly muscular and neuronal

nAChRs [3], long term exposure of primary cortical neurons to gymnodimine decreased the expression levels of the M2 subtype of mAChRs. Since the effect of gymnodimine on mAChRs has not yet been evaluated, it remains possible that an inhibitory effect of the toxin on M2 mAChRs and the consequent disinhibition of presynaptic Ach release could participate in the increase in Ach levels after GYM treatment of 3xTg-AD neurons since M_2 mAChR blockade causes elevated ACh release in vivo [27]. Gymnodine has been postulated to act as a α7nAchRs antagonist [3, 4] and different studies have shown that Aβ binds to α7nAchRs and coimmunoprecipitates with them in postmortem samples from AD patients [46]. In the same way, α7nAchRs antagonists compete with Aβ for the binding to α7nAchRs [47, 48]. Moreover, other α7nAchRs antagonists such as MLA or α-bungarotoxin, decreased the Aβ-induced phosphorylation of tau, prevented GSK-3β activation [49] and blocked Aβ-induced stimulation of ERK-MAPK activity trough α7 nAchR [50]. Several specific α7nAChRs antagonists have been used for the specific study of the structure and mechanism of action of GYM. BTX is a specific nAchR antagonist that has been used in competition assays with gymnodimine for the binding to nAChRs-enriched membranes of *Torpedo marmorata [45].* Besides, MLA has been used for the study of the structure and binding of the toxin to nAchR [4], indicating the involvement of α7nAchRs in the mechanism of action of gymnodimine.

The results described in this work indicate that GYM showed a positive effect over the two main pathological hallmarks of AD, namely hyperphosphorylation of tau and Aβ accumulation. In this sense, GYM decreased intracellular Aβ levels and the hyperphosphorylated isoforms of the tau protein recognized by the AT8 and AT00 antibodies respectively, but it failed to decrease the levels of $A\beta_{40\text{-}42}$ in the extracellular medium. The lack of effect of gymnodimine on extracellular Aβ could be partially

explained by the fact that the detection method for the peptide in serum-containing samples may difficult the determination of the final extracellular Aβ concentration as indicated in the manufacturer's protocol. Moreover, we found here that the effects of the toxin on intracellular Aβ accumulation and tau phosphorylation are linked to an increase in the levels of the inactive isoform of GSK-3β without modifications on total GSK-3β levels and a decrease in phospho ERK1/2 levels, thus decreasing the activity of two of the main kinases involved in Aβ-induced abnormal tau phosphorylation [49-51].

In view of the effects of gymnodimine on Aβ and tau pathology our hypothesis is that the decrease in the phosphorylated tau isoforms in the presence of the toxin may be secondary to the intracellular Aβ reduction and mediated by an inhibitory action of the toxin on GSK-3β and ERK1/2 kinases after binding of the phycotoxin to α7 nAchR. Further studies will be needed to clarify whether the effects reported here are due to a direct action of GYM over the kinases implicated in AD pathology or is a consequence of the action of the toxin on α7nAchRs involving different regulation steps that need to be investigated.

In the last years both α7nAchRs agonist [35, 52-54] and also several antagonist have been described to ameliorate AD pathology [35, 53, 55], and since nAChRs are ligand-gated ion channels critical for learning and memory and involved in the development of AD [7, 56], the results presented here indicate that gymnodimine may constitute a valuable compound to design new disease-modifying drugs in Alzheimer´s disease and related neurodegenerative disorders.

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ACKNOWLEDGMENTS:

This work was funded with the following FEDER cofunded-grants: SAF2009-12581 (subprograma NEF), AGL2009-13581-CO2-01, TRA2009-0189, AGL2010-17875. From Xunta de Galicia, Spain: GRC 2010/10, and PGIDT07CSA012261PR, PGDIT 07MMA006261PR, PGIDIT (INCITE) 09MMA003261PR, 2009/XA044, 2009/053 (Consell. Educación), 2008/CP389 (EPITOX, Consell. Innovación e Industria, programa IN.CI.TE.), 10PXIB261254 PR. From EU VIIth Frame Program: 211326 - CP (CONffIDENCE), 265896 BAMMBO, 265409 μAQUA, and 262649 BEADS. From the Atlantic Area Programme (Interreg IVB Trans-national): 2008-1/003 (Atlantox) and 2009-1/117 Pharmatlantic. Eva Alonso is recipient of a predoctoral fellowship from Fondo de Investigaciones Sanitarias (pFIS), Ministerio de Sanidad y Consumo, Spain.

Conflict of interest:

The authors do not express any conflict of interest.

FIGURE LEGENDS.

Figure 1:

Effect of GYM on the glutamate-induced neurotoxicity and the calcium response elicited by glutamate in cortical neurons. A) Glutamate-induce neurotoxicity in primary cortical neurons from 3xTg-AD and NonTg mice. Pretreatment of the neurons with GYM protected against glutamate-induced neurotoxicity as evaluated by the MTT reduction assay. B) Chronic exposure of cortical neurons from 3xTg-AD mice to 50 nM gymnodimine did not modify the calcium response induced by 50 μM glutamate. Data are mean ± SEM of 3 independent experiments, each performed in duplicated. * $p < 0.05$.

Figure 2:

Effect of GYM treatment on Ach levels. A) Representative Western blot bands indicating ChAT enzyme expression in NonTg, 3xTg-AD and GYM-treated 3xTg-AD neurons. B) Quantification of the data obtained in panel A as obtained from 3 independent experiments, showing an increase in the ChAT levels in 3xTg-AD treated neurons versus non treated 3xTg-AD cells. C) Ach levels in NonTg, 3xTg-AD and 3xTg-AD GYM treated neurons. Chronic GYM treatment increased intracellular Ach levels in primary cortical cultures exposed to 50 nM GYM from 3 to 7 div. D) AchE activity in NonTg, 3xTg-AD and 3xTg-AD GYM treated neurons. GYM pretreatment did not modify Ach esterase activity in 3xTg-AD treated neurons versus non treated ones. Data are mean ± SEM of 3 independent experiments. * $p < 0.05$.

Figure 3:

Effect of chronic 50 nM GYM treatment in the steady-state expression of α7nAchRs, M1 mAchRs and M2 mAchRs. A) Representative experiment showing Western blot bands for M1mAchRs levels in NonTg, 3xTg-AD and 3xTg-AD treated neurons. B)

Quantitative analysis showing no effect of GYM on M1 mAchRs levels in 3xTg-AD neurons as obtained from 3 independent experiments. C) Representative Western blot bands showing the effect of GYM in the steady-state level of M2 mAchRs. D) Quantification of the data in figure C as obtained from 3 independent experiments indicating a decreased expression of M2 mAchRs after treatment of 3xTg-AD neurons with GYM respect to 3xTg-AD non treated neurons. E) Representative Western blot bands showing α7nAchRs levels in NonTg and 3xTg-AD neurons treated and non treated with 50nM GYM. F) Quantification of the Western blot data shown in panel E as obtained from 4 independent experiments showing no effect of GYM over 3xTg-AD neurons. Western blot data are presented as percentages of NonTg neurons in the histograms. Data are mean ± SEM of 3 independent experiments.

Figure 4:

GYM treatment decreases intracellular Aβ accumulation. A) Representative Western blot bands indicating intracellular Aβ levels in NonTg, 3xTg-AD and 3xTg-AD neurons treated with 50 nM GYM as probed with the 6E10 antibody. B) Quantification of Western blot bands intensities for intracellular Aβ levels as obtained from 3 independent experiments, each performed in duplicate. GYM pretreatment reduces 6E10 immunoreactivity in 3xTg-AD treated neurons versus non treated 3xTg-AD neurons. Data are presented as percentages of NonTg neurons in the histograms. *** $p < 0.001$ compared with 3xTg-AD non treated neurons. C) Effect of the incubation with GYM in the extracellular Aβ levels.

Figure 5:

Chronic GYM exposure decreases tau phosphorylation. A) Representative Western blot bands showing levels of phospho-tau residues 199/202 recognized by the AT8 antibody in NonTg, 3xTg-AD and 3xTg-AD neurons treated with 50 nM GYM. B)

Quantification of the Western blot data of AT8 immunoreactivity, showing reduction in AT8 levels in 3xTg-AD neurons treated with GYM versus non treated 3xTg-AD neurons. C) Representative Western blot bands showing levels of phospho-tau residues 212/214 recognized by the AT100 antibody. D) Quantification of AT100 levels in NonTg, 3xTg-AD and 3xTg-AD neurons pretreated with 50 nM GYM showing a decrease in AT100 expression produced by the pretreatment with GYM. Data are presented as percentages of NonTg neurons in the histograms and include results from 4 separate experiments, each performed in duplicate. * $p < 0.05$ compared to 3xTg-AD non treated neurons for AT8 and *** $p < 0.001$ versus 3xTg-AD non treated neurons for AT100.

Figure 6:

Chronic GYM exposure increases phosphorylated glycogen synthase kinase expression. A) Representative experiment showing Western blot bands of phoshoGSK-3β and total GSK-3β immunoreactivity in NonTg, 3xTg-AD and 3xTg-AD neurons treated with 50 nM GYM. B) Corresponding histogram of the quantification of phosphoGSK-3β and total GSK-3β expression and the effect of GYM pretreatment of 3xTg-AD neurons over the inactive and total isoform levels of the enzyme, indicating an increase in the levels of phosphoGSK-3β in 3xTg-AD neurons after GYM treatment. Means are presented as percentages of NonTg neurons in the histograms and include results from 3 separate experiments, each performed in duplicate. * $p < 0.05$ compared to 3xTg-AD non treated neurons.

Figure 7:

Chronic GYM treatment decreases phospho-extracellular signal-regulated kinase1/2 expression. A) Representative Western blot bands of phosphoERK1/2 antibody immunoreactivity in NonTg, 3xTg-AD and 3xTg-AD neurons treated with 50 nM GYM. B) Quantification of data in panel A showing a decrease in the pERK1/2 levels in 3xTg-AD treated neurons versus the pERK1/2 levels in non treated 3xTg-AD neurons. Means are presented as percentages of NonTg neurons in the histograms and include results from 4 separate experiments, each performed in duplicate. ** $p < 0.01$ compared to 3xTg-AD non treated neurons.

TABLES

Table 1. List of antibodies and dilutions used

Antibody	**Immunogen**	**Host**	**Dilution**	**Source**
6E10	Aa 1-16 of Aβ	mouse	1:1000	Signet
AT8	Peptide with phospho-S199/S202/T205	mouse	1:1000	Pierce
AT100	Peptide with phospho-S212/T214	mouse	1:1000	Pierce
Phospho GSK-3β	Phosphoepitopes Ser9 of GSK-3β	rabbit	1:10000	Millipore
GSK-3β	Recognizes 47 kD GSK-3β protein	rabbit	1:1000	Millipore
Phospho ERK1/2	Recognizes 42-44 kD phospho ERK1/2	mouse	1:1000	Cell signaling
ChAT	Recognizes 68 kD active ChAT	rabbit	1:1000	Millipore
α7	α7 subunit nicotinic receptor	Null	1:1000	Millipore
M1	M1 subtype muscarinic receptor	Rabbit	1:1000	Santa Cruz
M2	M2 subtype muscarinic receptor	Mouse	1:100	Santa Cruz
Actin	C-terminal actin fragment, clone C4	mouse	1:20000	Millipore

Table 2.

Effect of different gymnodimine concentrations on mitochondrial function as assessed with the MTT assay in NonTg neurons exposed to different toxin concentrations from 3 to 7 days in culture. Means are presented as % of control non treated neurons and include results from 3 separate experiments, each performed in duplicate

[GYM], nM	NonTg (% of control)
0	100 ± 5.9
2.5	97.0 ± 4.9
5	102.8 ± 0.3
10	105.6 ± 5.9
25	105.1 ± 1.09
50	102.9 ± 1.4
100	101.8 ± 1.7

Figure 1

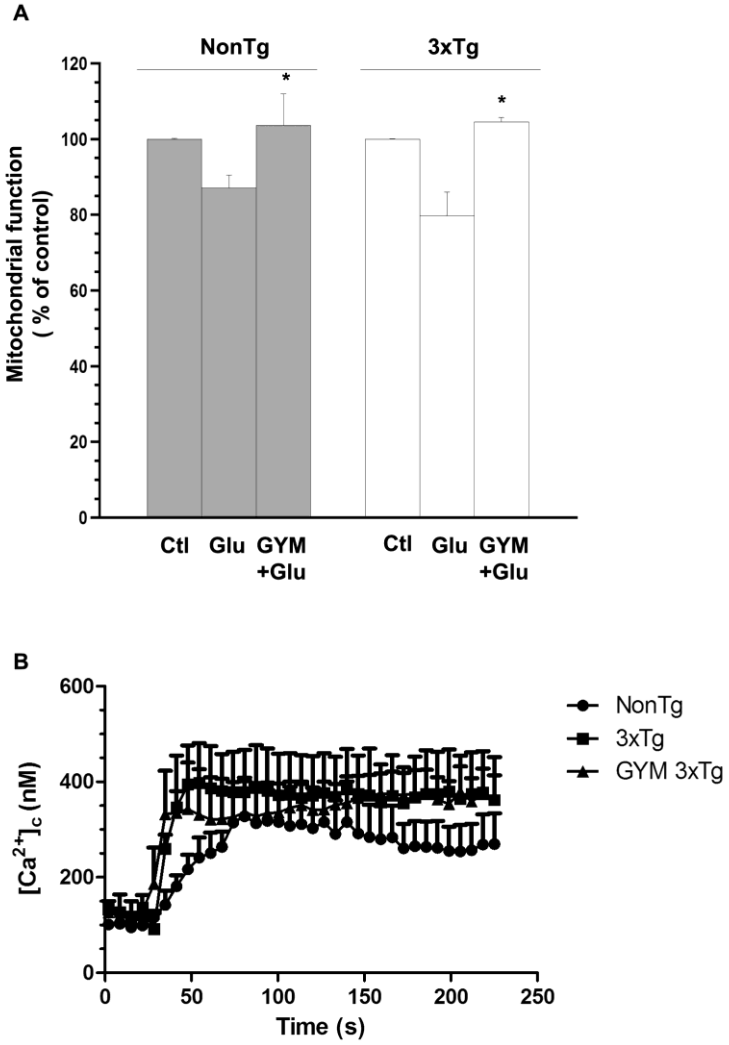

A
NonTg
3xTg
Mitochondrial function
(% of control)
120
100
80
60
40
20
0
*
*
Ctl
Glu
GYM
+Glu
Ctl
Glu
GYM
+Glu
B
600
400
200
0
[Ca2+]c (nM)
0
50
100
150
200
250
Time (s)
NonTg
3xTg
GYM 3xTg

Figure 2

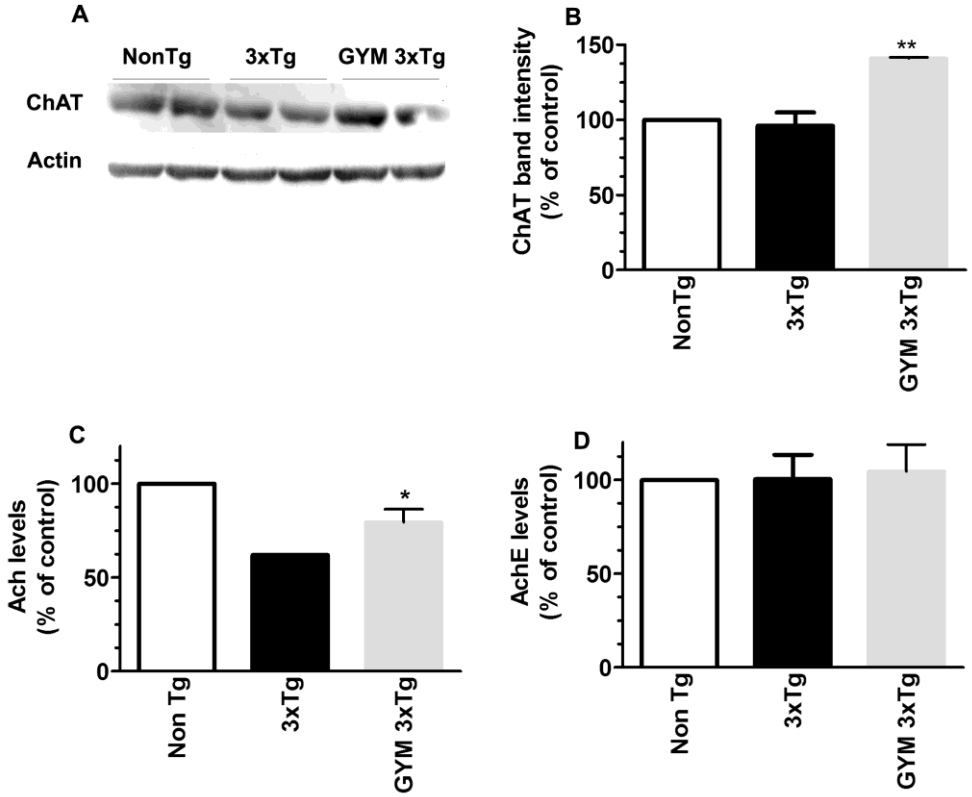

A
NonTg
3xTg
GYM 3xTg
ChAT
Actin
B
ChAT band intensity (% of control)
150
100
50
0
**
NonTg
3xTg
GYM 3xTg
C
Ach levels (% of control)
100
50
0
*
Non Tg
3xTg
GYM 3xTg
D
AchE levels (% of control)
100
50
0
Non Tg
3xTg
GYM 3xTg

Figure 3

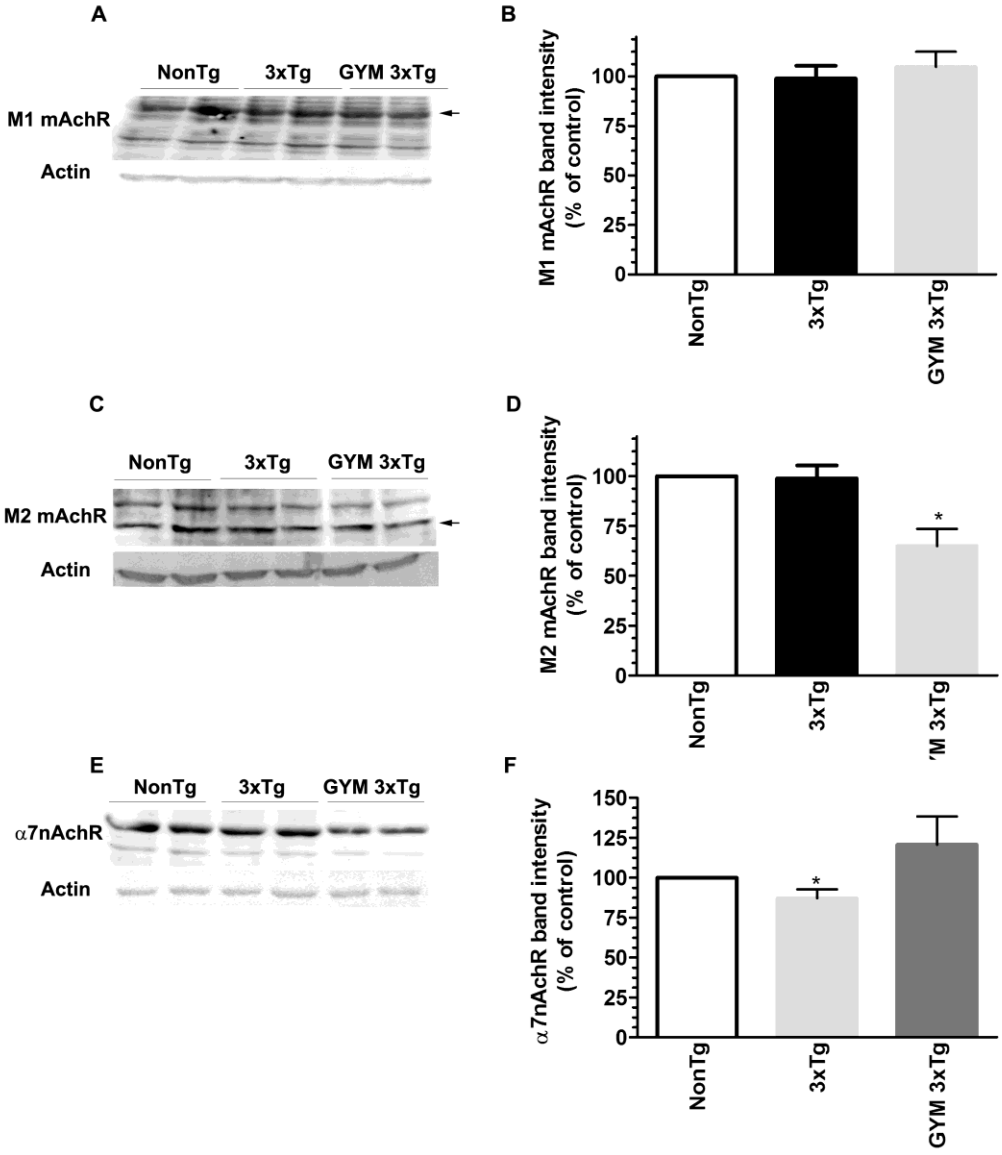

A
NonTg
3xTg
GYM 3xTg
M1 mAchR
Actin
B
M1 mAchR band intensity (% of control)
100
75
50
25
0
NonTg
3xTg
GYM 3xTg
C
NonTg
3xTg
GYM 3xTg
M2 mAchR
Actin
D
M2 mAchR band intensity (% of control)
100
75
50
25
0
*
NonTg
3xTg
'M 3xTg
E
NonTg
3xTg
GYM 3xTg
α7nAchR
Actin
F
α7nAchR band intensity (% of control)
150
125
100
75
50
25
0
*
NonTg
3xTg
GYM 3xTg

Figure 4

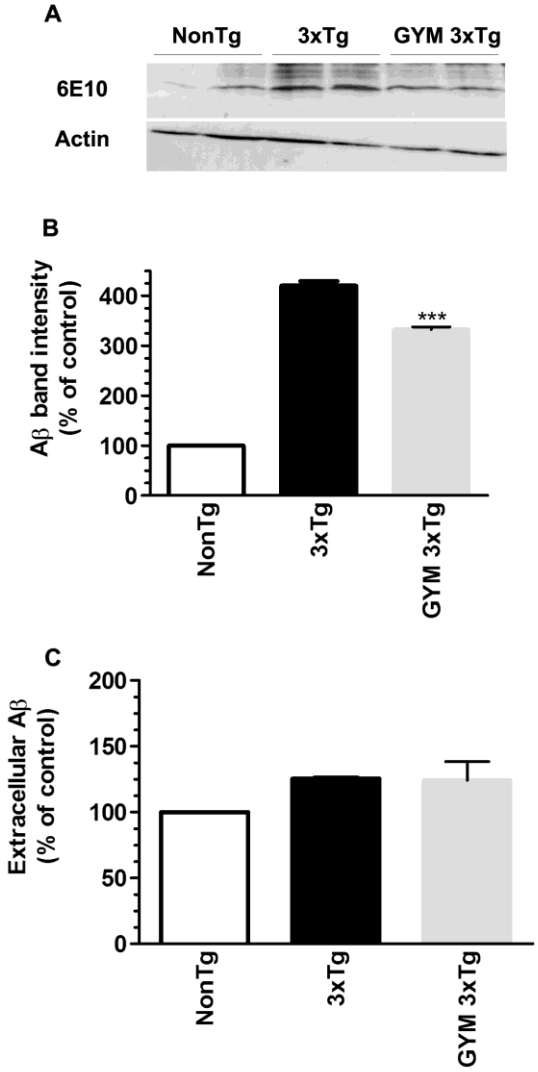

A
NonTg
3xTg
GYM 3xTg
6E10
Actin
B
Aβ band intensity (% of control)
400
300
200
100
0
***
NonTg
3xTg
GYM 3xTg
C
Extracellular Aβ (% of control)
200
150
100
50
0
NonTg
3xTg
GYM 3xTg

Figure 5

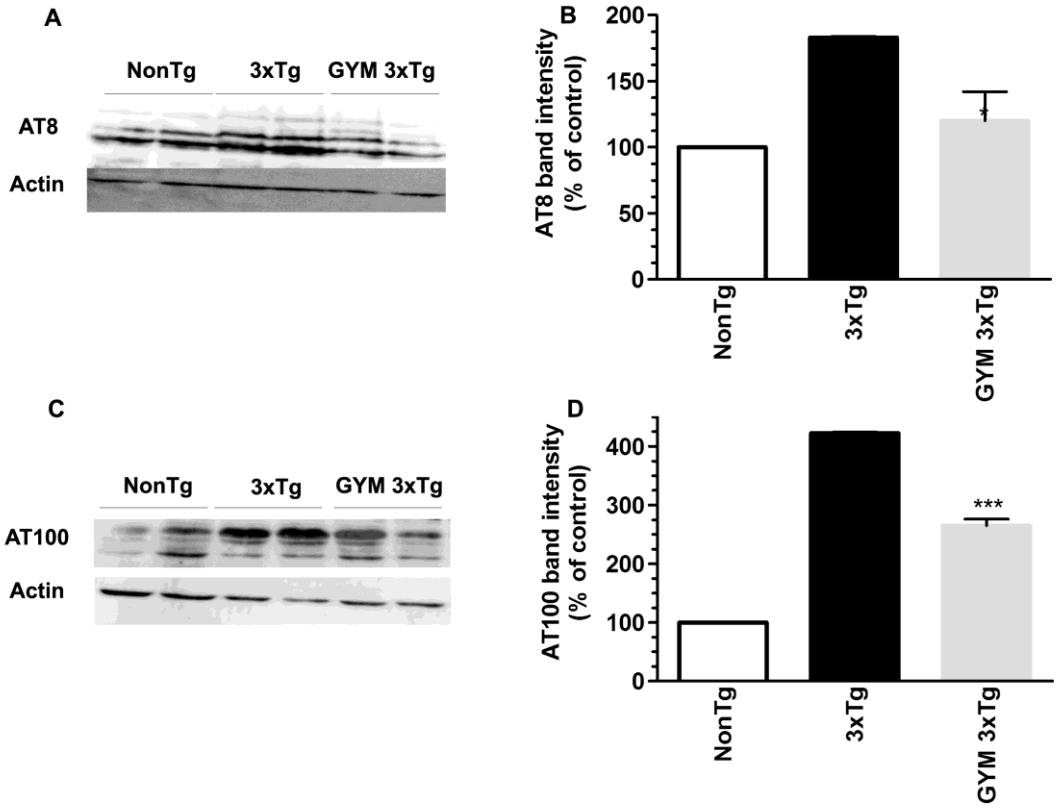

A
NonTg
3xTg
GYM 3xTg
AT8
Actin
B
AT8 band intensity (% of control)
200
150
100
50
0
*
NonTg
3xTg
GYM 3xTg
C
NonTg
3xTg
GYM 3xTg
AT100
Actin
D
AT100 band intensity (% of control)
400
300
200
100
0
***
NonTg
3xTg
GYM 3xTg

Figure 6

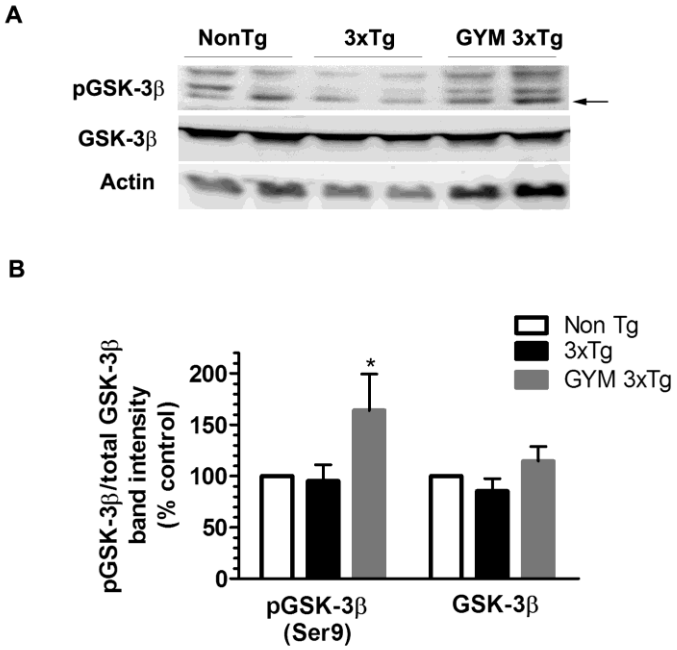
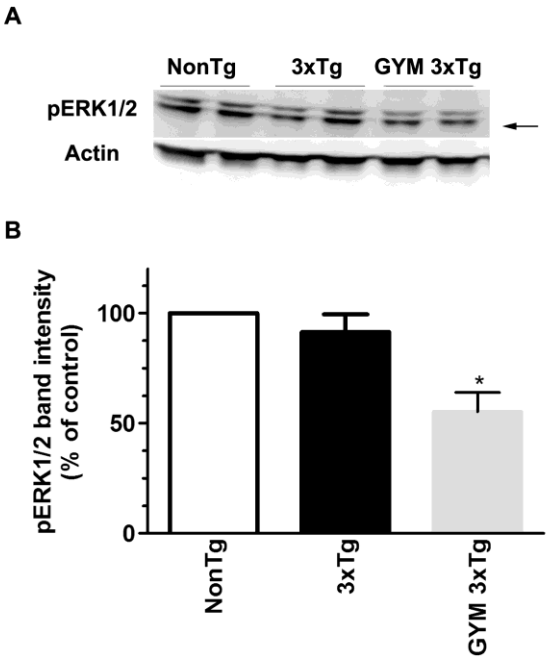

A
NonTg
3xTg
GYM 3xTg
pGSK-3β
GSK-3β
Actin
B
Non Tg
3xTg
GYM 3xTg
pGSK-3β/total GSK-3β band intensity (% control)
200
150
100
50
0
*
pGSK-3β (Ser9)
GSK-3β

Figure 7


A
NonTg
3xTg
GYM 3xTg
pERK1/2
Actin
B
pERK1/2 band intensity
(% of control)
100
50
0
*
NonTg
3xTg
GYM 3xTg