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Ca^{2+}-Myristoyl Switch in Neuronal Calcium Sensor-1: A Role of C-Terminal Segment

Viktoriia E. Baksheeva[1], Aliya A. Nazipova[2], Dmitry V. Zinchenko[3], Marina V. Serebryakova[1], Ivan I. Senin[1], Sergei E. Permyakov[2], Pavel P. Philippov[1], Yi Li[4], Andrey A. Zamyatnin Jr.[1,5], Evgeni Y. Zernii[*,1] and Gjumrakch Aliev[#,6,7]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, 119992, Russia*

[2]*Institute for Biological Instrumentation of the Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia*

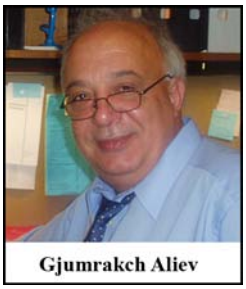
[3]*Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russia*

[4]*Department of Human Sciences, Texas A&M University-Kingsville, Kingsville, USA*

[5]*Institute of Molecular Medicine, Sechenov First Moscow State Medical University, Moscow, 119991, Russia*

[6]*"GALLY" International Biomedical Research Consulting LLC, San Antonio, 78229, USA*

[7]*School of Health Science and Healthcare Administration, The University of Atlanta, Johns Creek, 30097, USA*


Gjumrakch Aliev

Abstract: NCS1 (neuronal calcium sensor-1) is a Ca^{2+}-myristoyl switch protein of the NCS protein family involved in synaptic plasticity and neurotransmission *via* Ca^{2+}-dependent regulation of dopamine D2 receptor and associated G-protein coupled receptor kinase (GRK)-2. Overexpression of NCS1 in synaptic terminals results in accumulation of membrane-bound protein and its redundant regulatory activity associated with neurological disorders. Here, we have demonstrated that bovine photoreceptors contain NCS1 that is capable of a partially irreversible interaction with isolated photoreceptor membranes and implicated in Ca^{2+}-dependent binding and regulation of GRK1 *in vitro*. Using NCS1-recoverin C-terminal chimeric construct (NR), it was found that the Ca^{2+}-myristoyl switch of NCS1 is affected by its C-terminal segment downstream the fourth EF-loop of the protein, which is variable within the NCS family. NR retains structural stability and sensitivity to Ca^{2+}, but interacts with photoreceptor membranes with lower affinity in a Ca^{2+}-dependent fully reversible manner and displays altered GRK1 modulation. These data combined with fluorescent probing of surface hydrophobicity of NCS1, NR and recoverin suggest that the C-terminal segment of NCS1 regulates reuptake of myristoyl group under Ca^{2+}-free conditions and participates in organization of the target-binding pocket of the protein. We point out a putative role of NCS1 in photoreceptors as a modulator of GRK activity and propose targeting of the C-terminal segment of NCS1 as an appropriate way for selective suppression of excessive membrane accumulation and aberrant activity of the protein in neurons associated with central nervous system dysfunctions.

Keywords: C-terminal segment, Ca^{2+}-myristoyl switch, G-protein coupled receptor kinase, membrane interaction, neurological disorders, neuronal calcium sensor-1, neuronal calcium sensor protein family, retinal neurons.

INTRODUCTION

Intracellular calcium signaling pathways play a crucial role in the regulation of neuronal survivability and function. The diversity of cell responses to Ca^{2+} signals is provided by a family of neuronal calcium sensor (NCS) proteins [1, 2]. The human genome encodes 14 NCS genes, some of which are expressed throughout the nervous system, while expression of others is restricted to certain types of neurons [3]. NCS proteins are capable of transmitting Ca^{2+}-signals to dozens of target proteins thereby contributing to reception [4], neurotransmission [1, 5], neuronal survival and growth [6], long-term and short-term synaptic plasticity [7, 8]. Despite a wide range of produced effects, NCS proteins display sequence and structural similarity. They are built of N-terminal and C-terminal domains each containing a pair of Ca^{2+}-binding EF-hand motifs. EF1 is non-functional due to the absence of negatively charged residues in the first and the third positions of the Ca^{2+}-binding loop [1, 9]. EF3 binds Ca^{2+} in all NCSs while EF2 and EF4 are functional or non-functional depending on the specific protein. Most NCS proteins maintain the N-terminal myristoyl group which is

[*]Address correspondence to this author at A.N. Belozersky Institute of Physico-Chemical Biology MSU, 119992 Leninskye gory 1, b. 40, Moscow, Russia; Tel: +7-495-939-23-44; E-mail: zerni@belozersky.msu.ru

[#]Alternatively, address correspondence to this author at "GALLY", International Biomedical Research Institute Inc., 7733 Louis Pasteur Drive, #330, San Antonio, TX, 78229 USA; Tel: +440-263-7461;
E-mails: aliev03@gmail.com, GAliev@uofa.edu

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involved in the so-called Ca^{2+}-myristoyl switch mechanism [9, 10]. In the absence of calcium (at resting intracellular Ca^{2+} levels) the myristoyl group is buried in the hydrophobic pocket within the NCS molecule. The binding of Ca^{2+} to the protein induces a major conformational change resulting in exposure of amino acid residues of the hydrophobic pocket and extrusion of the myristoyl group, that increases membrane affinity of the protein [9, 11, 12]. Although NCSs do not universally exploit this mechanism, the myristoyl group is normally involved in membrane targeting, which is critical for their co-localization with transmembrane or membrane-anchored signaling partners [1, 2]. The hydrophobic groove that is formed upon myristoyl release generally represents a target-recognition site [9, 11, 13-15]. However, each NCS possesses a unique target-binding interface where responsive element(s) of the target protein is positioned in a somewhat different way. It was proposed that the myristoyl group provides for folding of Ca^{2+}-free NCS proteins into unique structures. Upon Ca^{2+}-induced displacement of the myristoyl group each protein exposes a specific set of previously masked hydrophobic residues intended for selective target recognition [9]. Recently it was shown that the C-terminal segment, which varies considerably in sequence within the NCS family, might also play a significant regulatory role. This region is located downstream of EF4 and comprises one or two alpha helices. The C-terminal segment contributes to stabilization of the Ca^{2+}-loaded form of NCS proteins, regulates the position of their myristoyl group and/or forms contacts with target proteins [14, 16-19]. The diversity of existing data along with C-terminal sequence variability indicates that regulatory function of the C-terminal segment could be a general feature of NCS proteins [20].

NCS1 (frequenin) is considered to be a progenitor of the NCS family as it is highly conserved among animal species and yeast and ubiquitous throughout the nervous system. NCS1 is expressed in most neuron types, as well as in developing heart muscle cells and mast cells [3]. In the central nervous system (CNS) NCS1 is usually localized in synapses, where it regulates synaptic function and development [21]. NCS1 is also associated with the *trans*-Golgi network and was reported to be a multifunctional regulator of secretion and phosphoinositide synthesis through its interaction with 1-phosphatidylinositol 4-kinase [22, 23]. The latter belongs to a strikingly wide range of NCS1 membrane-bound protein targets, including dopamine receptor of D_2 subtype (D2R) [24], voltage-gated Ca^{2+}-channels of N- and P/Q-types [25, 26], Ca^{2+}-dependent activator protein of secretion [27], transforming growth factor β receptor-1 (TGFβR1) [28], interleukin-1 receptor accessory protein-like 1 (IL1RAPL1) [29] and G-protein coupled receptor (GRK) kinases [24, 30]. NCS1 malfunction is associated with a number of CNS disorders such as cocaine addiction [31], X-linked mental retardation [29], autistic spectrum disorder (ASD) [32] and schizophrenia [33, 34]. For instance, in ASD patients, a point mutation R102Q in NCS1 causes structural deficit in its C-terminal segment and impairs the dynamic exchange between membrane-bound and cytoplasmic pools of the protein [35]. As a result, NCS1 accumulates on membranes in a Ca^{2+}-independent manner resulting in aberrant regulation of its targets such as IL1RAPL1, a protein involved in Ca^{2+}-dependent vesicle release [29]. Schizophrenia and bipolar disorder are characterized by NCS1 overexpression [33]. Since NCS1 inhibits D2R phosphorylation by GRK2 and attenuates internalization of the receptor [24], its excess was supposed to lead to redundant inhibition of GRK2 and altered dopamine signaling which is widely considered to underlie cognitive deficits in schizophrenic patients [36].

NCS1 has three functional EF-hands (EF2-EF4) and binds Ca^{2+} with nanomolar dissociation constant (K_d = 90 nM) which is comparable with basal Ca^{2+}-levels in living cells [2]. In the presence of Mg^{2+} the affinity of NCS1 for Ca^{2+} shifts to submicromolar range (K_d = 440 nM), which was suggested to avoid undesirable permanent activation of the protein [37]. Ca^{2+} is not necessarily required for its membrane association *in vitro* and in neural cell lines [38, 39]. Before the three-dimensional structure of Ca^{2+}-free NCS1 was resolved, the N-terminal alpha-helix of the protein had been speculated to be stuck in conformation with constitutively exposed myristoyl group regardless of the presence of calcium [40]. However, a recent nuclear magnetic resonance (NMR) study revealed that the myristoyl group of NCS1 being exposed in the Ca^{2+}-bound protein becomes sequestered within the hydrophobic pocket in its apo-form (see Fig. **7**) [15]. Using living cell models it was demonstrated that NCS1 dynamically associates with membranes at resting Ca^{2+} levels but binds irreversibly in response to Ca^{2+} influx [35]. Thus, the existence Ca^{2+}-myristoyl switch in NCS1 was confirmed but its exact mechanism is yet to be determined.

The C-terminal segment of NCS1 was suggested to be critical for structural integrity and function of the protein. In Ca^{2+}-bound NCS1 the C-terminal segment lacks secondary structure and is flexible enough to occupy the exposed hydrophobic groove. In a way, the C-terminal segment imitates target proteins by interacting with the exposed hydrophobic surface of NCS1 and provides global stability of its Ca^{2+}-loaded form [18]. It was suggested that upon binding target proteins such as D2R, the C-terminal segment becomes displaced but its exact conformation in this state has not been resolved [41]. Moreover, the C-terminal segment of NCS1 was suggested to directly interact with target proteins, as in the case of IL1RAPL1 [29]. The functional significance of the C-terminal segment in NCS1, while revealed *in vitro* remains to be confirmed under *in vivo* conditions [42].

In the present study, we addressed a role of the C-terminal segment in NCS1 focusing on regulatory activity of the protein in retinal photoreceptor cells. Phototransduction cascade in rod outer segments (ROS) represents a striking example of the Ca^{2+}/NCS regulated signaling system. Photoreceptors express several members of the NCS family including recoverin which controls the life-time of photoexcited rhodopsin *via* Ca^{2+}-dependent regulation of its associated kinase (GRK1) [11, 43]; however, the specific function of NCS1 in these cells remains unknown [44]. We demonstrated the presence of NCS1 in bovine retinal and ROS extracts and investigated functional properties of the protein specific to photoreceptors. Furthermore, using phosphorylation of visual receptor rhodopsin by GRK1 as a convenient model of Ca^{2+}/NCS-modulated process we studied an impact of the C-terminal segment of NCS1 on

regulatory activity of the protein. To this end, we obtained a chimeric protein representing NCS1 with its C-terminal segment replaced by the corresponding peptide of recoverin (NR) and characterized the effect of replacement on Ca^{2+}-myristoyl switch-dependent properties of NCS1 such as membrane interaction and regulation of GRKs. Our findings point to a putative role of NCS1 in photoreceptors and suggest a regulatory function of its C-terminal segment. We suggest targeting of the C-terminal segment as a promising way for selective suppression of the NCS1 redundant activity in neurons associated with CNS dysfunctions.

MATERIALS AND METHODS

Materials

RNA purification kit and DNA gel extraction kit were purchased from Qiagen. All reverse transcription and PCR reagents and enzymes were from Evrogen, Russia. [γ-^{32}P]-adenosine triphosphate (ATP) was provided by the Institute of Bioorganic Chemistry of the Russian Academy of Sciences. Glutathione sepharose and goat anti-rabbit IgG peroxidase conjugate were from GE Healthcare Life Sciences. Protein and DNA molecular weight markers and restriction endonucleases *HindIII* and *NdeI* were from Thermo Fisher Scientific Inc. Other chemicals were from Fluka, Panreac, Sigma, Amresco and Serva and were at least of reagent grade. In all solutions ultrapure water was used. To avoid calcium contamination of protein samples only plastics or quartz ware were used instead of glassware.

The concentration of water stock solution of 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) was evaluated using molar extinction coefficient ε_{385nm} of 16,790 $M^{-1}cm^{-1}$ [45].

Construction of Recombinant Clones

To obtain bovine NCS1 gene total mRNA was purified from *Bos taurus* brain tissue. Reverse transcription and PCR amplification were performed using two specific oligonucleotide primers, containing restriction endonucleases sites *HindIII* and *NdeI* (underlined) correspondingly: 5'-GGGCATATGGGGAAATCCAACAGCAAGTTG-3' and 5'-GGGAAGCTTATACCAGCCCGTCGTAGAGGG-3'. To obtain wild-type NCS1 (*wt*NCS1) the resulting amplicon was cloned to pET-22b(+) expression vector for *Escherichia coli* using the above-mentioned restriction endonucleases. GST-NCS1 fusion protein was obtained by sub-cloning of NCS1 gene into the pGEX-5x-1 expression vector as previously described [17, 46, 47].

The chimeric construct "NR" representing NCS1 in which the residues downstream D176 were exchanged with K180-L202 sequence of recoverin (Fig. **4A**) was obtained on the basis of the *wt*NCS1 gene and previously described recoverin-encoding plasmid pET-11d [48]. The chimeric gene was constructed by overlap PCR [49], using NCS1-specific forward primer 5'-GGGCATATGGGGAAATCC AACAGCAAGTTG-3', recoverin-specific reverse primer 5'-GGGCCATGGTCAGAGTTTCTTTTCCTTCAG-3' and overlap forward primer 5'- GAAGGCTCCAAGGCTGACA AGGAAATTCTGCGACTGCG-3'. The resulting PCR product was cloned into pET-22b(+) vector using *HindIII* and *NcoI*.

Plasmid encoding GST-tagged $GRK1^{1-25}$ fragment was described previously [46].

All recombinant DNA constructs were verified by automated sequencing.

Protein Expression and Purification

Recombinant myristoylated NCS1 and recoverin were obtained according to the previously developed protocol [17, 50]. NCS1, NR and wild-type recoverin concentrations were measured spectrophotometrically by Bradford assay [51] or using molar extinction coefficients at 280 nm of 21,430 M^{-1} cm^{-1}, 20,065 $M^{-1}\cdot cm^{-1}$ and 24,075 $M^{-1}\cdot cm^{-1}$, respectively [17, 52]. The degree of NCS1 myristoylation exceeded 95% according to analytical high-performance liquid chromatography [17]. N-terminally GST-tagged NCS1 was expressed in BL21(DE3)CodonPlus *E. coli* cells for 4 h at 37°C and was purified from bacterial lysate by glutathione affinity chromatography [47]. Myristoylated NR was generated by co-expression with N-myristoyl transferase from yeast in BL21(DE3) CodonPlus *E. coli* cells for 4 h at 37°C. NR was obtained using hydrophobic chromatography as previously developed for NCS1 purification, which was modified due to altered hydrophobic properties of the chimera (see Fig. **6**). The bacterial cytosol extract was adjusted to 2 mM ethylene glycol tetraacetic acid (EGTA) and was applied to a phenyl sepharose column equilibrated with 20 mM tris-(hydroxymethyl)-aminomethane (Tris)-HCl buffer (pH 8.0), containing 1 mM dithiothreithol (DTT) and 2 mM EGTA. The elution of NR was performed with the same buffer which instead of EGTA contained 4 mM Mg^{2+}.

GRK1 was extracted from bovine rod outer segment preparations as described previously [53]. GST-$GRK1^{M1-S25}$ expression and purification were performed according to the previously published procedure [46].

Preparation of Antibodies

Polyclonal (monospecific) antibodies against wild type NCS1 were generated by rabbit immunization and purified according to the standard procedure [54].

Preparation of Retinal and ROS Extracts

For the extraction of cytosol and membrane-anchored retinal proteins, bovine retinas frozen at 70°C were thawed in 1 ml/retina volume of a buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM ethylene diamine tetraacetic acid, 1 mM phenylmethanesulfonyl fluoride, and incubated for 1-2 h at 4°C upon stirring. The resulting suspension was centrifuged at 27000 g. The pellet was resuspended in 1 initial suspension volume of the above-mentioned buffer, containing 250 mM NaCl. The extraction was performed on ice for 1 h and followed by centrifugation at 27000 g. Both supernatants were mixed and adjusted to 3 mM $CaCl_2$. ROS extract was obtained similarly from isolated ROS, prepared from frozen bovine retinas as described previously [55].

Affinity Purification of NCS1-Interacting Cytosol Proteins from Retinal Extract

The purification of proteins, which associate with NCS1 in a Ca^{2+}-dependent manner, from retinal cytosol extract was carried out by previously described optimized approach [28]. Ten micrograms of either GST-NCS1 or GST was immobilized on glutathione sepharose beads by 2 h incubation at 4 °C with gentle stirring. The beads were then washed 5 times using 10 ml of a buffer containing 20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM DTT to remove the non-bound protein. The last supernatant was removed and 10 ml of previously obtained retinal cytosol extract was applied to both NCS1 and GST (control) coupled to sepharose. The suspension was incubated overnight at 4°C and washed 5 times with the above-mentioned buffer. The bound protein was then eluted with buffer containing 20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 1 mM DTT and 5 mM EGTA.

Mass-Spectrometry

The proteins from the eluted Ca^{2+}-free fraction were separated by polyacrylamide/sodium dodecyl-sulphate gel electrophoresis (SDS-PAGE). The gel was stained by Coomassie Brilliant Blue R250, and the protein band corresponding to GRK1 (~67 kDa) was excised from the gel and washed with 200 µl 40% acetonitrile/0.1 M NH_4HCO_3 for 20 min at 37°C to remove the staining. The gel fragments were then dehydrated in 100 µl acetonitrile. After the acetonitrile was removed, 4 µl of buffer containing 15 µg/mL modified trypsin (Promega) in 0.05 M NH_4HCO_3 was applied to the gel. The hydrolysis was performed at 37°C overnight. The resulting peptides were purified by addition of 8 µl of 0.5% trifluoroacetic acid in 10% acetonitrile, thorough mixing and brief centrifugation. Two microliters of each supernatant was applied to a matrix-assisted laser desorption/ionization (MALDI) target plate, mixed with 0.5 µl of 20 mg/ml 2,5-dihidroxybenzoic acid in 20% acetonitrile/0.5% trifluoroacetic acid and air-dried. Peptide mass fingerprints were measured using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)/TOF mass spectrometer (UltrafleXtreme Bruker Daltonics, Germany), equipped with Nd ultraviolet laser in positive-ion mode with a reflector. Monoisotopic masses were measured in 700-4500 m/z diapason with a peptide tolerance of 50 ppm. To analyze mass spectra FlexAnalysis 3.3 software (Bruker Daltonics, Germany) was used. GRK1 identification based on mass fingerprints of digestion-generated peptides was performed by searching the NCBI protein database using MASCOT search software (Matrix Science) for tryptic peptides.

Fluorescence Measurements

Fluorescence measurements were carried out using a Cary Eclipse spectrofluorimeter (Varian Inc.) with a Peltier-controlled cell holder and quartz cells. Buffer conditions: pH 7.3, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH, 100 mM KCl, 1 mM DTT and either 1 mM EGTA (apo-form), 1 mM $CaCl_2$ (Ca^{2+}-bound form) or 1 mM $MgCl_2$ and 1 mM EGTA (Mg^{2+}-bound form). Protein and bis-ANS concentrations were 5-21 µM and 1.2 µM, respectively. Protein and bis-ANS fluorescence were excited at 280 nm and 385 nm, respectively. LogNormal software (IBI RAS, Pushchino) was used to correct and lognormally fit all spectra for spectral sensitivity of the instrument [56]. The positions of the fluorescence spectrum maxima (λ_{max}) were obtained from these fits.

To perform spectrofluorimetric temperature scans a stepwise manner permitting the sample to equilibrate at each temperature was used [57]. The average heating rate was 0.5°C/min. Temperature was controlled directly inside the cuvette using a Cary temperature probe (Varian Inc.). The cell compartment was purged with dry nitrogen to avoid condensation of water vapor at temperatures below room temperature. The fraction of conversion from the native to the thermally denatured protein state was calculated as reported previously [58].

GRK1 Assay

GRK1 activity in the presence of NCS proteins was assayed using as readout rhodopsin phosphorylation according to the previously described procedure, with modifications [47, 59]. The assay mixture contained 50 µM recoverin, NCS1 or NR, 10 µM rhodopsin (urea-washed photoreceptor membranes) and 0.3-0.5 units of GRK1. The kinase activity was assayed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM [γ-^{32}P]ATP, 1 mM DTT, 3 mM $MgCl_2$, containing either 250 µM $CaCl_2$ or 10 µM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid bromine salt and 0.3 µM $CaCl_2$ (50 nM free Ca^{2+} buffer). Free Ca^{2+}-concentration in the buffers was calculated using Webmaxc Standard software (Stanford University). The reaction (15 min) was initiated by light illumination and terminated by addition of sample buffer for SDS-PAGE. The proteins were separated by polyacrylamide gel electrophoresis and γ-^{32}P emission was registered by phosphorimaging radioautography.

GRK1$^{1-25}$-Binding Assay

Interaction of NCS1 with GRK1$^{1-25}$ was tested using analytical affinity chromatography (pull-down assay) [46]. A 50 µg amount (in 50 µl) of the GST-tagged GRK1$^{1-25}$ fragment was immobilized on glutathione sepharose beads in 20 mM Tris-HCl buffer (pH 8.0). The beads were washed 3 times with wash buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT and 0.05% Tween 20) to remove the non-bound GRK1 fragment. Next, 100 µM of NCS1 or recoverin was applied to the washed pellet. The suspension was incubated for 1 h at 4°C in the above-mentioned buffer with addition of either 2 mM Ca^{2+} and 2 mM Mg^{2+} or 5 mM EGTA. The non-bound protein was removed with wash buffer. Bound proteins were eluted by SDS-PAGE sample buffer and analyzed by SDS-PAGE.

Photoreceptor Membrane Binding Assay

To obtain photoreceptor membranes, cytosol and membrane-associated proteins were removed from isolated ROS by washing with 5 M urea [60]. The binding of proteins to urea-washed photoreceptor membranes was performed according to a procedure developed previously for recoverin [17, 50, 61]. Briefly, 10 µl of ROS membranes were incubated for 15 min (Eppendorf Thermomixer, 1200

rev./min) in 50 µl of a solution containing 40 µM NCS, 20 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$ and either 2 mM $CaCl_2$ or 5 mM EGTA. The mixture was centrifuged at 14500g for 20 min and the membrane pellets were recovered and dissolved in 50 µl of sample buffer for SDS-PAGE. To assess binding reversibility the membrane pellets, containing bound Ca^{2+}-loaded proteins, were additionally incubated in 50 µl of solution containing 20 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$ and 5 mM EGTA for 15 min. The supernatant was mixed with 25 µl of SDS-PAGE sample buffer and the membrane pellet resuspended in 75 µl of the same buffer. The obtained protein fractions were analyzed by SDS-PAGE. Binding was estimated by quantitative densitometry of Coomassie-stained protein bands, using GelAnalyzer v.2010a software.

RESULTS

The initial aim of the present study was to determine a role of the NCS1 C-terminal segment in Ca^{2+}-myristoyl switch-dependent properties of the protein such as membrane binding and regulation of GRKs. Since NCS1 was previously identified in bovine photoreceptors by immunohistochemistry [44], we focused on the activity of the protein in these neurons. In particular, the G protein-coupled receptor (GPCR) rhodopsin/GRK1 tandem was chosen as a potential experimental model for examination of an impact of the C-terminal segment on Ca^{2+}-dependent regulatory activity of NCS1. The model has the following advantages: (i) rhodopsin phosphorylation by GRK1 is triggered by light, (ii) all components of the reaction could be easily purified from retinal tissue; (iii) rhodopsin yield in photoreceptor membrane preparations is remarkably high in comparison with GPCRs in other cell types (over 80% of total membrane protein). Yet, prior to utilizing this experimental model for the intended aims it was necessary to confirm the presence of NCS1 in retinal and ROS extracts and to verify the capability of the protein to recognize GRK1 within these extracts and to regulate the activity of the enzyme *in vitro*.

Phosphorylation of Rhodopsin by GRK1 as a Ca^{2+}/NCS1-Regulated Process

Immunoblotting using polyclonal antibodies against recombinant *wt*NCS1 revealed positive reaction both in retinal and ROS extracts (Fig. **1**). To verify whether NCS1 is capable of recognizing GRK1 within these extracts we applied a procedure previously developed for the proteomic identification of NCS1-interacting proteins [28]. The NCS1 gene was cloned into the bacterial expression vector pGEX-5x-1 to obtain N-terminally GST-tagged NCS1 chimera (GST-NCS1). Using glutathione sepharose pull-down assay, the fraction of photoreceptor proteins capable of interacting with GST-NCS1 in a Ca^{2+}-dependent manner was obtained. Indeed, this fraction was immunopositive for GRK1 (Fig. **2A**). The presence of GRK1 in the fraction of NCS1-interacting proteins was further confirmed by MALDI-TOF mass spectrometry (Fig. **2B**).

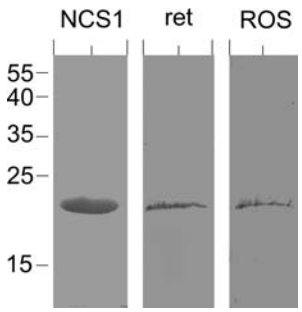

Fig. (1). Detection of NCS1 in retinal extracts. Western blotting of bovine retinal extract (”ret”) and isolated bovine rod outer segments extract (“ROS”) using polyclonal anti-NCS1 antibodies. The purified recombinant NCS1 is used as a control (“NCS1”).

The effect of NCS1 on GRK1 activity was then examined in a rhodopsin phosphorylation assay conducted in the reconstructed photoreceptor system that included rhodopsin-containing photoreceptor membranes, purified GRK1 and *wt*NCS1. In the presence of saturating Ca^{2+}-concentration NCS1 inhibited GRK1 activity by 75% i.e. almost as efficiently as the well-known GRK1 modulator recoverin (Fig. **3A**). Notably, in Ca^{2+}-free/Mg^{2+} conditions NCS1 also demonstrated a moderate suppressing effect on GRK1 activity. Recoverin was previously shown to inhibit GRK1 *via* interaction with N-terminal 1-25 portion of the enzyme [11, 46]. Using pull-down experiments with immobilized GST-$GRK1^{1-25}$ construct we demonstrated that NCS1 is also capable of binding to this GRK1 fragment (Fig. **3D**), suggesting that enzyme inhibition occurs in a recoverin-like manner.

Association with cellular membranes was shown to be crucial for NCS1 compartmentalization with most of signaling partners including GRKs. To investigate Ca^{2+}-dependent binding of NCS1 to photoreceptor membranes we applied equilibrium centrifugation assay which was modified in order to monitor the reversibility of membrane binding this is important in the case of NCS1. In the presence of Ca^{2+} NCS1 entered the photoreceptor membrane fraction only ~1.8 times less efficiently than recoverin (Fig. **3B**). Yet, in contrast to recoverin, a significant NCS1 pool was observed in association with membranes in Ca^{2+}-free/Mg^{2+} conditions. To assess irreversibility of this interaction, membrane-bound Ca^{2+}-NCS1 and Ca^{2+}-recoverin were additionally incubated in a buffer containing Ca^{2+}-chelator. It was revealed that only one-half of membrane-bound NCS1 was eluted into the soluble fraction by addition of 5 mM EGTA, while 83% of recoverin dissociated from the membranes under these conditions (Fig. **3C**). Thus, NCS1 interaction with photoreceptor membranes has low Ca^{2+}-sensitivity and occurs in a partially irreversible manner, i.e. in a similar fashion to what was observed in previous studies [15, 38, 40].

In summary, our data indicate that NCS1 maintains its common activity profile during interaction with photoreceptor membranes and regulation of rhodopsin phosphorylation by GRK1. For this reason this photoreceptor-derived system could be used as an experimental model for examining the regulatory role of the C-terminal segment of NCS1 in its general membrane-binding and GRK modulating mechanisms.

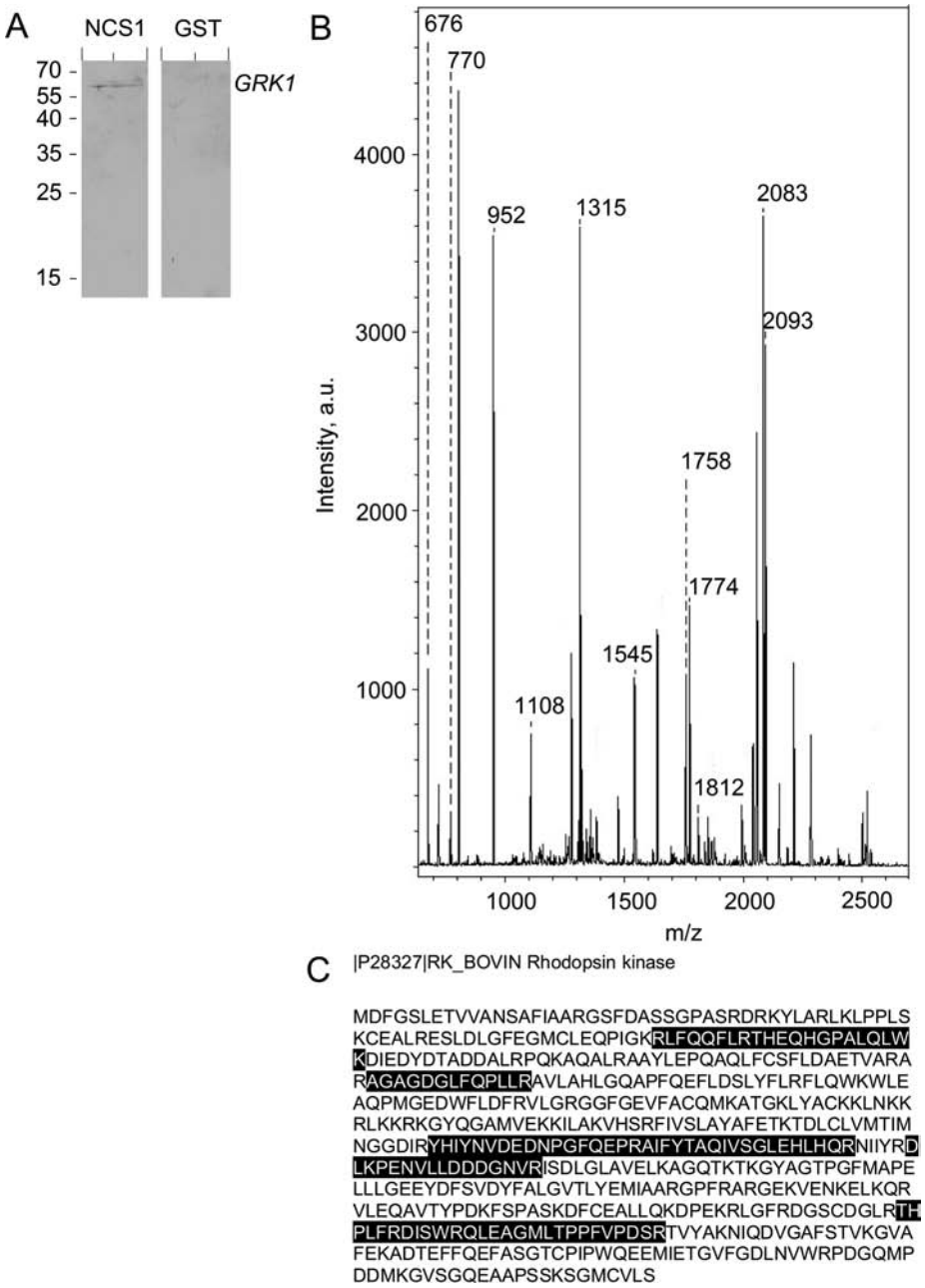

Fig. (2). Identification of GRK1 as a bovine retinal extract protein capable of Ca^{2+}-dependent interaction with NCS1. (**A**) Western blotting of GRK1 in the fraction of the retinal proteins interacting with GST-NCS1 or GST (control) in Ca^{2+}-dependent manner. (**B**) MALDI-TOF mass-spectrometry analysis of the protein with apparent molecular weight of 67 kDa which interacts with immobilized GST-NCS1 in the presence of Ca^{2+}. The peaks of the peptides, matching GRK1 sequence, are labeled with corresponding m/z value. The matched peptides in GRK1 primary structure are shown in black boxes.

Design of NCS1-Recoverin C-Terminal Chimera (NR) and Characterization of its Structural Stability

Previous studies of the role of the C-terminal segment of NCS proteins relied on characterization of their C-terminally truncated forms [16-18]. However, this approach is not suited for studies of NCS1 since its C-terminal truncation leads to global structural deficiency of the protein [18]. Therefore, to get an insight into the functional role of the C-terminal segment of NCS1 we created a genetic construct encoding bovine NCS1, in which its C-terminal fragment beginning from D176 residue was replaced by C-terminal segment K180-L202 of bovine recoverin (Fig. **4A**). The resulting "NR" construct was co-expressed in *E. coli* with N-myristoyl transferase from yeast. NR was purified from bacterial lysates using hydrophobic chromatography developed previously for NCS1 and recoverin, which was modified due to the altered hydrophobic properties of the chimera (see Fig. **6**). Structural integrity of the NR chimera was examined by its tryptophan fluorescence (Fig. **4B-D**). Temperature dependencies of fluorescence spectrum maximum position (λ_{max}) of NCS1 and NR revealed a pronounced red shift at elevated temperatures reflecting

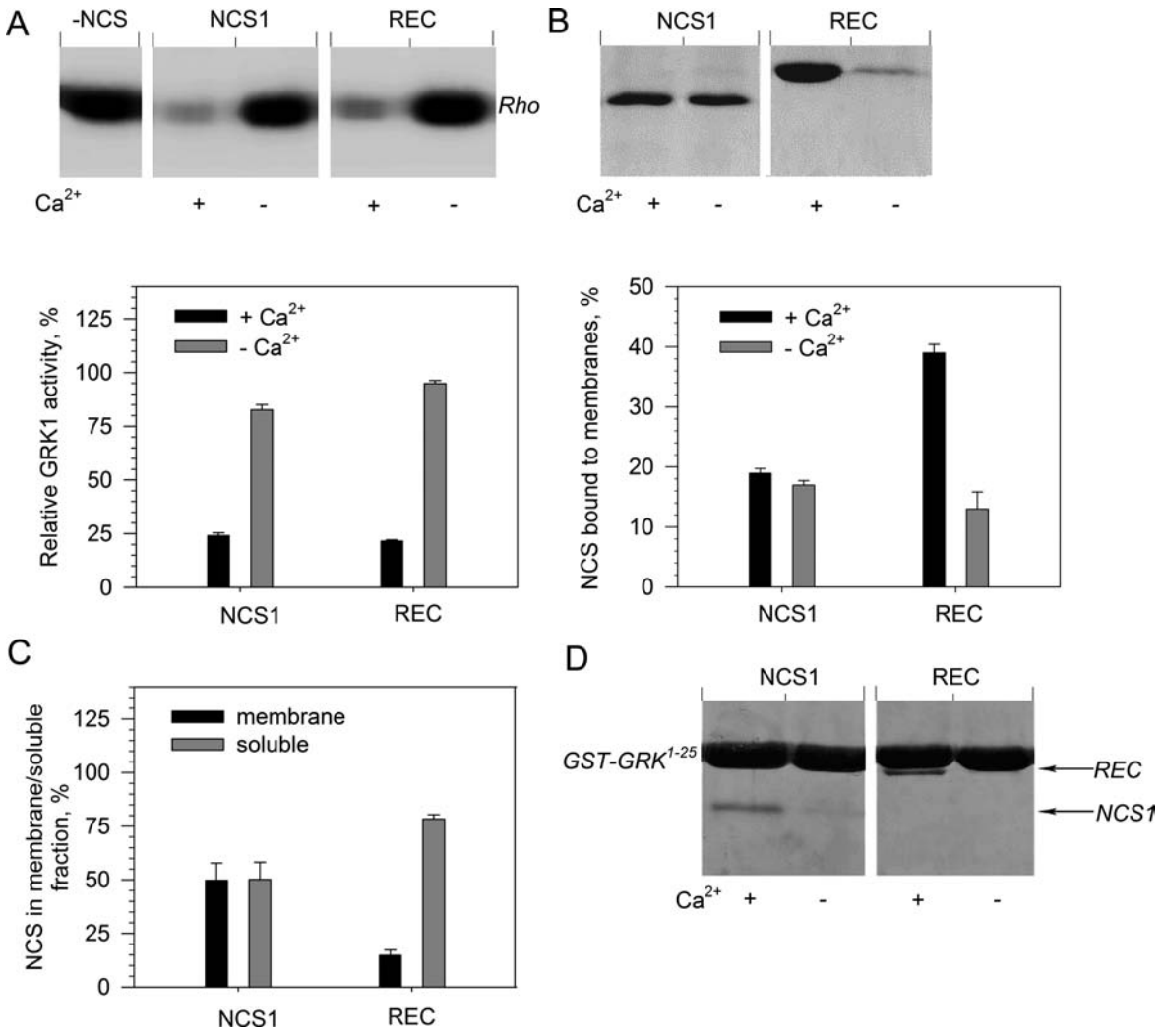

Fig. (3). Functional characteristics of photoreceptor NCS1. (**A**) Ca^{2+}-dependent GRK1 inhibition. Rhodopsin phosphorylation by GRK1 in the presence of 50 μM NCS1 or recoverin at high Ca^{2+} (250 μM) or at low Ca^{2+} (50 nM) concentration was visualized by ^{32}P-radioautography. The "-NCS" lane represents phosphorylated rhodopsin in the absence of NCS proteins. The relative GRK1 activity was estimated from the data obtained for at least three independent experiments. (**B**) Binding to photoreceptor membranes. NCS1 or recoverin were incubated with photoreceptor membranes in the presence of either 2 mM Ca^{2+} or 5 mM EGTA, the membranes were precipitated and the pellet was analyzed by SDS-PAGE. Weight fractions of membrane-bound proteins were estimated from the data obtained for at least three independent experiments. (**C**) Reversibility of NCS1 and recoverin membrane association. The membrane pellets, containing bound Ca^{2+}-loaded NCS1 and recoverin were additionally incubated in 5 mM EGTA, membranes were precipitated and the resulting supernatants and membrane pellets were analyzed by SDS-PAGE. Weight fractions of the membrane-bound and soluble proteins were estimated from the data obtained for at least three independent experiments. (**D**) Ca^{2+}-dependent interaction with GST-GRK1$^{1-25}$. NCS1 or recoverin co-precipitated with immobilized GST-GRK1$^{1-25}$ in Ca^{2+}-dependent manner were visualized by SDS-PAGE.

thermally-induced protein unfolding. Thermal stability of NR is very close to that for NCS1, with difference in the mid-transition temperatures not exceeding 5°C at least for apo- and Mg^{2+}-bound forms of the proteins (see Table **1** and Fig. **4D**). The metal-binding-induced increase in thermal stability of NR chimera was analogous to that for NCS1, indicating that NR retains metal-binding capability. Meanwhile, the 2-3 nm blue shift with regard to NCS1 fluorescent spectra of Mg^{2+}- and Ca^{2+}-bound forms of NR at temperatures below 30°C (Fig. **4C**, **D**) is indicative of a less polar and mobile environment of its Trp residues, consistent with their partial burial in the hydrophobic interior of the protein.

Summing up, replacement of the C-terminal segment of NCS1 with the corresponding C-terminal region of recoverin does not affect structural stability of NCS1, but leads to limited changes in the local environment of its Trp residues. Overall, the NR construct can be considered suitable for further functional tests.

Membrane-Binding and GRK1 Regulation by NCS1-Recoverin C-Terminal Chimeric Protein (NR)

One of the main features of NCS1 is the characteristic operation of its Ca^{2+}-myristoyl switch mechanism that provides membrane association and target recognition ability

Table 1. Mid-transition temperatures ($T_{1/2}$,°C) for thermal unfolding of apo-, Mg^{2+}- and Ca^{2+}-loaded forms of *wt*NCS1 and NCS1-recoverin chimera, estimated from the experiments shown in Fig. (4B, D) according to ref [58].

Protein Form	$T_{1/2}$, °C	
	NCS1	NR
Apo-form	39	39
Mg^{2+}-bound form	60	56
Ca^{2+}-bound form	n.d.*	84

*Not determined due to high thermal stability of the protein.

of the protein [15]. In NCS1, the myristoyl group is reversibly exposed in a Ca^{2+}-dependent manner in solution [15, 18] and, in the presence of phospholipid membranes, becomes permanently embedded in the membrane [38]. This effect results in partially irreversible interaction of the protein with membrane structures (see Fig. **3B**, **C**), which is commonly observed in different models [15, 38, 40]. Our next goal was to study the role of the C-terminal segment in such a distinctive membrane association mechanism of NSC1. Using a modified equilibrium centrifugation assay (see hereinbefore), Ca^{2+}-bound NR was found to interact with urea-washed photoreceptor membranes although its membrane affinity was ~1.3 times lower comparing to NCS1 (Fig. **5A**). Under Ca^{2+}-chelating conditions more than 85% of membrane-bound NR passes into the soluble fraction, which contrasts to NCS1 behavior and somewhat resembles the membrane binding profile of recoverin (Fig. **5B**). Thus, replacement of the C-terminal segment of NCS1 with the corresponding fragment of recoverin results in decreased membrane affinity and complete loss of irreversibility of membrane binding of the protein, suggesting a role of this segment in promoting Ca^{2+}-independent membrane localization of NCS1.

Another common feature of Ca^{2+}-myristoyl switch of NCS proteins is Ca^{2+}-induced exposure of the unique set of previously masked hydrophobic residues that comprise a specific site for binding of target proteins such as GRKs. To get insight into the role of the NCS1 C-terminal segment in modulation of GRKs we monitored Ca^{2+}-dependent regulatory activity of NR in phosphorylation of rhodopsin by GRK1, using the above described reconstructed system. Since the C-terminal segment of recoverin was shown to stabilize the protein complex with GRK1 [17], it was logical to assume that its introduction into NCS1 would improve enzyme inhibition. However, no stabilizing effect of C-terminal replacement on NCS1 activity towards GRK1 was found. Instead, in the presence of calcium NR exerted a 2-fold reduction of the GRK1 inhibitory effect in comparison to NCS1 (Fig. **5C**). In addition, the chimera acquired the ability to inhibit the enzyme by 37% under Ca^{2+}-free conditions.

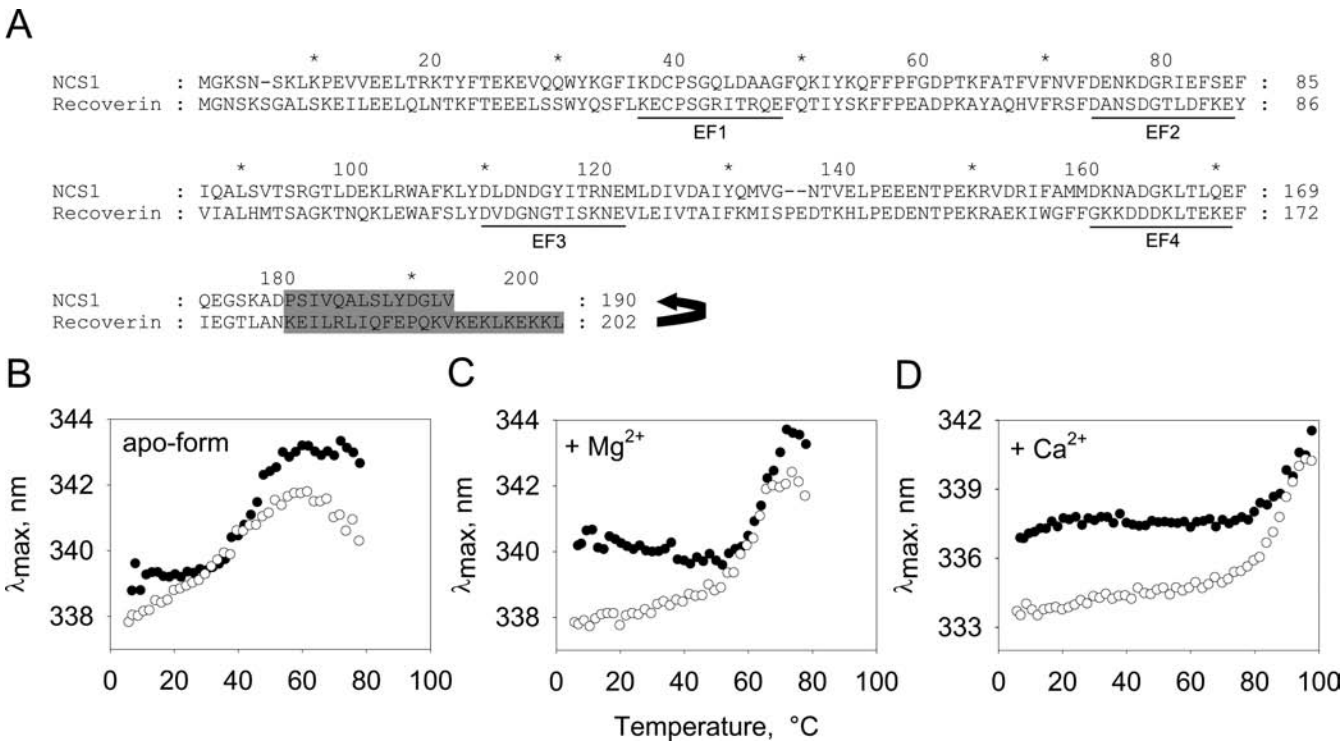

Fig. (4). Impact of replacing the C-terminal segment on NCS1 thermal stability. (**A**) Design of NCS1-recoverin C-terminal chimera ("NR"). Alignment of bovine NCS1 and recoverin was performed using Needleman-Wunsch alignment algorithm [79]. EF-hand loops are underlined; the C-terminal segments exchanged to obtain NR chimera are indicated in gray. (**B-D**) Thermal unfolding of NCS1 (*solid circles*) and NR chimera (*open circles*) was monitored by their tryptophan fluorescence spectrum maximum positions (λ_{max}). Buffer conditions: pH 7.3, 10 mM HEPES-KOH, 100 mM KCl, 1 mM DTT and either 1 mM EGTA (**B**), 1 mM $MgCl_2$ and 1 mM EGTA (**C**), or 1 mM $CaCl_2$ (**D**). Protein concentration was 14-21 μM. Excitation wavelength was 280 nm.

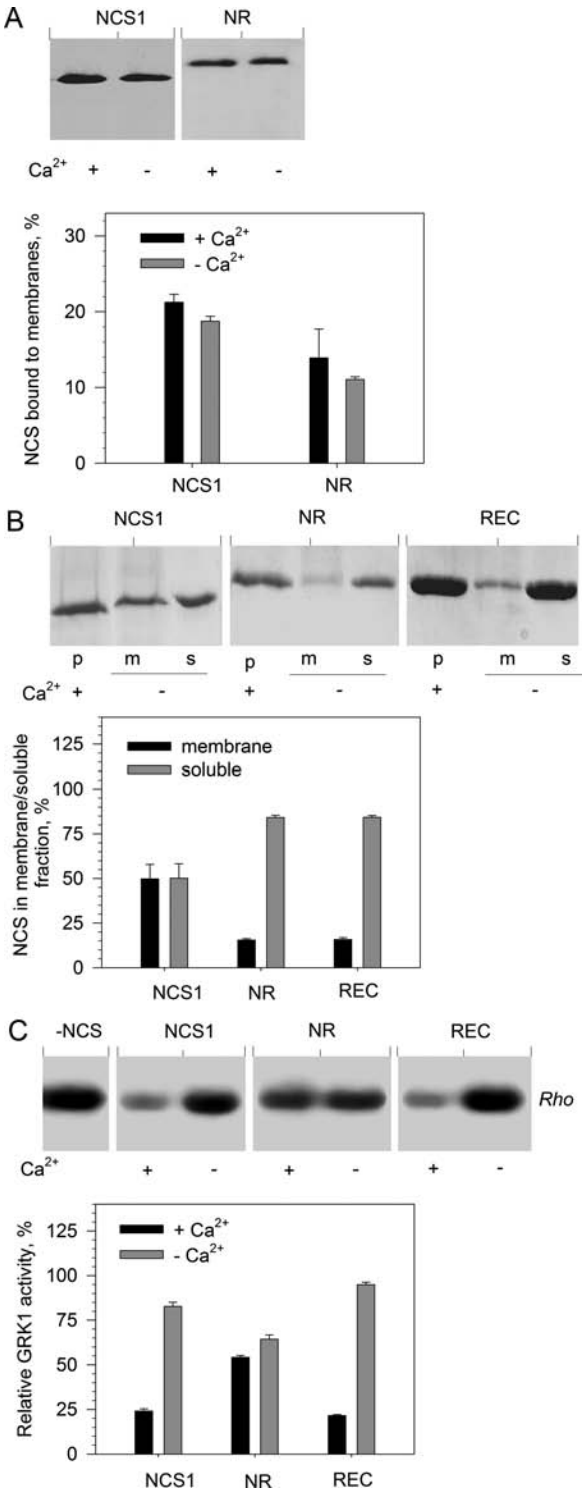

Fig. (5). Impact of C-terminal segment replacement on NCS1 association with photoreceptor membranes and GRK1 regulation. (**A**) Binding to photoreceptor membranes. NCS1 or NR were incubated with photoreceptor membranes in the presence of either 2 mM Ca^{2+} or 5 mM EGTA, the membranes were precipitated and the pellet was analyzed by SDS-PAGE. Weight fractions of membrane-bound proteins were estimated from the data obtained from at least three independent experiments. (**B**) Reversibility of NR membrane association. The membrane pellets, containing bound Ca^{2+}-loaded NCS1, NR or recoverin ("p"), were additionally incubated in 5 mM EGTA, membranes were precipitated and the resulting supernatants ("s") and membrane pellets ("m") were analyzed by SDS-PAGE. Weight fractions of the membrane-bound and soluble proteins were estimated from the data obtained for at least three independent experiments. (**C**) Ca^{2+}-dependent GRK1 inhibition. Rhodopsin phosphorylation by GRK1 in the presence of 50 μM NCS1, NR or recoverin at high Ca^{2+} (250 μM) or at low Ca^{2+} (50 nM) concentration was visualized by ^{32}P-radioautography. The "-NCS" lane represents phosphorylated rhodopsin in the absence of NCS proteins. The relative GRK1 activity was estimated from the data obtained from at least three independent experiments.

Taken together, these data indicate that replacement of the C-terminal segment of NCS1 with the corresponding region of recoverin provides NCS1 with recoverin-like reversible Ca^{2+}-myristoyl switch, but disturbs to some degree its ability to regulate GRK1 in a Ca^{2+}-dependent manner.

Probing of Surface Hydrophobic Sites in NR Chimera

The functional properties of NCS proteins are generally governed by solvent accessibility of their hydrophobic sites, used for both myristoyl binding and target recognition. The presence of the hydrophobic sites are potentially able to accommodate hydrophobic regions of respective target molecules can be monitored using fluorescent dyes such as ANS and bis-ANS (for review, see [62]). ANS probing is commonly applied for examination of putative surface hydrophobic sites of NCSs [37, 63, 64]. A pronounced increase in fluorescence quantum yield of bis-ANS/ANS, along with a blue shift of its fluorescence emission spectrum is detected upon noncovalent interaction of the dye with protein hydrophobic surfaces. To gain an insight into the structural rearrangements accompanying the C-terminal replacement in NCS1, Mg^{2+}- and Ca^{2+}-bound forms of NCS1, NR and recoverin were probed by bis-ANS fluorescence (Fig. **6**). The apo-forms of the proteins were not analyzed, since NCS1 is considered to be either Mg^{2+}- or Ca^{2+}-associated under physiological conditions [37]. The Mg^{2+}-bound NCS1 exhibits the lowest affinity to bis-ANS, while recoverin and NR are much more hydrophobic (Fig. **6A**). The fluorescence intensity of bis-ANS increases upon Ca^{2+}-binding to recoverin, which likely reflects an exposure of its N-terminal hydrophobic groove [64, 65] (Fig. **6B**). The same effect is less pronounced for NCS1, consistent with the previous observation that the exposed hydrophobic groove of Ca^{2+}-loaded NCS1 is occupied by its C-terminal segment [18]. The replacement of the C-terminal segment of NCS1 with the respective recoverin region increases surface hydrophobicity of the Ca^{2+}-bound protein (Fig. **6B**), suggesting that the C-terminal fragment of recoverin does not shield the hydrophobic groove within the NR chimera. Based on these observations, one may conclude that the C-terminal segment of NCS1 affects exposure of its hydrophobic sites, potentially important for myristoyl binding and GRK recognition. Thereby the data presented point out a key role of the C-terminal segment of NCS1 in maintenance of its functional status.

DISCUSSION

NCS1 is a Putative Modulator of Photoreceptor GRKs

In the current study we investigated a role of the C-terminal segment in NCS1 focusing on regulatory activity of the protein in retinal photoreceptor cells. Photoreceptor neurons represent a highly differentiated sensor system built around visual receptor rhodopsin. The receptor is incorporated in packed membrane disks (photoreceptor membranes) folded within the outer segment of the rod cell (ROS) [66]. Consequently, components of the visual cascade are presumably membrane-associated proteins. Alterations of Ca^{2+} levels in response to light stimuli trigger regulatory feedback performed by NCS proteins recoverin and guanylate cyclase activating proteins [67, 68]. Recoverin is implicated in modulation of rhodopsin desensitization by inhibiting rhodopsin kinase (GRK1) at high calcium and releasing the enzyme at low calcium levels *in vitro* and *in vivo* [60, 69]. The regulation of membrane-bound GRK1 by recoverin is governed by Ca^{2+}-myristoyl switch of the latter that provides reversible Ca^{2+}-dependent interaction of the protein with photoreceptor membranes [10, 50]. NCS1 represents another Ca^{2+}-myristoyl switch NCS protein previously identified by immunohistochemistry in inner segments and synaptic terminals of photoreceptor neurons [44]. Consistently we have identified NCS1 in bovine retinal extracts by immunoblotting (Fig. **1**). Interestingly, the ROS extract was also immunopositive for NCS1, although ROSs were not stained by anti-NCS1 antibodies in the retinal sections [44]. Furthermore we demonstrated that NCS1 binds to isolated photoreceptor membranes and the binding is weakly sensitive to Ca^{2+}, i.e. occurs a in similar fashion to that observed in the case of membranes of the other cell types [15, 38]. Prior to the current study, the function of NCS1 in photoreceptor cells remained unclear, although there were indications that it can regulate GRKs including GRK1 [24, 30]. Using a previously developed proteomic approach [28] we found that NCS1 is indeed capable of recognizing GRK1 in bovine retinal extract (Fig. **2**). Furthermore, we showed that NCS1 can inhibit GRK1 in a recoverin-like manner, i.e. *via* interaction with the enzyme's N-terminal recoverin-binding site (Fig. **3A**, **D**) [11, 46]. Although the exact patterns of co-localization of NCS1 with GRK1 in photoreceptor cells remain to be determined, their interaction can be suggested to occur in photoreceptor inner segments. Thus, GRK1 is expressed in the inner segments

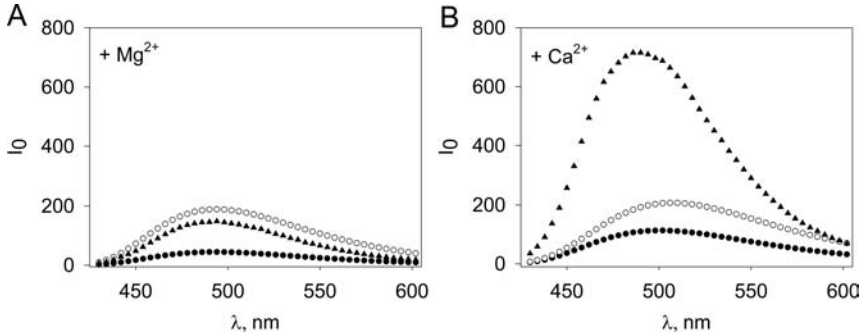

Fig. (6). Impact of replacing the C-terminal segment of NCS1 on its interaction with the hydrophobic fluorescent probe bis-ANS at 20-25°C. Fluorescence emission of 1.2 μM bis-ANS in the presence of 4.8-5.0 μM of *wt*NCS1 (*solid circles*), NR (*open circles*) or recoverin (*triangles*) was excited at 385 nm. Buffer conditions: pH 7.3, 10 mM HEPES-KOH, 100 mM KCl, 1 mM DTT and either 1 mM $MgCl_2$ and 1 mM EGTA (**A**), or 1 mM $CaCl_2$ (**B**).

and subsequently transported to the outer segment by prenyl-binding protein δ [70]. Along with newly synthesized rhodopsin, the inner segment contains the other GPCRs that are known to undergo phosphorylation by GRK1 at least under *in vitro* conditions [71]. Since the proven GRK1 regulator recoverin is likely to remain inactive in the inner segment due to its low calcium sensitivity [72], we suggest that NCS1 might acts as an inhibitor of undesirable phosphorylation of GPCR(s) by GRK1 in this compartment. It is also possible that photoreceptor NCS1, being a multifunctional protein, regulates some other targets including GRKs. For instance, the well-known NCS1 target GRK2 has been detected in photoreceptor inner segments [73]. Nevertheless, phosphorylation of rhodopsin by GRK1 clearly represents a Ca^{2+}/NCS1-regulated process and therefore was chosen as a convenient experimental model for the *in vitro* study of an impact of the C-terminal segment on Ca^{2+}-dependent regulatory activity of NCS1.

Conformational Dynamics of the C-Terminal Segment Regulate Interaction of NCS1 with Photoreceptor Membranes

To investigate a role of the C-terminal segment in NCS1 we engineered a chimeric protein NR representing NCS1 with the C-terminal segment replaced by the corresponding amino acid sequence of recoverin. Previous studies revealed that truncation of the C-terminal segment of NCS1 leads to drastic conformational destabilization of the protein [18]. By contrast, NR does not significantly differ from *wt*NCS1 in thermal stability, indicating that the C-terminal segment of recoverin can substitute the corresponding region of NCS1. This observation is in agreement with the fact that NR retains the ability to bind Ca^{2+} and Mg^{2+} as seen in our fluorescence studies (Fig. **4B-D**).

The first major difference between NCS1 and NR is the quite distinct Ca^{2+}-dependent membrane binding profiles of the proteins. In general, under *in vitro* conditions NCS1 interacts with membranes in a partially irreversible manner, and was considered until recently as a neuronal calcium sensor without functional Ca^{2+}-myristoyl switch [38]. The myristoyl group is necessary for NCS1 membrane association [39] and therefore was suggested to be exposed both in the Ca^{2+}-bound and Ca^{2+}-free forms of the protein. In addition, N-terminal alpha-helix of NCS1 contains unique residues that are required for constitutive membrane association of the protein [40]. Meanwhile, NMR studies demonstrated that in solution the myristoyl group of Ca^{2+}-free NCS1 is constrained in the hydrophobic pocket [15]. This contradiction was somewhat explained by experiments on fluorophore-tagged NCS1 in living cells [35]. It was found that NCS1 interaction with membranes at resting Ca^{2+} is a dynamic equilibrium process where NCS1 cycles between cytosol and membrane-bound fractions. By contrast, in Ca^{2+}-saturating conditions NCS1 is irreversibly bound to membranes. Consistently, overstimulation of NCS1 expression in cell lines causes general accumulation of the protein on the membrane surface [74]. In our experiments, association of NR with photoreceptor membranes is weakly sensitive to Ca^{2+}, i.e. it occurs similarly to *wt*NCS1 (Fig. **5A**). At the same time, NR displays reduced membrane affinity as compared to NCS1 both at high and low Ca^{2+} concentration. Furthermore, membrane binding of the chimera becomes fully reversible under Ca^{2+}-chelating conditions, similar to recoverin and other Ca^{2+}-myristoyl switch proteins (Fig. **5B**). These data suggest that the C-terminal segment of NCS1 is involved in the mechanism regulating the position of the myristoyl group in Ca^{2+}-free/Ca^{2+}-bound protein forms, thereby governing affinity and reversibility of association of the protein with membranes.

C-terminal Segment is Involved in Ca^{2+}-Myristoyl Switch of NCS1

Structural mechanisms underlying the observed effects of the C-terminal segment replacement on membrane-binding properties of NCS1 can be suggested considering the available NCS1 atomic-resolution structures [15, 18] and our results of the NCS1/NR bis-ANS fluorescence hydrophobicity probing. In NMR structure of Ca^{2+}-free NCS1 the myristoyl group is buried in the hydrophobic pocket of the C-terminal domain of the protein and the C-terminal segment is positioned on the surface of the molecule covering the pocket (Fig. **7A**). Such a position of the C-terminal segment is stabilized by hydrophobic contacts of its inward-facing non-polar residues with the hydrophobic core and myristoyl group of the protein. Thus, the C-terminal segment of Ca^{2+}-free NCS1 may serve as a gate protecting the hydrophobic pocket, thereby interfering with reuptake of the myristoyl group and making membrane binding poorly reversible upon Ca^{2+}-release. According to our bis-ANS fluorescence studies (Fig. **6A**), Ca^{2+}-free NR is characterized by increased NCS1 surface hydrophobicity. We assume that the C-terminal segment of recoverin in the Ca^{2+}-free chimera fails to shield the hydrophobic pocket of NCS1 and does not impede capturing the myristoyl group. In this case, membrane-bound NR will be able to take up the myristoyl group upon Ca^{2+}-release and shift the equilibrium towards the soluble form of the protein. These observations may explain why NR displays lower membrane affinity in the absence of Ca^{2+} as compared to NCS1 and, when bound to membranes, undergoes dissociation following Ca^{2+} release (Fig. **5A**, **B**).

NCS proteins are generally characterized by the ability to undergo global conformational changes upon Ca^{2+}-binding, leading to exposure of the residues of their hydrophobic pocket [75]. According to our data, Ca^{2+}-bound NCS1 displays much lower accessibility to the hydrophobic dye bis-ANS as compared to recoverin (Fig. **6B**). This could be explained by the fact that the hydrophobic groove formed upon Ca^{2+}-binding is less surface accessible in NCS1 than in recoverin, since it is masked by the C-terminal segment (Fig. **7B**) [18]. In contrast, NR demonstrates an increased surface hydrophobicity of the Ca^{2+}-bound protein, indicating that the C-terminal segment of recoverin is not capable of covering the hydrophobic groove. Previously it was suggested that blocking of the hydrophobic groove by the C-terminal segment similarly occurs in membrane-bound Ca^{2+}-NCS1 when its myristoyl group is inserted into the membrane [18]. Thus, it is the competition between the C-terminal segment and myristoyl group that could provide partially irreversible membrane binding of the protein. We suggest that in NR this competition is disturbed enabling spontaneous myristoyl

group recapture by the hydrophobic groove, thereby decreasing affinity of the Ca^{2+}-bound protein to membranes, as observed in our experiments (Fig. **5A**).

C-Terminal Segment of NCS1 is Crucial for GRK1 Regulation

The above-mentioned speculation may partly explain the second major finding of our study, namely a decreased ability of NR to inhibit GRK1 in the presence of Ca^{2+} as compared to *wt*NCS1. The inhibition occurs in a recoverin-like manner since Ca^{2+}-loaded NCS1 efficiently binds to N-terminal 1-25 peptide of GRK1, most likely *via* conserved NCS amino acid residues of the protein's hydrophobic groove [11, 15]. In Ca^{2+}-NR, the above suggested spontaneous myristoyl group reuptake may then compete with GRK1 binding, thereby suppressing enzyme inhibition (Fig. **5C**). Another possibility is that in the presence of Ca^{2+} the C-terminal segment of NCS1 participates in shaping of a unique GRK1-binding site in the protein. In this case, the replacement of the C-terminal segment alters the target-binding site of the protein, which would result in decreased affinity and inhibitory activity of the chimera towards GRK1. Interestingly, in contrast to recoverin, NCS1 is able to marginally inhibit GRK1 in Ca^{2+}-free conditions and this effect is somewhat enhanced in NR. We suggest that this effect could be associated with the ability of both NCS1 and NR to bind to membranes in the absence of calcium (Fig. **5A**). Yet the exact molecular mechanism of GRK1 regulation by Ca^{2+}-free/Ca^{2+}-bound NCS1 and the role of the C-terminal segment in this interaction remains to be investigated.

C-terminal Segment Targeting is a Prospective Approach for Selective Suppression of the Excessive Activity of NCS1 Associated with Neurological Dysfunctions

As discussed above the Ca^{2+}-myristoyl switch provides two main aspects of NCS activity, namely: membrane association, which is required for their compartmentalization

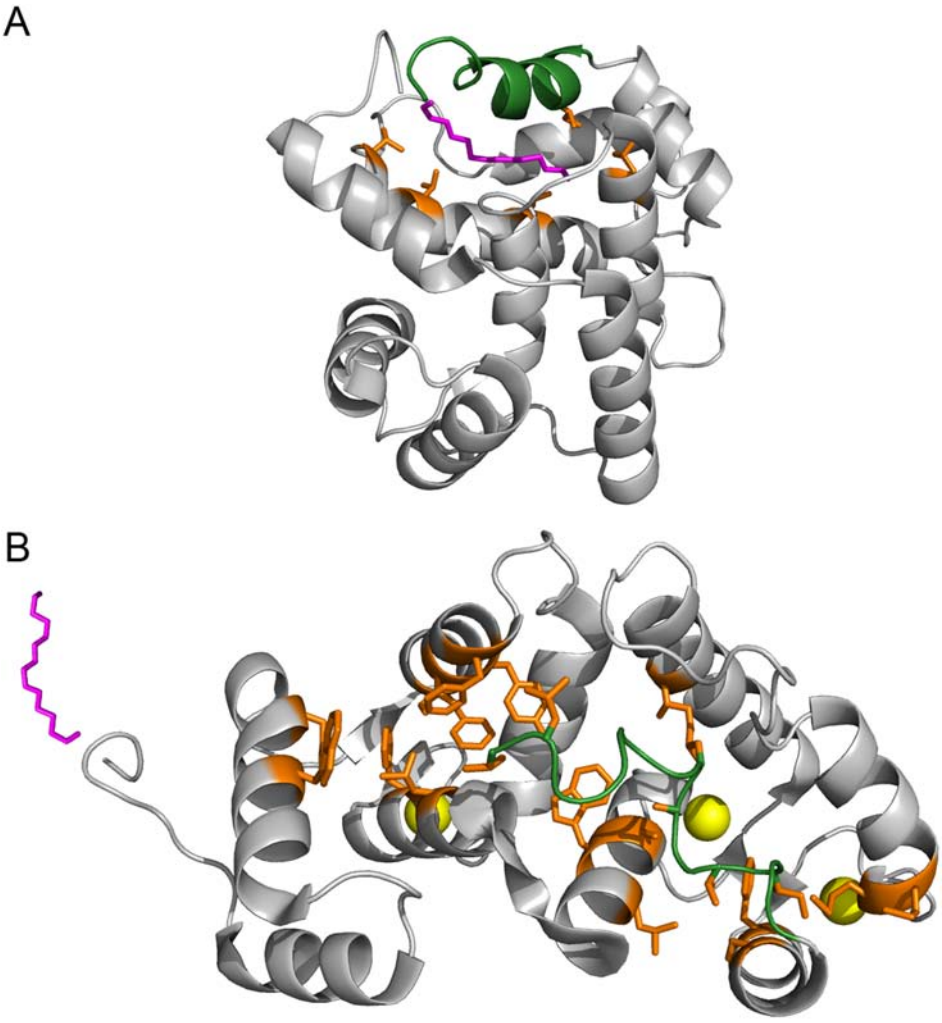

Fig. (7). Hydrophobic contacts involving the C-terminal segment of NCS1. The structures were generated from PDB 2L2E [15] and PDB 2LCP [18] using PyMOL 1.41 software (DeLano Scientific). The N-terminal myristoyl group and the C-terminal segment of NCS1 are indicated in magenta and green, respectively. Calcium ions are shown as yellow spheres. (**A**) The structure of Ca^{2+}-free NCS1. The residues Ile179, and Ala182 of the C-terminal segment and Leu101, Val125, Val132, Val136 or the hydrophobic pocket, interacting with constrained myristoyl group, are shown as orange sticks [15]. (**B**) The structure of Ca^{2+}-loaded NCS1. The residues of the C-terminal segment interacting with the exposed hydrophobic groove and residues of the hydrophobic groove of NCS1 are shown as orange sticks [18].

with signaling partners; exposure of target-binding sites. Our data suggest that the characteristic operation of NCS1 Ca^{2+}-myristoyl switch is substantially provided by its C-terminal segment. Indeed, the replacement of the C-terminal segment in NCS1 alters its Ca^{2+}-myristoyl switch resulting in a pronounced suppressive effect on both membrane binding and target regulation abilities of the protein. This effect may have an important practical application, since increased NCS1 membrane accumulation and its aberrant activity are associated with a number of neurological disorders [34]. In ASD the point mutation R102Q causes the protein to bind irreversibly to cell membranes at resting Ca^{2+} levels [35], thereby altering regulation of synaptic vesicle release [76]. Furthermore, this mutation facilitates NCS1 association with D2R *in vitro* [41]. Although mutations in the NCS1 gene have not been described in schizophrenic patients so far, overexpression of the protein has been observed in cases of this disorder [33]. Since NCS1 is known to attenuate dopamine-induced D2R internalization *via* a mechanism involving a reduction of receptor phosphorylation by GRK2 [24], its excessive functioning would lead to altered dopamine receptor signaling, which is widely regarded to underlie psychotic symptoms and impairments of cognitive function inherent in schizophrenia [34, 77, 78]. For these reasons diminishing of the NCS1 membrane pool and consequently impediment of its interaction with signaling partners including GRKs seems to be a viable strategy against NCS1 hyperfunction, associated with the above-mentioned psychoneurological diseases. Based on the present data we suggest that its C-terminal segment could be targeted to achieve selective pharmacological suppression of NCS1 activity in the presence of other co-localized NCS proteins. Indeed, the structure of the C-terminal segment of NCS1 is unique within the NCS family and is crucial for function of the protein. Nevertheless, future studies are necessary to verify feasibility of this approach considering NCS1 activity under conditions characteristic for the targeted neuronal systems.

CONCLUSION

We have demonstrated that NCS1 is present in retinal and ROS extracts where it can recognize GRK1 in the presence of Ca^{2+}. NCS1 irreversibly interacts with isolated photoreceptor membranes and inhibits GRK1 in a recoverin-like manner. Replacement of the C-terminal segment in NCS1 with the corresponding sequence of recoverin disrupts irreversibility and reduces affinity of membrane binding as well as down-regulates GRK1-targeted functional activity of the protein, without essential disturbance of its structure and metal cation-binding properties. Possible biomedical applications of these data are suggested.

LIST OF ABBREVIATIONS

ASD = Autistic Spectrum Disorder

bis-ANS = 4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid

D2R = Dopamine Receptor of D_2 Subtype

DTT = Dithiothreithol

EF(1-4) = EF-Hand Ca^{2+}-Binding Domains

EGTA = Ethylene Glycol Tetraacetic Acid

GPCR = G-Protein-Coupled Receptor

GRK = G-Protein-Coupled Receptor Kinase

GST = Glutathione-S-Transferase

HEPES = 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

IL1RAPL1 = Interleukin-1 Receptor Accessory Protein-Like 1

MALDI-TOF = Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

NCS = Neuronal Calcium Sensor

NCS1 = Neuronal Calcium Sensor-1

NMR = Nuclear Magnetic Resonance

ROS = Rod Outer Segments

SDS-PAGE = Polyacrylamide/Sodium Dodecyl-Sulphate Gel Electrophoresis

Tris = Tris-(Hydroxymethyl)-Aminomethane

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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