Cellular and Molecular Biology ™ **55**, OL1138-OL1150
DOI 10.1170/130

ISSN 1165-158X
2009 Cell. Mol. Biol.™

MODULATION OF MUSCARINIC SIGNALING IN PC12 CELLS OVEREXPRESSING NEURONAL CA^{2+} SENSOR-1 PROTEIN

M.M. GUIMARÃES[1, 2], H.J. REIS[1, 3], L.P. GUIMARÃES[1], D.S. CARNEIRO[1, 3], F.M. RIBEIRO[4], M.V. GOMEZ[1], A.JEROMIN[5] AND M.A. ROMANO-SILVA[1]✍

1 Laboratório de Neurociência, Departamento de Saude Mental, INCT de Medicina Molecular, Faculdade de Medicina da UFMGAv Alfredo Balena, 190 - Centro -Belo Horizonte-MG CEP 30130-100 – Brazil ✍ Email address: romano-silva@ufmg.br. Phone number: +55 31 3248 9134. Fax number: +55 31 3409 2983
2 Departamento de Ciências Básicas, Faculdade de Ciências da Saúde, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Rua da Glória, 187, Diamantina. 39100-000 Minas Gerais, Brazil.
3 Laboratório de Neurofarmacologia, Departamento de Farmacologia, Instituto de Ciência Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, 31270-901, Minas Gerais, Brazil.
4 Cell Biology Research Group, Robarts Research Institute London, Ontario, Canada.
5 Center for Learning and Memory, University of Texas at Austin, Austin, TX, USA.

Received April 26th, 2009; Accepted June 20th, 2009; Published August 5th, 2009

Abstract – It has been suggested that overexpression of neuronal Ca^{2+} sensor-1 (NCS-1) protein is implicated in the pathophysiology of neurodisorders such as schizophrenia, bipolar disturbance and X-linked mental retardation. The mechanism by which NCS-1 would be involved in the causes and/or consequences of these neurodisorders is still far from elucidation. Independent evidence has pointed NCS-1 as a key regulator of synaptic efficacy by altering the expression and activity of voltage-gated channels, inhibiting internalization of dopaminergic receptors, and altering phosphoinositide metabolism. In this study, we examined the possible participation of NCS-1 protein in signal transmission dependent on muscarinic receptor activation, using PC12 cells stably expressing NCS-1 (PC12-NCS-1). Carbachol (CCH; 300μM) was able to evoke glutamate release more efficiently from PC12-NCS-1 (15.3±1.0nmol/mg of protein) than wild type cells (PC12-wt; 8.3±0.9nmol/mg of protein). This increase of glutamate release induced by CCH was independent on extracellular Ca^{2+} influx. Additionally, a larger increase of cytoplasmic levels of $InsP_3$ (663.0±63.0 and 310.0±39.0% of fluorescence in A.U.) and $[Ca^{2+}]_i$ (766.4±40.0 and 687.8±37.1nmol/L) was observed after CCH stimulus of PC12-NCS-1 compared with PC12-wt. Clearly distinction between intracellular Ca^{2+} dynamics was also observed in PC12-NCS-1 and PC12-wt. A larger increase followed by fast decay of $[Ca^{2+}]_i$ was observed in PC12-NCS-1. A plateau with a delayed decay of $[Ca^{2+}]_i$ was characteristic of PC12-wt $[Ca^{2+}]_i$ response. Both enhancement of $InsP_3$ production and glutamate release observed in PC12-NCS-1 were blocked by atropine (10μM). Together, our data show that overexpression of NCS-1 in PC12 cells induces an enhancement of intracellular second messenger and transmitter release dependent on CCH response, suggesting that muscarinic signaling is "up-regulated" in this cell model.

Key words: PC12 cells, NCS-1, Calcium signaling, glutamate release.

INTRODUCTION

NCS-1 is an EF-hand-containing Ca^{2+}-binding protein that was originally identified in *Drosophila* and named as frequenin. Many studies have shown that overexpression of this protein leads to facilitation of post-synaptic excitation and neurotransmitter release in various models, such as neuromuscular junction of Xenopus, pheochromocytoma PC12 cells and Calyx of Held (25, 30, 33, 44, 35). The role of NCS-1 in cellular transmission seems to involve a complex and dynamic system. Depending on the cell model that overexpresses NCS-1, alterations of divergent functional activities have been reported, such as regulation of K^+ channels (Kv4) and Ca^{2+} channels (N and P/Q channels) activity (12, 44, 47, 49), interaction and activation of phosphatidylinositol 4-kinase-β (PI4K-β) activity (10, 42, 52), regulation of GRK2 and dopamine receptor desensitization (14), regulation of cellular membrane trafficking and neurite outgrowth (10, 11, 12, 16, 43).

The independent regulatory functions reported above could be participating in a major adaptative process controlled by NCS-1 activity. For example, studies in humans and monkeys

have suggested a possible participation of NCS-1 in the adaptative response related to brain disorders, including retardation syndrome, schizophrenia, and bipolar disorder (2, 5, 18, 41). Increased levels of NCS-1 have been independently reported in samples from two brain bank collections of postmortem prefrontal cortex from schizophrenic patients (2, 18). Additionally, immunohistochemical experiments in Rhesus monkeys (*Macaca mulatta*) have shown that NCS-1 and D2 receptor co-localize at ultrastructural level in the prefrontal cortex (27). Reported data that corroborate this evidence showed that NCS-1 overexpression in PC12 cells potentiates neurosecretion of dense-core granules and changes dopaminergic D2 receptors plasma membrane expression by inhibiting D2 receptor desensitization, indicating that NCS-1 overexpression could potentiate signal transduction dependent on dopaminergic pathway (2, 14, 31). Furthermore, it was reported potentiated exocytosis in PC12 cells overexpressing NCS-1 after activation of purinergic signaling (28). These results, together with evidence demonstrating NCS-1 role in increasing PIP_2 production by activation of PI4K-type II (19, 51), lead to the hypothesis that NCS-1 behaves as a molecular key of signaling adaptation dependent on G protein receptors activation. However, the intracellular mechanisms of NCS-1 overexpression in cholinergic signaling remain unclear. In this context, our general objective is to understand the role of NCS-1 overexpression in intracellular adaptative response related to muscarinic activation. In particular, we are interested in understanding the role of NCS-1 overexpression in cholinergic-signaling modulation using a neuroendocrine PC12 cell model. We found that NCS-1 overexpression in PC12 cells significantly increased PIP_2 hydrolysis/$InsP_3$ production, intracellular Ca^{2+} levels, and glutamate release induced by CCH, suggesting the participation of NCS-1 in adaptative response of muscarinic signal transmission.

MATERIALS AND METHODS

Reagents

DMEM high glucose, penicillin/streptomycin (P/S), horse serum (HS), fetal bovine serum (FBS), and nerve growth factor-7S (NGF) were from Gibco-Invitrogen Corporation (CA-USA); HRP-coupled goat anti-rabbit secondary antibody, HRP-conjugated anti-goat secondary antibody, and HRP-coupled goat anti-mouse secondary antibody were purchased from Molecular Probes-Invitrogen (CA-USA). NaCl, KCl, $CaCl_2$, HEPES, glucose, $MgSO_4$, atropine, MOPS (3-morpholinopropane-1-sulfonic acid), EGTA (glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) and EDTA (2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid) were purchased from Merck (Darmstadt, Germany); TRANSFAST™ were from Promega Corporation (OR-USA). CCH, BAPTA-AM, U73122 and Indo1-AM, β-NADP (β-nicotinamide adenine dinucleotide phosphate-sodium salt), L-glutamic dehidrogenase and L-glutamic acid, Nonidet P/40, pyrophosphate sodium fluoride, β-glycerophosphate, phenylmethylsulfonyl fluoride, benzamidine and leuleptin were obtained from Sigma (MO-USA). Pluronic F127, sodium orthovanadate and G418 were obtained from Calbiochem-Novabiochem Corporation (CA-USA). Mouse monoclonal antibody against actin was purchased from Chemicon-Millipore (MA-USA). Goat polyclonal primary antibody against muscarinic receptor type 1 (M1R), and rabbit polyclonal primary antibody anti-PLC-β were purchased from Santa Cruz Biotechnology (CA-USA).

Cell culture

Wild type PC12 cells (PC12-wt) and PC12 cells stably overexpressing NCS-1 (PC12-NCS-1) were kindly provided by Dr. A. Jeromin (Center for Learning and Memory, University of Texas at Austin, Austin, TX, USA). PC12-NCS-1 exhibited a 3-fold increase in NCS-1 expression, as compared to PC12-wt, as previously described (19). Both PC12-wt and PC12-NCS-1 were maintained in high glucose DMEM supplemented with 1% penicillin/streptomycin (P/S), 5% fetal bovine serum (FBS) and 5% horse serum (HS). The medium was changed every 2 days, and cells were moved to a new flask every week. In order to secure NCS-1 stable overexpression, PC12-NCS-1 cells were maintained in growth medium supplemented with 400µg/mL G418 (19).

$PH_{PLC\delta 1}$-EGFP plasmid transient transfection

For perfusion experiments, PC12-wt and PC12-NCS-1 cells were plated (1 x 10^5 cells) onto 22 mm coverslips and differentiated for 7 days with 100 ng/mL of NGF-7S in high glucose DMEM supplemented with 2.5% FBS, 2.5% HS and 1% P/S. For PC12-NCS-1 cells, the differentiation medium was supplemented with 400 µg/mL G418. The differentiated cells were transiently transfected with 1µg of $PH_{PLC\delta 1}$-EGFP plasmid kindly provided by Dr. A. Jeromin and constructed as previously described (45, 46). Transfection was mediated by the liposome method, using TRANSFAST™ reagent on coverslip in high glucose DMEM without serum and P/S. Fluorescence microscopy experiments were performed 48h after transfection.

Perfusion procedures

A coverslip containing the transfected cells was used as the base for an open 400µL bath superfusion chamber. Cells were perfused by means of a valve attached to a chambered superfusion reservoir with continuous laminar flux of 3.0mL/min. After perfusing KRH medium for 1-3 minutes, cells were stimulated with 300µM CCH diluted in KRH in the presence of 2.0mM $CaCl_2$.

$PH_{PLC\delta 1}$-EGFP fluorescence imaging

Experiments were performed at room temperature (20-25°C). The chamber was mounted onto the stage of an inverted microscope (Zeiss Axiovert 100) with a water

immersion objective (40x, 1.2 NA), coupled to a Bio-Rad MRC 1024 laser scanning confocal system running the software Timecourse 1.0. Confocal laser images were recorded at a rate of 1Hz. Cells transfected with $PH_{PLC\delta1}$-EGFP were imaged by excitation at 488nm of an argon laser line, and the emission signal was detected with a 522/35 bandpass filter.

A region of interest (ROI) in the cytoplasm was selected for each cell and the average pixel intensity inside the ROI was registered in order to collect a time series of fluorescence variations during the experiment. $PH_{PLC\delta1}$ was expressed as the ratio of fluorescence intensity of EGFP to baseline (fluorescence expressed in %). This self-ratio method (F/F_o) was used to correct factors such as dye concentration, excitation intensity, and detector efficiency**.** Differences are considered significant if $p < 0.05$ using an unpaired Student´s *t* test, and all values are displayed as mean±SEM. Background fluorescence was automatically subtracted from all measurements. Each experiment was carried out in duplicate or triplicate, on at least four separate days.

Intracellular Ca^{2+} single cell fluorescence detection

Differentiated PC12 cells on glass coverslips were incubated at room temperature for 45 min. with 10µM Indo1-AM and 1% pluronic F127 followed by an additional 15 min. in dye-free media for desesterification.

Loaded cells were imaged by excitation with a 351nm argon laser line and resulting emission at 405 and 460nm were separated and simultaneously collected through band pass filters using two separate photomultipliers. The ratio image (F405/F460) was automatically generated by the software and recorded on a microcomputer. Fluorescence ratios (R=F405/F485) inside the cytosolic ROI were used to calculate free $[Ca]_i$ according to the equation (9): $[Ca]_i = K_d * \beta[(R-R_{min})/(R_{max} - R)]$, using either *in vitro* K_d of 250nM. The β value (ratio of the free/bound Indo-1 fluorescence at 485nm) was approximately 3.0. Each experiment was repeated in triplicate, on at least four separate days. Only data from responsive cells was submitted for analyses and statistical study.

Western blot analysis

Total cellular protein was extracted by incubating PC12-wt and PC12-NCS-1 cells during 1h/4°C in lysis buffer (MOPS pH:7.0, 2.0mM EGTA, 5.0mM EDTA, 30mM sodium fluoride, 40mM β-glycerophosphate, 20mM pyrophosphate, 1.0mM sodium orthovanadate, 1.0mM phenylmethylsulfonyl fluoride, 3.0mM benzamidine, 10µM leupeptin, 0.5% Nonidet P-40) followed by 20 min./4°C centrifugation at 14,000 rpm. Total cellular proteins present in supernatants of PC12-wt and PC12-NCS-1 cells were quantified by Bradford staining method. Protein homogenates (50µg) of PC12-wt and PC12-NCS-1 were fractionated in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Germany). Membranes were incubated for 2 h at room temperature in phosphate-buffered saline (PBS) with 5% non-fat milk, containing goat polyclonal primary antibody against muscarinic receptor type 1 (M1R) and mouse monoclonal antibody against actin, at concentrations of 1:100 and 1:3,000, respectively. Binding of primary antibodies was detected by incubating membranes during 1.0 h. at room temperature in PBS containing 1:2,500 HRP-conjugated anti-goat secondary antibody and 1:7,000 HRP-conjugated goat anti-mouse secondary antibody. Detection was performed with a chemiluminescence ECL-kit system (Amersham Biosciences, Germany). For phospholipase C-β (PLC-β) detection, the membrane was subject to the same protocol except for the rabbit polyclonal primary antibody anti-PLC-β at concentration of 1:500 and HRP-coupled goat anti-rabbit secondary antibody at concentration of 1:20,000.

Glutamate release and measurement

Differentiated PC12-NCS-1 and PC12-wt cells plated in 60mm culture dishes were incubated with agonists and drugs during 15 min. at 37°C in atmosphere containing 5% CO_2. CCH (300µM) and CCH plus atropine (10µM) were prepared in KRH containing 2.0mM $CaCl_2$. To test the effects of extracellular Ca^{2+} on glutamate release, CCH (300µM) was prepared in KRH containing 2.0mM EGTA.

To test glutamate release dependence on intracellular Ca^{2+} PC12-wt and PC12-NCS-1, cells were pre-incubated for 20 min. with BAPTA-AM (20µM) in KRH containing 2.0mM $CaCl_2$. Then, both PC12 cells were stimulated with CCH (300µM) in KRH containing 2.0mM EGTA.

Glutamate released was detected by measuring the increase in fluorescence resultant from production of NADPH in the presence of NADP and glutamate dehydrogenase, using a fluorimetric assay method as respectively described and modified for this present study (28, 32). In brief, supernatant (2.0mL) containing glutamate released from PC12 cells was added to a curvet and incubated with NADP (1.0mM) and 50 units of glutamate dehydrogenase (EC1.4.1.3.). Fluorescence at 360nm/450nm (excitation/emission) was measured (spectrophotofluorimeter Photon Technology International, Canada). After stabilization of the fluorescence signal at a plateau level, 5 nmol of glutamate was added as an internal standard. Data was expressed as nmol of glutamate per milligram of protein. Differences were considered significant at $p<0.05$ using non-paired Student's t-test (Sigma-Stat, Jandel Corporation).

RESULTS

It has been known that NCS-1 overexpression modulates dopaminergic and purinergic signaling in PC12 cells (3, 19, 35, 51). We hypothesize that NCS-1 overexpression can also modulate cholinergic signaling in this PC12 cell model. Evidence from the literature shows that PC12 cells could release glutamate and differentiated PC12 cells have been extensively used as an *in vitro* model for neurotoxicity and neurodegenerative studies (6, 29, 37, 39, 50). Therefore, we tested whether glutamate release dependent on cholinergic activation was altered in PC12-NCS-1, as compared to PC12-wt. For this purpose, PC12-wt and PC12-NCS-1 cells were stimulated for 15 min with 300µM CCH, and tested for glutamate released in the extracellular fluid. Our results showed that cholinergic activation of both PC12-NCS-1 and PC12-wt cells leads to an increase in glutamate release. Importantly, PC12-NCS-1 showed greater amounts of glutamate release as compared to PC12-wt cells (15.3±1.0 and 8.3±0.9 nmol/mg of protein, respectively, **Fig. 1)**.

In order to assess whether glutamate release induced by CCH is dependent on extracellular and intracellular Ca^{2+}, PC12-wt and PC12-NCS-1 cells were stimulated in the presence of either 2.0mM EGTA or 10μM BAPTA-AM plus 2.0mM EGTA. In the absence of extracellular Ca^{2+} (CCH + EGTA), glutamate release induced by CCH was reduced, as compared to CCH without EGTA. However, glutamate release in the presence of EGTA remained significantly higher in PC12-NCS-1 cells than in PC12-wt cells (10.1±0.5 and 5.7±0.4nmol/mg of protein, respectively) (**Fig. 1**). In the absence of extracellular and intracellular Ca^{2+} (CCH + EGTA + BAPTA-AM), CCH-evoked glutamate release was abolished in PC12-wt and PC12-NCS-1 cells (**Fig. 1**).

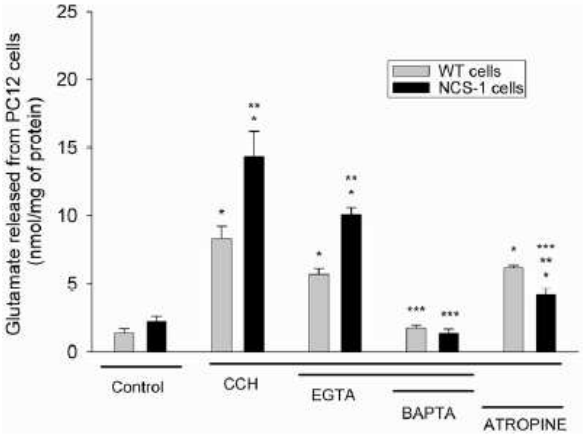

Figure 1. Alterations at glutamate release in PC12-NCS-1 and PC12-wt cells provoked by CCH stimulation. Differentiated PC12 cells were submitted to 5 min/37°C glutamate release assay induced by 300μM CCH or 300μM CCH in the presence of Ca^{2+} buffers or muscarinic antagonist. Dark and light bars were referent to PC12-NCS-1 and PC12-wt cells respectively. Grouped light and dark par of bars are correspondent respectively to basal conditions of glutamate release, 300μM CCH isolated stimulus, 300μM CCH in the presence of 2.0mM EGTA, 20min 10μM BAPTA-AM pre treatment followed by 300μM CCH stimulation in the presence of 2.0mM EGTA and, finally, 300μM CCH in concomitant presence of 10μM atropine was represented at the last group of bars. Glutamate release was expressed in nmol/mg of protein as average of at least three independent experiments. Statistical significance was considered as $p < 0.05$. * basal conditions compared with stimulus; ** PC12-wt response compared with PC12-NCS-1 cells response, *** CCH stimulation compared with experimental conditions (CCH plus EGTA, BAPTA plus CCH /EGTA or CCH plus atropine).

In order to assess the cholinergic origin of the glutamate facilitation release, both PC12-wt and PC12-NCS-1 cells were stimulated with 300μM CCH in the presence of 10μM atropine, which is a muscarinic receptor antagonist. In PC12-wt cells, CCH-stimulated glutamate release was not significantly inhibited by atropine, as compared to glutamate release induced by CCH alone (6.2±0.1 and 8.2±0.9nmol/mg of protein, respectively) (**Fig. 1**). On the other hand, glutamate release was more strongly inhibited by atropine in PC12-NCS-1 than in PC12-wt cells (4.2±0.3 and 6.2±0.1nmol/mg of protein, respectively). These results indicate that increase in glutamate release caused by NCS-1 over-expression is mainly due to a muscarinic component.

It has been previously suggested that NCS-1 overexpression could increase the efficiency of exocytosis by a mechanism entailing activation of PI4Kβ and consequent increase of PIP_2 production (19, 42). To investigate the mechanism involved in glutamate release facilitation by NCS-1 overexpression, we took advantage of $PH_{PLC\delta1}$-EGFP domain and confocal time course images to assess the dynamics of PIP_2/$Insp_3$ hydrolysis/formation in PC12-wt and PC12-NCS-1 stimulated with 300μM CCH. In undifferentiated PC12-wt and PC12-NCS-1 cells, no translocation of $PH_{PLC\delta1}$-EGFP from the plasma membrane to the cytosol was observed after stimulation with 300μM CCH (data not shown). In differentiated cells, 300μM CCH induced $PH_{PLC\delta1}$-EGFP translocation to the cytosol in 88% of PC12-wt cells and 87% of PC12-NCS-1 cells (**Table 1**). A complete loss of plasma membrane-cytosol fluorescence gradient, which represents the maximum detectable translocation of $PH_{PLC\delta1}$-EGFP to the cytosol, was observed in 67% of PC12-NCS-1 cells, in contrast to 17% of PC12-wt. Partial translocation of $PH_{PLC\delta1}$-EGFP from the plasma membrane to the cytosol was observed in 71% of PC12-wt and in 20% of PC12-NCS-1 cells (**Table 1**). These results indicate that the level of $PH_{PLC\delta1}$-EGFP translocation in response to CCH stimulation in PC12-NCS-1 is higher than in PC12-wt cells. Spatial (**Fig. 2A**) and temporal (**Fig. 2B**) information concerning the dynamics of $PH_{PLC\delta1}$-EGFP stimulated with 300μM CCH was analyzed. The temporal analysis of ROI outlined in cytosol showed a similar onset for $PH_{PLC\delta1}$-EGFP translocation in PC12-wt and NCS-1 cells. However, translocation was slower in wt cells than in NCS-1 cells (**Fig. 2B**). The average membrane/cytosol fluorescence ratio after maximum translocation of $PH_{PLC\delta1}$-EGFP stimulated by CCH (300μM) was 1.60±0.10 in PC12-wt cells (n=22) and 0.83±0.07 in PC12-NCS-1 cells (n=20) ($p < 0.01$),

Table 1. Percentage of cells exhibiting distinct pattern of $PH_{PLC\delta1}$-EGFP localization after stimulation of the cells with 300μM CCH.

PC12 cells	$PH_{PLC\delta1}$-EGFP in the cytosol	$PH_{PLC\delta1}$-EGFP in the cytosol and membrane	$PH_{PLC\delta1}$-EGFP in the membrane
WT	17% (17 cells)	71% (70 cells)	12% (11 cells)
NCS-1	67% (50 cells)	20% (15 cells)	13% (10 cells)

the predominance of $PH_{PLC\delta1}$-EGFP cytosolic accumulation in the latter compared to the former (**Fig. 2C**).

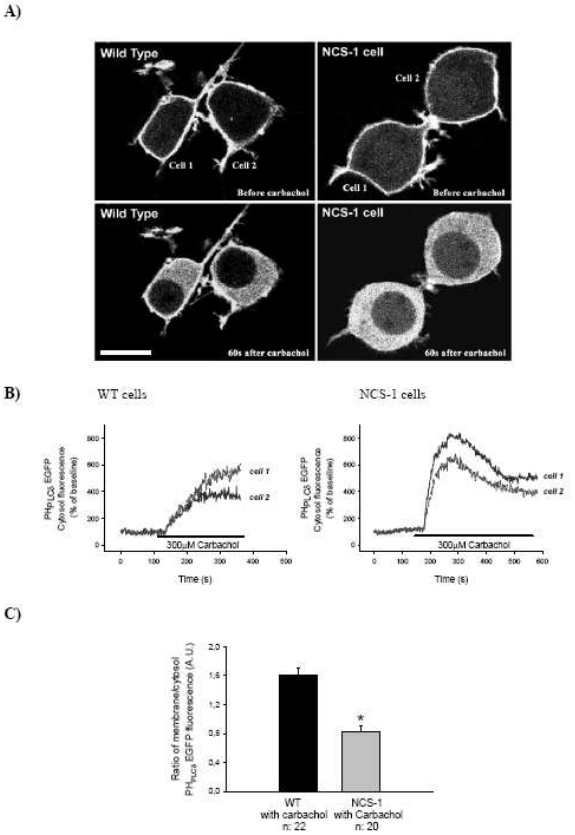

Figure 2. Distinct dynamic of $PH_{PLC\delta1}$-EGFP after carbachol stimulation in PC12-wt and PC12-NCS-1 cells. PC12 cells transfected transiently with $PH_{PLC\delta1}$-EGFP were submitted to 300μM carbachol stimulation in HBS solution with 2.0mM Ca^{2+}. **A:** Spatial alterations at $PH_{PLC\delta1}$-EGFP distribution before (upper) and after (**lower**) stimulation of cells with 300μM carbachol. Distinct patterns can be observed at PC12-wt (left) and PC12-NCS-1 transfected with $PH_{PLC\delta1}$-EGFP (right). Scale bar: 20μm. **B:** Temporal analyses of $PH_{PLC\delta1}$-EGFP dynamics collected in a defined ROI in cell cytosol. Each line corresponds to isolated cell. **C:** ratio of cytosolic $PH_{PLC\delta1}$-EGFP fluorescence intensity (in A.U.) at membrane and cytosol of PC12 cells at maximum response to 300μM carbachol stimulation. Statistical analysis performed by *t-student* test was considered significant for $p < 0.05$.

To confirm that $PH_{PLC\delta1}$-EGFP translocation stimulated by CCH was due to muscarinic activation, cells were perfused with 300μM CCH in the presence of 10μM atropine, a non-specific muscarinic receptor antagonist. When translocation of $PH_{PLC\delta1}$-EGFP induced by CCH achieved a plateau, cells were perfused with 10μM atropine in the presence of 300μM CCH (**Fig. 3**). In both PC12-NCS-1 and PC12-wt cells, the $PH_{PLC\delta1}$–EGFP domain returned to the plasma membrane, with consequent reduction of cytosolic fluorescence (**Fig. 3**), which indicates that CCH effects on phosphoinositide dynamics in PC12-wt and PC12-NCS-1 cells were induced by muscarinic activation. Western blot analyses showed muscarinic receptor type 1 protein expression in both PC12-wt and PC12-NCS-1 cells (**Fig. 3, inset**). This muscarinic receptor could be correlated with activation of breakdown of phosphoinositides, production of $InsP_3$, and regulation of intracellular Ca^{2+} dynamics.

In order to prove the participation of PLC in the increase of $PH_{PLC\delta1}$-EGFP translocation observed in NCS-1 cells, a selective inhibitor of PLC, U73122 (10μM) was added to the incubation medium prior and during stimulation of cells with 300μM CCH. In both PC12-wt and PC12-NCS1 cells, $PH_{PLC\delta1}$-EGFP translocation was impaired by the PLC inhibitor, as shown in **Fig. 4**. Maximum $PH_{PLC\delta1}$-EGFP translocation after stimulation of PC12-NCS-1 and PC12-wt cells with 300μM CCH in the presence of 10μM U73122 was respectively 314.6±34.8 n=11; 221,0±16.4 % of basal fluorescence (n=21, $p < 0.01$). These results suggest that facilitation of $PH_{PLC\delta1}$-EGFP translocation induced by muscarinic activation in PC12-NCS-1 cells might be associated to pathways regulated by PLC. The **inset** (**Fig. 4**) is a western blot showing PLC-β protein expression in both PC12-wt and PC12-NCS-1 cells. Moreover, immunofluorescence experiments determined PLC-β expression in

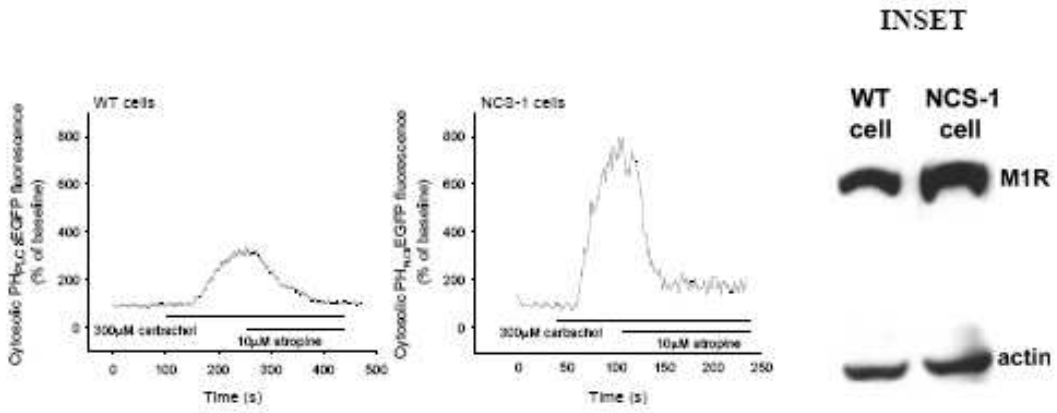

Figure 3. $PH_{PLC\delta1}$-EGFP translocation induced by carbachol is dependent on muscarinic signaling activation both at PC12-wt and PC12-NCS-1 cells.
PC12 cells transfected transiently with $PH_{PLC\delta1}$-EGFP were submitted to 300μM carbachol stimulation followed by infusion of 10μM atropine in continuously presence of 300μM carbachol (both diluted in HBS solution with 2.0mM Ca^{2+}). Curves indicate temporal analyses of $PH_{PLC\delta1}$-EGFP dynamics collected in a defined ROI in cell cytosol. Each line represents isolated cell and, at least, three independent experiments were performed. The inset shows western-blot analysis of the expression of muscarinic receptor MR-1 (upper bands) and actin (lower bands) at PC12-NCS-1 and PC12-wt cells.

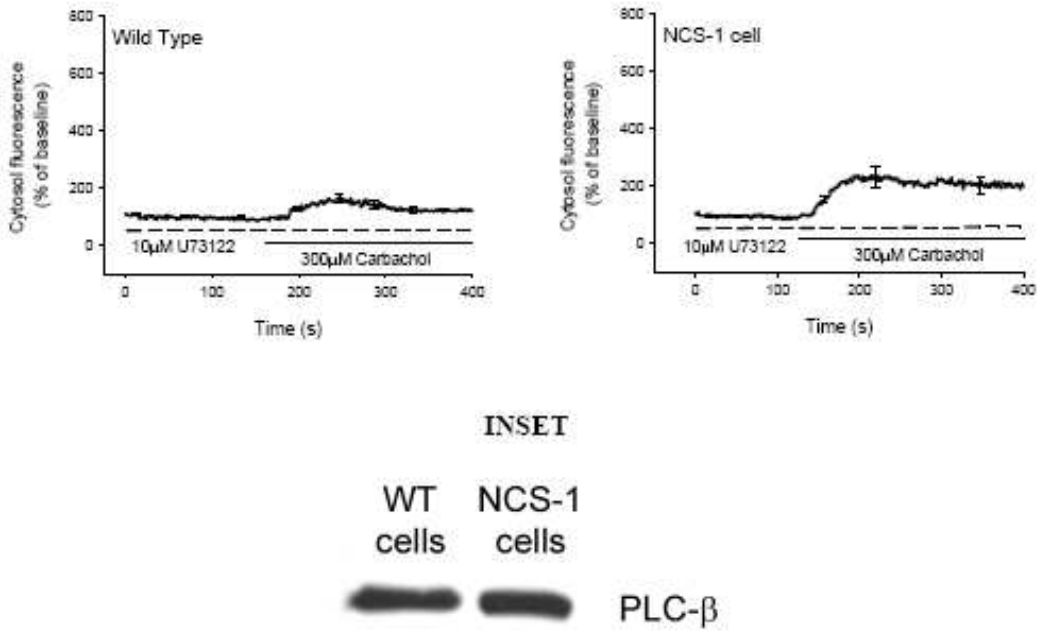

Figure 4. Participation of PLCβ on $PH_{PLC\delta1}$-EGFP translocation at PC12-wt and PC12-NCS-1 cells.
PC12 cells transiently transfected with $PH_{PLC\delta1}$-EGFP were pre incubated for 30 min./37°C with 10μM U73122, a selective PLC inhibitor, in HBS solution containing 2.0mM Ca^{2+}. The cells were stimulated with 300μM carbachol in continuous infusion 10μM U73122. Curves indicate temporal analysis of $PH_{PLC\delta1}$-EGFP translocation before and after carbachol stimulation in the presence of 10μM U73122. The curves indicate average values (means±S.E.M.) of $PH_{PLC\delta1}$-EGFP fluorescence intensity in a selected cytosolic region (in % of baseline) from at least 3 separate experiments. The inset shows western-blot analysis of the expression of PLC-β at both PC12-wt and PC12-NCS-1 cells.

both PC12-wt and PC12-NCS-1 (data not shown).

To determine whether facilitation of $PH_{PLC\delta1}$-EGFP translocation observed in NCS-1 cells was dependent on extracellular Ca^{2+} influx, both control and NCS-1 PC12 cells overexpressing $PH_{PLC\delta1}$-EGFP were stimulated with 300μM CCH in the presence of 2.0mM EGTA. Under these conditions, translocation of $PH_{PLC\delta1}$-EGFP was not affected in either cell type (**Fig. 5A**). Maximal cytosolic translocation of $PH_{PLC\delta1}$-EGFP was significantly higher in PC12-NCS-1 than in PC12-wt cells (599.51±20.45 % and 467.08±87.00 % of basal fluorescence, respectively), indicating that external Ca^{2+} influx might not be essential for triggering the facilitation of $InsP_3$ formation in PC12-NCS-1 cells (**Fig. 5A**).

The requirement of intracellular Ca^{2+} increase to produce translocation of $PH_{PLC\delta1}$-EGFP was tested by loading PC12-wt and PC12-NCS-1 cells with 10μM BAPTA-AM, for 20 min./37°C. Both PC12-wt (n=14) and PC12-NCS-1 cells (n=7) loaded with BAPTA-AM exhibited an attenuated translocation of $PH_{PLC\delta1}$-EGFP from the plasma membrane to the cytosol following 300μM CCH stimulation (**Fig. 5B**), indicating, in this case, the dependence on cytosolic Ca^{2+} increase from intracellular compartments. These results highlight the relevance of $PIP_2/InsP_3$ dynamics for glutamate release facilitation observed in PC12-NCS-1 cells, since both mechanisms are independent from external, but dependent on internal Ca^{2+} (**Fig. 1**).

Johenning et al., (2002) (13) demonstrated that $InsP_3$ receptors ($InsP_3R$) subtypes I and III, but not type II, are expressed in PC12 cells differentiated for 7 days with 100ng/mL NGF. Previous results demonstrated that $InsP_3$ production stimulated by UTP induces higher intracellular Ca^{2+} mobilization from stores in PC12 cells overexpressing NCS-1 than in control cells (19). In order to confirm the contribution of Ca^{2+} from intracellular sources, cells were pre-treated for 20 min. with 100 μM 2APB, which is an unspecific inhibitor of Ca^{2+} release from intracellular stores (26). Imaging time series showed that 100μM 2APB reduced $PH_{PLC\delta1}$-EGFP translocation in both PC12-wt and PC12-NCS-1 cells stimulated with 300μM CCH (**Fig. 5C**). As shown in **Fig. 5**, maximum cytosolic $PH_{PLC\delta1}$-EGFP fluorescence induced by CCH in the absence of 2APB (PC12-wt 310±39%, and PC12-NCS-1 663±63% of baseline cytosolic fluorescence) was much higher than in cells treated with 2APB (PC12-wt 212±24% and PC12-NCS-1 252±43% of baseline cytosolic fluorescence). These results indicate that Ca^{2+} intracellular stores sensible to 2APB can be an important contributor to the enhancement of $PH_{PLC\delta1}$-EGFP translocation observed in NCS-1 cells.

To confirm whether Ca^{2+} influx and $[Ca^{2+}]_i$ could be altered in PC12-NCS-1 compared to PC12-wt cells, we measured temporal changes in CCH induced $[Ca^{2+}]_i$ in cells loaded with indo-1. Distinct profiles of intracellular Ca^{2+} dynamics were observed among PC12-wt and PC12-NCS-1 stimulated with CCH. PC12-wt cells exhibited an increase in $[Ca^{2+}]_i$ that was sustained during CCH perfusion (**Fig. 6A, left graph**). However, PC12-NCS-1 cells exhibited a rapid and transient increase in $[Ca^{2+}]_i$ (**Fig. 6A, right graph**). Maximum $[Ca^{2+}]_i$ increase was significantly higher in NCS-1 PC12 than in PC12-wt cells (766.4±40.0 and 687.9±37.1 nM respectively, $p<0.05$) (**Fig. 6B**). This faster and greater increase in $[Ca^{2+}]_i$ observed in PC12-NCS-1 cells might be correlated with facilitation of $PH_{PLC\delta1}$-EGFP translocation. The increase in intracellular $PH_{PLC\delta1}$EGFP translocation and intracellular Ca^{2+} dynamics observed in PC12-NCS-1 cells upon CCH stimulation indicate a facilitation in production of secondary messengers via PIP_2 cleavage, generating $InsP_3$ and Ca^{2+} mobilization from intracellular stores, which can be correlated to the facilitation of glutamate release observed in this cell model (**Fig. 1**).

DISCUSSION

The vast number of reports showing the wide range of cellular functions regulated by NCS-1 and the participation of NCS-1 in different neurotransmitter systems demonstrates the importance of NCS-1 for understanding the pathophysiology of many neuropsychiatry disorders. In this work, our objective was to examine the role of NCS-1 overexpression in cholinergic signaling, using a neuroendocrine PC12 cell model. To achieve this objective, we took advantage of specific fluorescent probes that allows examining the dynamics of intracellular messengers. $InsP_3$ production was researched through the high affinity $InsP_3$ probe, $PH_{PLC\delta}$-EGFP; changes in Ca^{2+} levels and profile were analyzed through the Ca^{2+} specific Indo probe. Our results demonstrated that PC12-NCS-1 cells

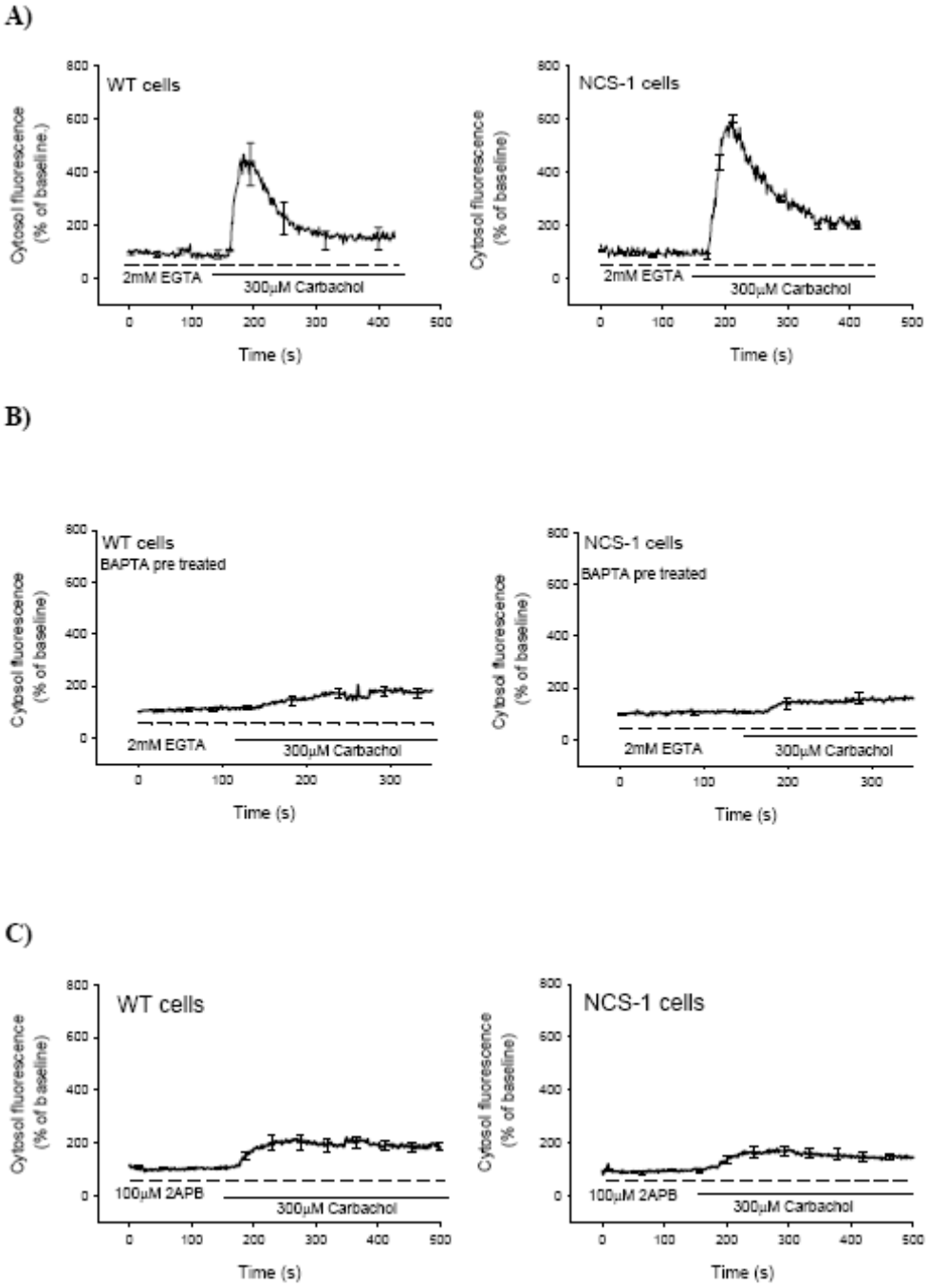

Figure 5. Contribution of extra/intracellular Ca^{2+} levels and 2APB-sensitive intracellular Ca^{2+} stores to carbachol induced alterations in translocation of $PH_{PLC\delta1}$-EGFP.

A: PC12-wt and PC12-NCS-1 cells transiently expressing $PH_{PLC\delta1}$-EGFP were perfused with HBS solution in the presence of 2.0mM EGTA. The cells were then submitted to stimulation with 300µM carbachol plus 2.0mM EGTA. **B**: Transiently $PH_{PLC\delta1}$-EGFP transfected PC12-wt and PC12-NCS-1 cells pre incubated with 10µM BAPTA-AM (20min/37°C) were submitted to 300µM carbachol stimulation in the presence of 2.0mM EGTA. **C**: PC12-wt and PC12-NCS-1 cells transiently transfected with $PH_{PLC\delta1}$-EGFP were pre incubated (20 min./37°C) with 100µM 2APB in HBS solution containing 2.0mM Ca^{2+} and stimulated with 300µM carbachol in the presence of 100µM 2APB. Time course curves indicate average values (means±S.E.M.) of $PH_{PLC\delta1}$-EGFP fluorescence intensity in a selected cytosolic region (in % of baseline) from at least 3 separate experiments.

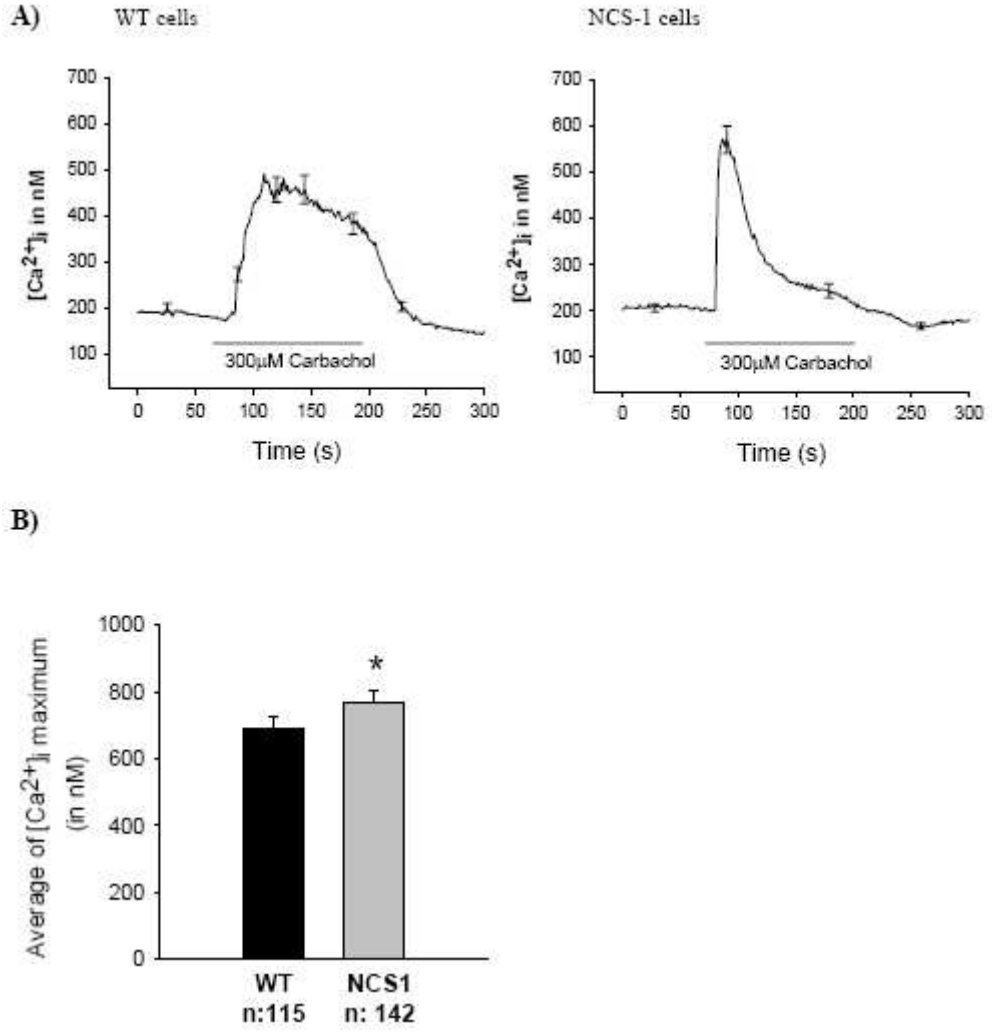

Figure 6. Temporal dynamics of intracellular Ca^{2+} concentration at PC12-wt and PC12-NCS-1 cells after carbachol stimulation.

The cells were pre loaded with 10µM Indo1-AM for 45min, 15 min to allow deesterification and then stimulated with 300µM carbachol. **A:** Distinct temporal patterns were identified at PC12-wt and PC12-NCS-1 cells after 300µM carbachol stimulation in the presence of 2.0mM Ca^{2+} at extracellular HBS solution. The results were expressed as the average curve of PC12-wt (n: 115) and PC12-NCS-1 (n: 142) responsive cells from, at least, 5 separate experiments. **B:** shows the average (mean±S.E.M.) of maximum intracellular Ca^{2+} concentration (in nM) observed after carbachol stimulation at PC12-wt e PC12-NCS-1 cells. Statistical significance was considered for $p < 0.05$.

stimulated with CCH exhibited a higher level of $InsP_3$ production and $[Ca^{2+}]_i$ peak, as compared to PC12-wt. *In vitro* studies in COS-7 and PC12 cells overexpressing NCS-1 have shown a direct interaction between NCS-1 and phosphatidilinositol 4-kinase-β (PI4Kβ) (3, 10, 31, 42). Furthermore, increased levels of radioactive $[^3H]$-$InsP_3$ in PC12 stable overexpressing NCS-1 in response of 100µM UTP stimulation have been reported (19). The goal of our studies was to associate the well documented effects of NCS-1 on PI4Kβ activation and $InsP_3$ production with activation of muscarinic receptors. Our results further confirm the role of NCS-1 in the mechanism of PIP_2 breakdown and $InsP_3$ production by using individual live cell fluorescence imaging. PC12-NCS-1 cells exhibited increased levels of $InsP_3$ production following CCH stimulation, since these cells presented higher levels of $PH_{PLC\delta}$-EGFP translocation to the cytosol.

The most relevant mechanism of NCS-1 interference in cellular function is its capacity to potentiate evoked exocytosis of dopamine mediated by purinergic agonists, such as ATP or UTP (19, 25). In our studies, we examined whether muscarinic activation of PC12-NCS-1

cells could be related to increased levels of glutamate release. The release of neurotransmitters, including dopamine and glutamate, has been implicated in hypoxia/ischemia-induced alterations in neuronal function and in subsequent tissue damage. PC12 cells have been extensively used as a dopaminergic model to study cell toxicity and its implications in neurodegenerative processes. Furthermore, differentiated PC12 cells have been used as an *in vitro* model to investigate neurotoxicity and oxidative stress induced by glutamate (7, 24, 38, 50). Moreover, glutamate is important to induce dopamine secretion from PC12 cells, suggesting that an integrative circuit of glutamate and dopamine signaling might play a role in the modulatory mechanisms of cellular toxicity (20). PC12 cells express metabotropic glutamatergic receptor type 1 (mGluR1) and the differentiation of these cells with NGF induces expression of functional NMDA receptors (15, 17, 21, 22). We showed for the first time here that CCH induced glutamate release by differentiated PC12 cell. Furthermore, PC12-NCS-1 cells released higher levels of glutamate as compared to PC12-wt (F**ig. 1**). Interestingly, although atropine significantly blocked glutamate release in PC12-NCS-1 cells, it did not block CCH-stimulated glutamate release in PC12-wt cells. These data indicate that NCS-1 overexpression facilitates glutamate release preferentially through a muscarinic signaling pathway. It is possible that cells overexpressing NCS-1 are able to respond faster to many types of GPCR stimulation. Based on our results as well as on reported data (3, 4, 12, 16, 36, 48, 49, 51), cells that stably overexpress NCS-1 can integrate different molecular components of the GPCR system, regulating plasma membrane Ca^{2+} channels, facilitating $InsP_3$ receptor activation, regulating PI4Kβ activity, increasing PIP_2 plasma membrane levels, and altering mobilization of the releasable pool of vesicles and its fusion to the plasma membrane. We propose here, that overexpression of NCS-1 in PC12 cells leads to an augmentation of muscarinic signaling, inducing increased levels of $InsP_3$ production and intracellular Ca^{2+} levels, leading to an increase in glutamate release. The significant blockage of glutamate release induced by atropine observed in PC12-NCS-1 cells, but not in PC12-wt cells, suggests a major role for NCS-1 overexpression in muscarinic pathway potentiation.

The prevalence of muscarinic response in PC12-NCS-1, in contrast to PC12-wt cells, can contribute to the distinct intracellular Ca^{2+} profiles exhibited by these cells. It is possible that the $[Ca^{2+}]_i$ decay observed in PC12-wt can have a component dependent on activation of nicotinic receptor. The small but significant CCH-stimulated increase in intracellular Ca^{2+} levels in PC12-NCS-1 cells, as compared to PC12-wt cells, suggests that facilitation of $InsP_3$ production in PC12-NCS-1 can be responsible for the higher peak of $[Ca^{2+}]$i. However, the most remarkable difference at the intracellular Ca^{2+} profile following CCH-stimulation was the rapid decay of $[Ca^{2+}]_i$ observed in PC12-NCS-1, as compared to the slow decay typical of PC12-wt. The participation of NCS-1 in facilitation of P/Q-type Ca^{2+} currents in calyx of the Held synapse and non-L type Ca^{2+} channels in adrenal chromaffin cells has been reported (40, 49). However, it has also been shown that NCS-1 is implied in the inhibition of P/Q-type channels via G-protein-coupled receptors (48, 49). Moreover, other Ca^{2+} channel types are also affected by NCS-1, which leads to inhibition of N- and L-type Ca^{2+} currents (34). Based on these results, it is possible that the rapid decay of intracellular Ca^{2+} concentration observed only in PC12-NCS-1 stimulated with CCH results from inhibition of Ca^{2+} channels dependent on NCS-1 action. Furthermore, it is important to take in consideration that CCH could also lead to increased expression of P/Q type Ca^{2+} channels β subunit, which also contributes to inhibition of plasma membrane Ca^{2+} channels, thus contributing to transmitter release regulation and short-term synaptic plasticity (23, 40, 53).

Although no clinical or experimental evidence has yet correlated NCS-1 overexpression to cholinergic alteration, experimental results have established a correlation between NCS-1 overexpression and neuropsychiatric disorders. More specifically, published studies using *postmortem* samples from either schizophrenic or bipolar patients have demonstrated the association of NCS-1 overexpression to these neuropsychiatric disorders. Immunohistochemistry analyses showed high levels of NCS-1 protein in the prefrontal cortices of these patients (2, 18). Interestingly, it was also reported that NCS-1 participates in associative memory and learning in *Caenorhabditis elegans* and in the enhancement of short-term plasticity in synapses of hippocampus primary culture (1, 8, 23, 40). Together, the data reported above reinforce the

need of further research of NCS-1 adaptative response in cholinergic signaling, since this pathway has great impact on the regulation of learning and memory.

In conclusion, the results presented here suggest that the association of NCS-1 and muscarinic/GPCR signaling is crucial for NCS-1 modulation of cellular function. We found that NCS-1 overexpression led to an increase in CCH-stimulated glutamate release in a mechanism that is atropine-sensitive. Furthermore, NCS-1 also led to an increase in $InsP_3$ formation and $[Ca^{2+}]_i$ upon CCH stimulation. We propose that this increase in production of secondary messengers is correlated with the facilitation of glutamate release, here observed. It is possible that NCS-1 facilitation of muscarinic-dependent intracellular signaling also regulates the release of other neurotransmitters.

Acknowledgements – We'd like to thank Mr. Carlos Serrano for English editing. The biosensor $PH_{PLC\delta1}$-EGFP used here was authorized by Dr. Tamas Balla, who we also thank. MVG and MAR-S are CNPq research fellows; DSC and MMG were holders of CNPq scholarships. Financial support was provided by CNPq Universal grant #471837/2004-0, Programa Institutos do Milênio/CNPq/FINEP and FAPEMIG grant # CBB-453/04. AJ current address: Allen Institute for Brain Science, Seattle, WA, USA.

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