Neurotransmitter Activation Sequesters Specific mRNAs Into Stress Granules

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Research Article

Keywords:

Posted Date: January 27th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1269940/v1>

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Abstract

Hormones and neurotransmitters can activate the Gαq / phospholipase Cβ1 (PLCβ1) signaling system eliciting cellular calcium responses. Aside from the plasma membrane, PLCβ1 has a cytosolic population that binds stress granule associated proteins preventing their aggregation, and activation of Gαq shifts PLCβ1 to the membrane releasing bound proteins and promoting the formation of stress granules. The cellular impact of stress granules formed upon routine Gαq protein signaling is unknown. Here, we have characterized Ago2 stress granules formed in response to neurotransmitter activation in cultured PC12 cells. We find these stress granules have a distinct protein composition, and unlike stress granules formed upon heat shock, contain only two mRNA transcripts, chromogranin B, which is involved in secretory function, and ATP synthase 5f1b, which is required for ATP synthesis. Our studies show an unexpected pathway where Gαq/PLCβ regulates the translation of specific proteins.

Introduction

Binding of extracellular ligands such as acetylcholine, serotonin and histamine, to their specific G protein coupled receptor will activate Gαq, one of the four major G proteins pathways [1]. Gαq, in turn, activates phospholipase Cβ, which catalyzes the hydrolysis of the signaling lipid phosphoinositide 4,5 biphosphate leading to an increase in intracellular calcium[2]. Along with this important membrane function, PLCβ1 has been found to have an atypical cytosolic population that binds to the promoter of RNA-induced silencing, C3PO, as well as several proteins involved in stress granules formation [3, 4]. Stress granules are halted ribosomal complexes that protect mRNAs under stress conditions such as arsenite treatment, heat / cold shock and osmotic stress [5–7]. Proteins that bind PLCβ1 include eIF5A, polyadenylate binding protein (PABPC1) and Ago2. Ago2 is additionally the main nuclease component of the RNA-induced silencing complex (RISC)[8] that degrades mRNA with the help of C3PO. When Ago2-bound mRNA pairs perfectly with a bound miR, Ago2 transitions to an active conformation to hydrolyze the mRNA. However, if pairing is imperfect, it will form a stalled complex resulting in stress granules [9–11].

Our recent study showed that reducing the cytosolic PLCβ1 population increases the number and size of particles containing Ago2 as well as the stress granule makers PABPC1 and G3BP1[12, 13]. Binding between PLCβ1 and stress granule proteins helps keep them disperse, while activation of Gαq promotes relocalization of cytosolic PLCβ1 to the plasma membrane, promoting release of bound proteins and the formation of stress granules. This mechanism suggests that Gαq may be connected to protein translation through cytosolic PLCβ1.

Here, we have characterized the composition of Ago2 stress granules formed in response to neurotransmitter activation in differentiated PC12, and compared these to traditional stress responses. PC12 cells have a large endogenous expression of Gαq and PLCβ1, and although not neuronal in origin, when treated with nerve growth factor, the cells adopt neuronal morphology and secrete particles mimicking synaptic vesicles [14, 15]. We find that Gαq activation produces stress granules that have a

distinct protein composition as compared to other stresses. Also, unlike heat shock that contain different mRNA and miRs, Gαq stress granules contain only two major mRNA transcripts. Our studies show a surprising connection between physiological G protein activation and protein translation.

Results

Previous studies followed the formation of stress granules associated with Ago2 and PABCP1 by carbachol activation in undifferentiated PC12 cells as well as two other cell lines [12], and here we extended these studies to differentiated PC12 cells. Here, we stimulated Gαq with carbachol, a stable form of acetylcholine, and followed the initial assembly of small particles of the stress granule marker, eGFP-G3BP, whose dimerization initiates stress granule formation [16]. These studies used time-lapse fluorescence imaging and assessed the number of fluorophores associated with a diffusing particle called Number and Brightness (N&B) [17] analysis which will allow us to monitor small aggregates that are missed by standard confocal imaging.

Figure 1A-D shows N&B results for eGFP-G3BP1 expressed in differentiated PC12 cells. These data show that fluorescence intensity associated with diffusing particles increases with the loss of PLCβ1 by down-regulation, by stimulation with carbachol which has been shown to promote relocalization of PLCβ1 to activated Gαq, or by osmotic stress which also reduces cytosolic PLCβ1a levels [4]. Additionally, we followed the formation of larger eGFP-G3BP1 aggregates in live cells when subjected to carbachol stimulation, heat shock or osmotic stress by measuring their size and number by confocal imaging using a 100x objective (Fig. 1E). As expected, we find a significant increase in the cumulative number and size of particles relative to control for all three conditions, which is consistent with stress granule formation [16]. The trends seen for eGFP-G3BP1 mirror previous studies of Ago2 under stress conditions [4], and in Fig. 1E we compare eGFP-G3BP1 with eGFP-Ago2 particles. Washing out the carbachol and viewing the cells 30 minutes after recovery show little disassembly of the stress granules (Fig. 1F). We note that G3BP1 particles formed in these cells under all conditions were stable for at least 30 minutes.

We characterized the proteins associated with Ago2 stress granules. These studies were carried out by pulling down Ago2 with a monoclonal antibody from the cytosolic fraction of cells subjected to carbachol stimulation, hypo-osmotic stress or heat shock. The proteins contained in these Ago2 complexes were then identified by mass spectrometry. We found that Ago2 complexes isolated from cells under control conditions showed the highest number of bound proteins (~125) accounting for more than ~95% of the total protein (**SI Table 1**). However, cells subjected to carbachol stimulation, heat shock and osmotic stress conditions had far less proteins bound to Ago2 (**SI Table 2-4**).

We grouped the Ago2 bound proteins into functional categories: mRNA associated, transcription factors, tRNA associated, RNA polymerase, stress proteins, heat shock and eukaryotic initiation and translation (Fig. 2A-D) and we note that several cytoskeletal proteins appear. As expected, different conditions had different protein distributions relative to control. Proteins bound to Ago2 in cells subjected to heat shock and carbachol stimulation show an increased percentage of transcription factors, which is consistent

with a halting of fundamental cellular events, whereas samples from cells subjected to osmotic stress show increased levels of proteins associated with tRNA, which is consistent with shifts in protein production to restore basal osmolality. Comparison of the most abundant proteins associated with Ago2 under each condition shows transcriptional enhancer factor TEF-5 to be the major binding partner followed by cytoskeletal and structural proteins (i.e. actin, desmoplakin, titin, dynein, nesprin), whose amounts shift slightly with each condition. Focusing on the ten most abundant proteins, Ago2 complexes formed under carbachol stimulation showed only one unique protein, polyubiquitin-B, while both Ago2 complexes under carbachol stimulation and osmotic stress contain Phe tRNA ligase, which is absent in control and heat shock samples.

We sequenced the RNAs contained in Ago2 particles characterized above (SI **Table 5-6**). We could not detect RNA in Ago2 complexes pulled-down from control cells consistent with the absence of stable stress granules under basal conditions. In contrast, Ago2 particles from cells stimulated with carbachol or subjected to heat shock contained measurable amounts of RNAs, and these were isolated and sequenced. We found these two conditions yielded very different results; in the carbachol-stimulated samples, 92 of the most abundant RNAs contained were from only two transcripts, *chromogranin A/B* (*Chga/b*) and *ATP synthase 5f1b* (*ATP5f1b*), while heat shock samples contained an assortment of different mRNAs and miRs. These results suggest that Ago2 particles formed in response to Gαq/PLCβ activation sequester specific transcripts, while those formed from heat are non-specific.

We extended the above studies by measuring changes in the total cellular levels by RT-PCR under different conditions. These studies included several controls. Carbachol can activate Gαi as well as Gαq but does not bind or activate PLCβ1, and to isolate the effect of Gαi, we stimulated with isoproterenol [18, 19]. We also measured changes in the levels of *tau* mRNA since this transcript is not found in Ago2 complexes in cells subjected to carbachol stimulation or heat shock, although protein levels of tau are influenced by the PLCβ1/Gαq pathway by a mechanism independent of stress granules [20].

In Fig. 3A, we compare cytosolic levels of *ATP5f1b*, *Chgb* and *tau* under control, carbachol stimulation, heat shock, osmotic stress and isoproterenol stimulation. Compared to controls, we find the heat shock and osmotic stress significantly reduced the levels of all four genes subjected to stress, while levels of *ATP5f1b* and *Chgb* are protected upon carbachol activation. These data are consistent with specific protection of *ATP5f1b* and *Chgb* in Ago2 stress granules formed upon Gαq activation. Activation of Gαi by isoproterenol, increases *ATP5f1b* and *Chgb* and does not affect *tau*, consistent with its role in promoting growth pathways [21].

We measured changes in the protein levels of Chgb and ATP5f1b in cells subjected to different environmental stress (Fig. 3B). Because changes in protein levels are subject to many post-synthetic modifications and regulatory mechanisms in addition to those that occur in translation, we added other conditions to better assess the role of carbachol stimulation of Gαq. In one series of studies, PLCβ1 was down-regulated to promote Ago2 particle formation without carbachol activation [4], and but this did not change protein levels. Additionally, no changes are seen in cells where cells were stimulated for 10 min or

30 min. However, heat shock, osmotic stress and stimulation of Gai by isoproterenol all impact protein levels. The lack of change in Chgb and ATP5f1b with carbachol stimulation as opposed to other stress conditions is suggest that that Gαq/PLCβ pathway only influences longer-term changes in mRNA through Ago2 stress granules and not short-term protein levels.

In a final series of studies, we assessed the protection of ATP5f1b with carbachol stimulation by monitoring changes in the redox state of cells, to assess mitochondria health and ATP production. These experiments measured changes in intrinsic fluorescence contributed mainly by NAD(P)H and FADH reflecting the optical redox state of mitochondria (see [22]). NADH, which is generated through the Krebs cycle, has a longer-lived fluorescence compared to other intrinsic fluorophores (see [23]). We measured the intrinsic fluorescence of cells under different stress conditions and found that while the lifetime of cells treated with carbachol is the same as control, cells subjected to osmotic stress, isoproterenol or heat trend towards higher values (Fig. 4). This higher lifetime is consistent with a transfer of ATP production to the Krebs cycle in response to reduced ATP synthase activity. These results support the idea that ATP5fb1 levels are protected with Gαq stimulation in contrast to other stresses.

Discussion

Mammalian Gαq and its analogs in other species play a key role in generating cellular responses by increasing levels of intracellular calcium [21]. In this study, we have uncovered a novel pathway in which Gαq may control specific mRNA translation. The data presented in this study suggest that in differentiated PC12 cells, Gαq activation by a neurotransmitter sequesters two mRNAs important for endocrine function and cell energy in Ago2 stress granules.

The basis of this pathway stems from the multifunctional nature of PLCβ1. PLCβ1 has several conserved structural domains surrounding the catalytic domain along with a long 400aa tail that is required for Gαq binding, and that can mediate binding to cytosolic partners (see [24]). One of these confirmed partners is the Promoter of RNA-induced silencing, C3PO [25, 26], which is inhibited by PLCβ1 binding [3, 27–29]. In recent work, we characterized the proteins bound to cytosolic PLCβ1 in undifferentiated PC12 cells and found roughly 30% of bound proteins are identified as stress granules proteins, including Ago2, PABPC, eIF5a and G3BP1 [4, 30]. These studies showed that reducing the cytosolic level of PLCβ1 either by down-regulation, osmotic stress or Gαq activation allowed for the release of bound proteins and promoted the formation of stress granules. While stress granules protect mRNA from degradation during stress conditions[5], it is surprising these particles form under routine physiological responses generated by Gαq. This observation prompted us to determine whether the stress granules formed upon Gαq activation had a specific physiological impact on cells.

Our previous studies using undifferentiated PC12 cells and two muscle cell lines, A10 and WKO-3M22, showed that Gαq activation promotes the assembly of stress granules as indicated by established markers, G3BP1 and PABPC, as well as Ago2 (see [4, 16, 31–34]). Those studies identified Ago2 as either a direct or indirect binding partner of PLCβ1 by pull down studies and by FRET using tagged proteins, and

showed Ago2 particles that grew in size and number under various stress conditions. Here, we show similar stress granule assembly as followed by the marker protein G3BP1 and that these particles that are stable for at least 30 minutes.

Formation of stress granules depends not only on the environmental conditions, but also on the nature of the bound miRs and mRNA [8, 13, 33, 35], and we reasoned that the binding of these species may be influenced by proteins that reside in stress granules. To this end, we characterized the proteins associated with Ago2 stress granules using a proteomics approach. Additionally, because previous studies suggested that different stresses form stress granules with distinct composition [31], here we identified proteins in Ago2-associated stress granules in cells subjected to carbachol stimulation, osmotic stress and heat. We find that the shifts in composition were surprisingly minor especially considering their different cumulative responses. All stress conditions found that TEF-5 is a major binding partner of Ago2. TEF-5 is a transcriptional enhancer factor that works through the hippo pathway to inhibit proliferation [36], and its association with Ago2 may serve to regulate TEF-5 translation to the nucleus. Comparing the top ten proteins in the Ago2 proteomics data sets, we find only one unique protein for Gαq stimulation upon carbachol addition, polyubiquitin-B, which impacts protein turn-over as well as various signaling cascades [37].

Under control conditions, we find that Ago2 is associated with many proteins but these complexes do not contain RNA indicating that they are not true stress granules. The observation that only two major transcripts (*ATP5f1b* and *Chgb*) are sequestered in Ago2 transcripts formed in response to neurotransmitter stimulation of Gαq was surprising. Our N&B studies found ~40% of G3BP1 aggregated with carbachol addition but we note that only 30% of the cells responded. By comparing the amount by total cellular *ATP5f1b* with the amount isolated in Ago2 particles per cell as determined by PCR where there was little loss in total Ago2, we find that at least 5% of this mRNA transcript is contained in stress granules. While this amount appears low, we also find that cells do not significantly recover after the stress is removed for 30 minutes implying a relatively long-term protection of these transcripts. Thus, if a larger cell population responds by increasing stimulation, then at least ~15% of the transcripts will be protected. By this argument, repeated stress would cause a significant population of transcripts to accumulate in Ago2 stress granules impacting cell function.

Maintaining cellular energy production during and after signaling is important, and the functional impact of preserving ATP5f1b during carbachol stimulation is seen though the preservation of the redox state (Fig. 4). Similarly, Gαq is found in many cell types with endo- and exocrine function and preservation of ChgB, which mediates vesicle formation, is expected. Of course, these studies focused on differentiated PC12 cells that have secretory function [15], and it is likely that other cell types will sequester other mRNAs upon Gαq stimulation.

PLCβ1 is associated with loss of proliferation and differentiation [38, 39] and these effects are likely to be due to its interaction with C3PO and Ago2, as well as mediating calcium signals. We have previously identified miRs associated PLCβ1 down-regulation in PC12 cells [40] and found that down-regulating

PLCβ1 levels results in substantial reduction in miR101a and miR101b, which may regulate ATP synthase 5B[41] and miR-22 levels by 20%, which regulates *Chgb*[42]. The loss of these miRs, along with the observation that loss of cytosolic PLCβ1 or sustained neurotransmitter activation of Gαq returns PC12 cells to the undifferentiated state, may indicate that the increased number of stress granules may sequester mRNAs important for defining cell phenotype.

Methods

Cell culture

PC12 cells were cultured in high glucose DMEM (Gibco) with 10% heat inactivated horse serum (Gibco), 5% fetal bovine serum (Atlanta Biologicals) and 1% Penicillin/Streptomycin (Gibco). Cells were treated with 0.1 ug/mL nerve growth factor (Bon Opus) for ~48 hours in media containing 1% heat inactivated horse serum and 1% antibiotic to induce differentiation. All cells were incubated at 37°C in 5% CO₂. Cells below passage 10 were used for all experiments.

Plasmids

EGFP-hArgonaute2 (eGFP-Ago2) was purchased from Addgene (plasmid # 21981) and was prepared in the laboratory of Philip Sharp (MIT). G3BP1-GFP was purchased from Addgene (plasmid # 119950) and was prepared in the laboratory of Jeffrey Chao (Friedrich Miescher Institute). siRNA(PLCβ1) was from Dharmacon (smartpool # L-092936-02-0005). Plasmid transfections and siRNA knockdowns were done using Lipofectamine 3000 (Invitrogen) in antibiotic free media. Medium was changed to one containing antibiotic (1% Penicillin/Streptomycin) 4-12 hours post-transfection.

Stress conditions

Cells were exposed to carbachol or isoproterenol stimulation at a final concentration of 5 μm for 10 minutes. For hypo-osmotic stress, the culture medium was diluted from 300 mOsm to 150 mOsm with 50% water for 5 minutes. For heat shock, cells were incubated at 42°C for 1 hour.

Number and Brightness (N&B) measurements

N&B defines the number of photons associated with a diffusing species by analyzing the variation of the fluorescence intensity in each pixel in the cell image, and full details about the method and analysis can be found in [17]. In these studies, we collected ~100 cell images viewing either free eGFP (control) or eGFP-G3BP1 at a 66 nm/pixel resolution and at a rate of 4 μs/pixel (see [12]). Number and Brightness (N&B) data were analyzed with SimFC4 software (www.lfd.uci.edu) where the brightness, B , in each pixel refers to the ratio of the variance, σ , over the average fluorescence intensity $\langle k \rangle$

$$B = \sigma^2 / \langle k \rangle$$
$$\langle k \rangle = \epsilon n$$

where n is the number of fluorophores. The variance in each pixel is obtained by rescanning the cell image ~100 times. The average fluorescence intensity, $\langle k \rangle$, is directly related to the molecular brightness, ϵ , in units of photons per second per molecule.

Particle analysis

Samples were imaged with a 100X/1.49 oil TIRF objective to microscopically count the number of particles per μm^2 formed under different conditions. For each condition, 10 to 20 cells were randomly selected, and z-stack measurements were taken (1.0 μm /frame). Analysis was performed with ImageJ and Fiji ImageJ software either by thresholding before analyzing, and averaging the number of particles per frame per measurement or by combining z-stack measurements to generate a 3D image for each sample before analyzing the number of particles per sample and averaging the results. Both methods produced identical results.

Mass spectrometry

Ago2 stress granules were purified from PC12 cells electroporated with eGFP-Ago2 then differentiated with NGF for ~48 hours using the protocol described by Wheeler *et al.* [43]. Six 150 mm dishes of cells were used per condition. Immunoprecipitation was done using GFP antibody (Santa Cruz Biotechnology, sc-9996) with protein A dynabeads and elution buffer (Invitrogen, cat #10006D). Following short gel electrophoresis and band excision, mass spectrometry was performed at UMass Medical School Mass Spectrometry Facility using the methodology previously described [12].

eCLIP transcript sequences

PC12 cells were transfected with eGFP-Ago2 using electroporation (Bio-Rad Gene Pulser Xcell) with one pulse in a 0.4 cm gap cuvette (Bio-Rad, cat #1652088) in complete media and plated at ~50% confluency to leave room for neurite growth. Cells were stressed and crosslinked using 254 nm ultraviolet light. Treated cells were collected from at least four 150 mm culture dishes per sample condition. Lysis was done on fresh, non-frozen cell pellets using 100 μL lysis buffer supplemented with RNase inhibitor and protease inhibitor tablets (Roche). A 10 μL portion of each lysate was used to extract total RNA (Zymo Clean and Concentrator) which was then tested for RNA integrity using RNA Fragment Analyzer services at University of Massachusetts Medical School Molecular Biology Core Lab. The remaining lysate was immunoprecipitated using mouse Ago2 antibody. Subsequent western blots, RNA cleanup and library construction was done using Eclipse Bioinnovation protocol v1.01R, storing samples at -80°C overnight when necessary. Sample quantity was evaluated by qPCR. Libraries were sequenced at UMass Medical School Deep Sequencing Core using Illumina HiSeq 4000 system. All reagents were supplied by Eclipse Bioinnovations unless otherwise specified.

RT-PCR: For each condition, one 100 mm dish was used for total RNA extraction. Cells were differentiated and stressed before isolating RNA using RNeasy Mini Kit (Qiagen, Cat #74104) with DNase digestion (Qiagen, cat #79254). CDNA synthesis was done using MiniAmp Thermal Cycler (Applied Biosystems).

Purified RNA sample concentrations were measured by Nanodrop and 1 µg RNA was incubated with dNTP (New England Biolabs, cat #N0447S), random hexamers (Thermo Scientific, cat #SO142), and nuclease free water (Invitrogen, cat #AM9930), at 65°C for 5 minutes. After the first incubation, 5x First Strand Buffer, DTT (Invitrogen cat #18080093) and RNase inhibitor (Invitrogen, cat #AM2694) were added to the mixture and incubated at 25°C for 2 minutes. After the second incubation, 1 µL Superscript III Reverse Transcriptase (Invitrogen, cat #18080093) was added and the mixture was incubated at 25°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. CDNA was stored at -20°C overnight or used immediately at a dilution of 1:20 in nuclease free water for RT-PCR experiments with Luna Universal Master Mix (New England Biolabs, cat #M3003L), nuclease free water, and custom ATP5f1b and CHGb primers (ITD). For positive and negative controls, 18S ribosomal subunit and tau primers were used (see Supplemental).

Western blotting: We used an establish procedure [40] with modifications. Samples were grown in 100 mm dishes plates and were treated with MG132 at a final concentration of 250 nM 24 hours before collection. Cells were stressed and then collected in 250 µl of NP-40 lysis buffer [150 mM NaCl, 50 mM Tris (pH=8.0), 1% NP40, protease inhibitor]. The primary antibodies used included: anti-ATP5f1b (Abcam, ab117991), anti-Chgb (Abcam, ab150354), anti-actin (Santa Cruz, sc-47778).

Fluorescence lifetime imaging measurements (FLIM)

Phase modulation FLIM measurements were performed on the dual-channel confocal fast FLIM (Alba version 5, ISS Inc.) using a two-photon titanium-sapphire laser and a Nikon Eclipse Ti-U inverted microscope as described[40]. The lifetime of the laser was calibrated each time before experiments by measuring the lifetime of Atto 425 in water with a lifetime of 3.61 ns at 80, 160, and 240 MHz. The samples were excited at 740 nm for intrinsic measurements, and emission spectra were collected through a 525/50 bandpass filter. For each measurement, the data were acquired until the photon count was >100.

Statistical analysis

Data were analyzed with the Sigma Plot 14 statistical packages, which included the student's *t* test and one-way analysis of variance (ANOVA).

Declarations

ACKNOWLEDGEMENTS This work was funded in apart from the Richard Whitcomb foundation.

AUTHOR CONTRIBUTIONS. LJ conceived and designed the experiments, carried out experimental work and analyzed data. AP assisted with data collection, SS conceived and designed the experiments, analyzed data and wrote the manuscript.

DATA AVAILABILITY. All primary and analyzed data are available upon request.

ADDITIONAL STATEMENTS. The authors declare no competing interests.

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Figures

Figure 1

Aggregation of G3BP1 in differentiated PC12 cells. Cells transfected with eGFP-G3BP1 were at **(A)** control conditions, $n=12$; **(B)** exposed to carbachol for 10 minutes, $n=30$; **(C)** treated with siRNA(PLC β 1), $n=28$; **(D)** subjected to hypo-osmotic stress (150 mOsm) for 5 minutes, $n=14$ from 3 independent experiments. The graphs in the top rows display N&B values for each pixel where the y-axis correspond to the brightness of the particle and the x-axis shows particle intensity. The pixels contained in the red boxes are the values found for free, non-aggregated eGFP. Points outside the red boxes, shown in green and blue, correspond to higher-order species of G3BP1 where the percent of pixels in these categories are given. Images in the middle rows show the pixels corresponding to free (red) and aggregated eGFP-G3BP1 (green and blue). Images in the bottom row are the corresponding fluorescence microscopy images. Total percent protein aggregation for each condition is reported in red text, while the portion of total aggregation derived from the green and blue boxes is reported in green and blue text, respectively. We note that a significant percent of cells in the carbachol (66%) and siRNA(PLC β 1) (54%) samples showed low ($\sim 2\%$) aggregation and only the percentages in the higher groups are shown. Scale bars = 10 μ m. **(E)** Cumulative values of the intensities (count) and size of eGFP-Ago2 or eGFP-G3BP1 particles in live cells under control conditions as determined by confocal imaging where the count values for Ago2 are reduced by 10 fold for scaling purposes. Also shown are eGFP-G3BP1 particle size and number in live cells immediately after carbachol treatment, hypo-osmotic stress or heat shock. For all conditions, $n = 11$ for eGFP-G3BP1 and $n = 12$ for Ago2 from 3 independent experiments and where SD is shown. **(F)** eGFP-G3BP1 particle data shown in **(E)** along with data taken for cells that were treated to a stress condition and then allowed to recover at 37°C for 30 minutes where $n = 11$ from 3 independent experiments and where SE is shown.

Protein distribution of Ago2 stress granules

Figure 2

Distribution of Ago2 stress granule proteins under different stress. PC12 cells ($\sim 1.2 \times 10^8$ cells/condition) over-expressing eGFP-Ago2 were exposed to **(A)** no stress, **(B)** carbachol activation (10 min, 5 μ M), **(C)** heat shock (1hr, 42°C), or **(D)** hypo-osmotic stress (150 mOsm, 5 min). Ago2 particles were pulled down for cytosolic fractions using a monoclonal GFP antibody. Purified stress granules were then subject to mass spectrometry analysis and results were filtered by the following categories: mRNA binding proteins, transcription factors, tRNA binding proteins, RNA polymerases, heat shock proteins, stress related proteins, and translation/initiation proteins, where the values in the segment corresponds to the percentage of the total amount of Ago2-bound protein.

Figure 3

Gene expression and protein levels of ATP5f1b and CHGb in cells under stress. (A) RT-PCR results showing the relative levels of gene expression in PC12 cells of *ATP5f1b*, *Chgb* and *tau* (as a negative control) in differentiated PC12 cells exposed to different stress conditions (carbachol stimulation, hypo-osmotic stress and heat shock, and isoproterenol as a Gai stimulant), where * denotes $p \leq 0.001$ and $n = 3-9$ from 3-5 independent experiments and SE is shown. (B) PC12 cells, either untransfected or treated with siRNA(PLCβ1) for 48 hrs, were stimulated with carbachol or isoproterenol, or subjected to heat shock or hypo-osmotic stress, followed treated with MG132 halt protein synthesis before by lysis and western blotting where $n = 8$ from 3 independent experiments, * denotes p values between 0.01 and <0.001 and SE is shown.

Figure 4

Intrinsic FLIM measurements to evaluate mitochondrial health. Untransfected, differentiated PC12 cells were exposed to carbachol, osmotic stress and heat shock before imaging at 740 nm excitation using a two photon laser, and measuring the autofluorescence lifetime by phase-modulation where $n = 12$ from 4 independent experiments. P values are from Student t-tests comparing the specific sample with control.

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