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Inositol 1,4,5-trisphosphate receptor movement is restricted by addition of elevated levels of O-linked sugar

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Abstract

The inositol 1,4,5-trisphosphate receptor (InsP₃R) is a versatile, ubiquitous intracellular calcium channel. Traditionally, visualizing the InsP₃R in live cells involves attaching a fluorescent marker to either terminal of the protein, but the termini themselves contain binding sites for accessory molecules and proteins. Using random transposition, constructs have been developed that express the type I InsP₃R with green fluorescent protein (GFP) inserted at various points within its sequence. We have used two of these constructs, one in the ligand-binding domain, and another in the regulatory domain, to investigate InsP₃R dynamics within the endoplasmic reticulum. We present evidence that endogenous calcium signaling is maintained when these constructs are expressed. In addition, by measuring the fluorescent recovery after photobleaching of a subcellular region, we demonstrate that treatment with 8 mM N-acetylglucosamine (GlcNAc), known to lead to increased O-linked GlcNAcylation of proteins, leads to a reduction in the ability of the InsP₃R to travel laterally within the endoplasmic reticulum. Each construct serves as the control for the other one, suggesting that this decrease in mobility is not specific to the insertion site of GFP within the InsP₃R. These constructs represent a new tool that will allow us to follow receptor turnover and translocation events.

Keywords

GFP; InsP₃R; FRAP; GlcNAc; Calcium

1. Introduction

Inositol 1,4,5-trisphosphate (InsP₃)¹ receptors (InsP₃R) are one family of regulated calcium (Ca²⁺) channel found in the endoplasmic reticulum (ER) membrane, and are the principle channel for intracellular Ca²⁺ release in most cells. They are tetramers, with each monomer formed of three functional domains: a cytosolic N-terminal ligand-binding domain, a regulatory domain, and a C-terminal six transmembrane-spanning pore forming domain [1]. There are three known subtypes, with a high percent sequence identity both within and

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¹Abbreviations: InsP₃= inositol 1,4,5-trisphosphate; InsP₃R= inositol 1,4,5-trisphosphate receptor; Ca²⁺= calcium; ER= endoplasmic reticulum; PIP₂= phosphatidylinositol 4,5-bisphosphate; PLC= phospholipase C; GFP= green fluorescence protein; GlcNAc= N-acetylglucosamine; SERCA= sarcoplasmic/endoplasmic reticulum calcium ATPase; HBS= HEPES buffered saline; FRAP= fluorescent recovery after photobleaching; ; A.U.= arbitrary units; GFP-InsP₃R= Type I InsP₃R with internally-inserted GFP.

between species- for example, the rat InsP₃R type I has 99 % sequence identity to both the mouse and human InsP₃R type I, and there is greater than 70 % sequence identity between all three human subtypes [2]. These subtypes vary in tissue expression and affinity for agonist and mediators, but all follow a general pattern of activation. InsP₃ is generated from its precursor membrane lipid phosphatidylinositol biphosphate (PIP₂) by enzymatic cleavage due to the action of phospholipase C (PLC) [3]. That InsP₃ is then free to diffuse through the cytosol, where it can bind to the InsP₃R. Upon InsP₃ binding, these channels open and permit Ca²⁺ efflux from the ER lumen [4-6]. Channel activity is mediated by both luminal and cytosolic Ca²⁺, as well as ATP, the presence of each serving to increase the open probability of these channels in the presence of InsP₃ [7-9].

InsP₃R signaling has been implicated in a host of cellular processes, including neuronal processing, development, and secretion [6, 10]. Regulation of activity can be achieved in many ways: phosphorylation, subtype expression, glycosylation and subcellular localization [6, 11-13]. There have been some preliminary investigations into the last mechanism [13, 14], but new tools are needed for further progress.

Green fluorescent protein (GFP) and its analogs are robust, and can self-assemble into the proper conformation (and thus retain their fluorescent properties) when fused to the termini of proteins, including the InsP₃R [14-18]. GFP has also been randomly inserted into the coding sequence of protein, and has been shown to maintain a fluorescent state while interfering minimally with endogenous protein function [19, 20].

Sheridan and Hughes (2004) previously reported a series of transposons capable of randomly inserting the coding sequence of fluorescent proteins into any gene of interest, thereby generating functional fluorescent fusion proteins with internal fluorescent insertions [20]. Using this technology, they generated 20 different fluorescent rat type I InsP₃Rs, each with GFP located at a different point along the peptide. We have used two of these constructs, one with GFP in the InsP₃-binding domain (pBFC016) and the other with GFP in the regulatory domain (pBFC013)(Fig. 1), to explore the relationship between N-acetylglucosamine (O-GlcNAc) and the InsP₃R. In parallel, these studies also verify that any effect seen upon manipulation of the InsP₃R is independent of the site of fluorophore insertion. With the ability to insert GFP in specific regions of the InsP₃R, we have a new tool that will allow us to investigate the effects of binding partners and post-translational modifications on the localization of the InsP₃R in intact cells.

2. Materials and methods

2.1 Cell culture

SH-SY5Y cells were plated on 22 mm² glass coverslips, at 0.5-1x10⁵ cells per well of a 6-well plate, and grown in a 1:1 mixture of F-12 Ham's and Minimum Essential Medium, supplemented with 10 % Fetal Bovine Serum, 1 % Minimum Essential Medium Non-Essential Amino Acids, and penicillin/streptomycin (Invitrogen). GFP-polycystin-2-expressing MDCK cells were grown on glass bottom culture dishes (MatTek) in Dulbecco's Modified Eagle Medium, supplemented with 10 % Fetal Bovine Serum, 10mM sodium pyruvate, and penicillin/streptomycin (Invitrogen).

2.2 Transfection

Cells were transfected with pGreenLantern (GIBCO, BRL), pBFC013 or pBFC016 (generous gifts of T. Hughes) using Fugene 6 or Fugene HD (Roche Applied Science) reagent in Opti-MEM I (Invitrogen), according to the manufacturer's instructions. Cells were left untreated or exposed to 8 mM GlcNAc and 8 mM mannitol for 72h [21] prior to imaging.

2.3 Immunofluorescence

Cells grown on coverslips were permeabilized and fixed with paraformaldehyde. Fixed cells were then exposed to rabbit anti-GFP (Molecular Probes) and mouse anti-SERCA2 (Affinity Bioreagents), or mouse anti-GFP (Molecular Probes) and rabbit anti-InsP₃R type I [13], as indicated, washed, then exposed to Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes), or Cy3 goat anti-mouse IgG (Biomed). After labeling, coverslips were affixed to glass slides using Pro-Long antifade solution (Molecular Probes), and imaged on a Zeiss LSM 510 confocal microscope with a 63X water-immersion objective, with excitation at 488 nm or 543 nm, in multitrack mode of the LSM software to minimize crosstalk.

2.4 Calcium imaging

Coverslips were incubated for 30 min in Hepes-buffered saline (HBS) containing (in mM): 130 NaCl, 5 KCl, 1.25 CaCl₂, 1.2 KH₂PO₄, 1 MgSO₄, 20 HEPES, pH 7.4 with NaOH, supplemented with 6 μ M rhod-2 AM (Molecular Probes). Cells were washed with HBS, and placed in an imaging chamber (Warner Instruments, Hamden, CT) bathed in HBS on the stage of a Zeiss LSM 510 confocal microscope. Rhod-2 and GFP fluorescence were excited using 568 nm and 488 nm laser, respectively, and emission was detected through 585 nm longpass and 505–550 nm bandpass filters. InsP₃-dependent Ca²⁺ release was stimulated by application of 1 μ M MgATP to the bath solution.

2.5 Fluorescent recovery after photobleaching (FRAP)

FRAP protocol, and calculation of diffusion constants were as described previously [17]. Briefly, for SH-SY5Y cells, coverslips containing transfected cells were placed in an imaging chamber; for MDCK cells, glass-bottomed dishes were placed directly on the stage. Cells bathed in HBS, excited with a 488 nm laser, and emission was recorded through a 505–530 nm bandpass filter. A circular region of interest was selected within a fluorescent cell. After recording stable baseline fluorescence, the region was bleached for 100 iterations at 100 % transmission, 6.1 A, and recovery of fluorescence was recorded for 40–60 frames at 0.2–0.5 Hz, 4% transmission.

2.6 Data Analysis and statistics

Immunofluorescence and FRAP data were collected and analyzed using Zeiss LSM 510 software. For FRAP experiments, all data were background subtracted, and any experiment with a baseline below 55 A.U. was discarded in order to effectively detect photobleaching. Data were plotted using SigmaPlot 9.0, fitted to a curve of exponential rise to maximum, and the time to half maximal recovery ($t_{1/2}$) was calculated. Using the radius of the bleached region and $t_{1/2}$, we were able to calculate the diffusion constants, as described previously [17]. To calculate the percentage recovery, we used the formula:

$$\% \text{Recovery} = (F_f - F_o(C_f/C_o) / F_i(C_f/C_o) - F_o(C_f/C_o))$$

where F is the fluorescence in the bleached spot and C is the fluorescence of an unbleached spot in the same cell at times i (just before bleaching), o (just after bleaching), or f (end of recording) (modified from [17]). After passing tests for normality and variance, a two-tailed t-test was performed to test for significance.

Ca²⁺ imaging data were background subtracted and analyzed using Igor Pro 5.0. Transfected cells were compared to non-transfected cells co-cultured on the same coverslips. Data are displayed as mean $\pm$ SE.

3. Results

3.1 GFP-InsP₃R is targeted to the endoplasmic reticulum

When GFP-InsP₃R constructs are transfected into HEK-293 cells, these GFP-InsP₃R constructs display a reticular pattern of fluorescence [22]. To confirm that this expression is localized to the ER, we transfected SH-SY5Y cells with two different constructs, pBFC013 or pBFC016. Cells transfected with GFP alone displayed a uniform cytosolic fluorescence (Fig. 2A). In contrast, both InsP₃R constructs displayed a reticular pattern of fluorescence, consistent with their predicted targeting to the ER (Fig. 2B,C). To further ensure that these constructs expressed in the correct organelle, we investigated co-immunofluorescence of GFP with known ER resident proteins. There is strong overlap of fluorescent signals of anti-GFP and anti-InsP₃R antibodies in transfected cells (Fig. 2D), confirming that GFP-InsP₃R remains intact when expressed, and that the GFP is not somehow removed from the InsP₃R. Non-transfected cells show no GFP signal, but still show a strong InsP₃R signal, consistent with endogenous InsP₃R expression in SH-SY5Y cells. InsP₃R are expressed as tetramers, and it is possible that GFP-InsP₃R subunits may interfere with the intrinsic quaternary structure and position of InsP₃R within transfected cells; that is, the GFP signal may overlap with the InsP₃R signal, and both may be improperly targeted. To test this, we repeated co-immunofluorescence experiments with a second ER marker, the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) (Fig. 2E). Again, GFP and SERCA signals showed a reticular pattern of fluorescence, and the two signals had significant overlap, suggesting the localization of GFP-InsP₃R to the ER.

3.2 GFP-InsP₃R-expressing cells retain endogenous calcium signaling

The functional unit of the InsP₃R is a tetramer [3]. It has been postulated that heterotetramers form in cells expressing more than one subtype [2, 12]. It is possible that the expressed GFP-InsP₃R could interfere with tetramer formation, the GFP acting to inhibit subunit interaction. It is equally possible that GFP-InsP₃R could form a heteromer with endogenous channel subunits and interfere with InsP₃-mediated Ca²⁺ signaling within transfected cells. To explore these possibilities, we loaded SH-SY5Y cells with rhod-2 AM, a Ca²⁺-sensitive fluorophore, and compared the Ca²⁺ release of control and GFP-InsP₃R expressing cells upon stimulation of the InsP₃ pathway through P₂Y purinergic receptor activation. The resulting Ca²⁺ release showed no difference in amplitude between non-transfected and co-cultured pBFC016- or pBFC013-expressing cells (Fig. 3B,C), and the percent of cells responding to the stimulus was similar (data not shown), suggesting that InsP₃-dependent Ca²⁺ signaling is intact. The green fluorescent emission remained constant over time, reflecting the stability of the transfected signal (Fig. 3B).

3.3 Fluorescence recovery after photobleaching (FRAP)

To investigate the diffusion of GFP-InsP₃R within the ER, we performed FRAP experiments. A region of interest was chosen in a transfected cell and bleached (Fig. 4A,B). Recovery was followed over time, and displayed an exponential rise to approximately 80 % of the original fluorescence (Fig. 4B). From the recovery data, diffusion constants were calculated: $0.022 \pm 0.003 \mu\text{m}^2/\text{s}$ for pBFC016-transfected, or $0.011 \pm 0.002 \mu\text{m}^2/\text{s}$ for pBFC013-transfected, unstimulated SH-SY5Y cells. By contrast, SH-SY5Y cells transfected with GFP alone displayed a much more rapid FRAP, and recovered to the same level as the rest of the cell (Fig. 4C). Changing the size of the bleached region, selecting an unbleached region proximal or distal to the bleached spot for comparison, or prolonging the length of recording did not significantly alter the recovery. Our results agree with several groups who demonstrated diffusion constants of $0.01 \mu\text{m}^2/\text{s}$ [16], $0.05 \mu\text{m}^2/\text{s}$ [14], and $0.03 \mu\text{m}^2/\text{s}$ [17], for C' or N'-terminally-tagged type I InsP₃R or N'-terminally-tagged type III InsP₃R, respectively, but differ from Fukatsu et al. (2004), who calculated a diffusion constant of

0.26 $\mu\text{m}^2/\text{s}$ using N-terminally-tagged GFP-InsP₃R type I in rat hippocampal neurons, over an order of magnitude faster. Different expression systems, methods of analysis and collection of data may account for some of this discrepancy.

3.4 Incubation with GlcNAc reduces GFP-InsP₃R mobility within the endoplasmic reticulum

Binding sites for accessory proteins and posttranslational modifications exist along the length of the InsP₃R [23, 24]. We have previously demonstrated that the type I InsP₃R undergoes O-linked GlcNAc glycosylation [25]. Upon O-GlcNAcylation the ability of the InsP₃R to open and release Ca^{2+} from the ER is reduced. To further investigate the role of GlcNAc on InsP₃R, we incubated GFP-InsP₃R-expressing SH-SY5Y cells for 72 h in the presence of 8 mM GlcNAc. After performing FRAP, we were surprised to see a reduced recovery of fluorescence in pBFC016-transfected cells treated with GlcNAc compared to untreated cells (0.80 ± 0.06 vs. 0.58 ± 0.06 , control vs. GlcNAc, $P = 0.02$; Fig. 5A,C). To confirm that this result was due to the effect of GlcNAc, and not a result of the site of GFP insertion, the experiments were repeated using pBFC013. Again, exposure to GlcNAc resulted in a reduced recovery of fluorescence to the bleached region (0.73 ± 0.04 vs. 0.57 ± 0.06 , control vs. GlcNAc, $P = 0.03$; Fig. 5B,D). In addition, some cells were observed to have less than 20 % fluorescence recovery after GlcNAc treatment, something not seen with untreated cells (Fig. 5C, D). Stimulating with forskolin, PKA phosphorylation of the InsP₃R has been shown to increase its open probability in the presence of InsP₃ [26]. Treating SH-SY5Y cells with forskolin to stimulate PKA in a manner consistent with InsP₃R phosphorylation did not significantly alter FRAP (data not shown), demonstrating the uniqueness of O-GlcNAc as a postranslational modification with effect on GFP-InsP₃R mobility.

To investigate whether or not GlcNAc treatment altered the mobility of all ER-resident proteins, or alters the ER itself, we repeated FRAP experiments on MDCK cells stably expressing GFP-tagged polycystin- 2, another transmembrane calcium channel found predominantly in the ER [27]. Experiments showed a diffusion coefficient, 0.010 ± 0.002 $\mu\text{m}^2/\text{s}$, similar to pBFC013, and no significant effect of GlcNAc on fluorescent recovery (0.93 ± 0.06 vs. 0.85 ± 0.07 , control vs. GlcNAc, data not shown). This suggests that the effect seen of GlcNAc is not due to any global effects on the ER or ER-resident proteins. Taken together, these results suggest a specific role for GlcNAc on the ability of InsP₃R to travel laterally through the ER membrane.

4. Discussion

Visualization of proteins by coupling to a fluorophore or fluorescent protein is a well established technique. Most commonly, the fluorophore is added to the N- or C- terminus. In the case of intra-molecular FRET, different fluorophores are added to each terminus of the same protein. Although effective in illuminating a protein of interest within cells, experiments performed with terminally-tagged constructs are potentially limited by the properties of the terminus of the protein. Many proteins, including the InsP₃R, possess domains at their termini that perform a variety of functions: localization, CaM binding, phosphorylation, protein-protein interaction [23, 24]. In addition, many proteins function as multimers or aggregates, and require binding to similar peptides to perform their physiological role. GFP is a 238 amino acid protein [28], and its presence can interfere with the targeting of accessory molecules or posttranslational modifications.

Random GFP insertion is a powerful tool. It has been used to insert GFP into the coding sequence of several different proteins [20, 22]. The N' and C' termini of GFP are proximal, which allows it to retain fluorescence while minimally interrupting proper protein folding when inserted into the middle of a coding sequence. In-frame insertions are easily screened

by the appearance of fluorescence in transfected cells. The use of random insertion by the transposon allows the identification of insertion sites that would not be predicted by rational design. By coupling GFP to the InsP₃R at multiple, independent locations, Sheridan and Hughes (2004) created 18 unique green fluorescent InsP₃R constructs. By performing experiments with multiple constructs in parallel, we have a means of verifying that any observation made is independent of the position of the fluorophore within our protein of interest.

We have demonstrated that internally fluorescent GFP-InsP₃R constructs are expressed and localized to the ER in SH-SY5Y cells, and that expressing cells maintain endogenous, InsP₃-mediated Ca²⁺ signaling. The latter is important, as the possibility of forming heteromers of native and GFP-InsP₃R, coupled with the overexpression of the fluorescent form, could lead to impaired cellular function, and possible alterations of the cytosolic membrane structures. Because we have retained endogenous agonist-stimulated Ca²⁺ release, it is likely that the detected diffusion constant is not due to a pathological condition of the cells themselves. It remains to be determined whether homomeric GFP-InsP₃R are themselves functional, but this has been difficult to assess. To date, no GFP-tagged InsP₃R construct has been conclusively demonstrated to be functional [14–18]. Current attempts are hampered by the ubiquitous expression of InsP₃R isoforms in all cell types [2], the necessity of InsP₃R for cell and organism survival [29], and the difficulty in maintaining InsP₃R knockout cells (B. DeGray, personal communication). A series of DT40 chicken B cell lines devoid of endogenous InsP₃R subtypes has been developed that can be maintained in culture [30]. These cells are promising as a vehicle for the study of exogenous InsP₃R functioning, but they are difficult to transfect and the agonists available for stimulating InsP₃-dependent Ca²⁺ signals are difficult to use. Regardless, the GFP-InsP₃R constructs used in this study do not interfere with endogenous InsP₃-dependent Ca²⁺ signaling nor would their diffusion within the ER membrane be expected to differ.

There is evidence for the presence of external factors in determining InsP₃R lateral mobility. Tateishi et al. (2005) showed that when permeabilized, GFP-InsP₃R expressing COS-7 cells displayed reduced FRAP, and by removing the 4.1N binding site, Fukatsu et al. (2004) showed the linking of InsP₃R to the actin cytoskeleton has an effect on mobility. Recently, several studies have been published which describe activation-induced clustering of InsP₃R to ER “hotspots”, demonstrating a physiological role for this mobility [14, 18]. We have shown that the lateral mobility of GFP-InsP₃R constructs within the ER is impaired when cells are treated with GlcNAc in a manner known to increase O-linked GlcNAc glycosylation of cytosolic proteins [21].

Elevated levels of GlcNAc-modified proteins have been detected in cells isolated from diabetic animals. Streptozotocin, a drug used to induce a diabetic phenotype in rats, binds to and inhibits O-GlcNAcase. This enzyme is responsible for removing GlcNAc from proteins [31] and has been used to remove GlcNAc from the type I InsP₃R [25]. Addition of the monosaccharide GlcNAc in the presence of the transferase that adds O-linked GlcNAc to proteins leads to a reduction in the open probability of the type I InsP₃R and consequentially reduced whole cell Ca²⁺ signaling [25]. We have uncovered a second effect of GlcNAc on InsP₃R, GlcNAc inhibits the ability of InsP₃R to diffuse through the ER membrane. Whether this effect is due to the O-GlcNAcylation of the InsP₃R itself or the contribution of another O- GlcNAcyated protein has not been determined. Although the specific O- GlcNAcyated residues have been identified for a selection of proteins [32, 33], as of yet no consensus binding motif has been established. In particular, the site(s) where the InsP₃R is O-GlcNAcyated have not been determined which means that the contribution of O-GlcNAc modification to InsP₃R mobility cannot yet be evaluated.

With the success of modifying the InsP₃R type I channel with a non-terminal GFP, the technique of GFP insertion may be applied to different isoforms of InsP₃R. With the use of different colored fluorophores, double transfection protocols may be performed to study the different spatial and temporal activation of InsP₃R subtypes within a single cell, as well as to study the possible heterotetramerization of InsP₃R subtypes and their contribution to intracellular Ca²⁺ signaling. GFP insertion is a potential tool for studying the importance of different sites along the InsP₃R. By targeting GFP to known modulation sites or protein interaction sequences, or residues critical for InsP₃ binding, we will be able to follow the effects on whole cell Ca²⁺ signaling without the need for drugs, and can directly compare control and transfected cells within the same culture.

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Fig. 1. GFP-InsP₃R constructs. A schematic of one subunit of the type I InsP₃R, with green dots representing the sites of insertion of GFP for pBFC016 and pBFC013.

Fig. 2. GFP-InsP₃R constructs are targeted to the endoplasmic reticulum. GFP fluorescence of *A*, GFP, *B*, pBFC016- or *C*, pBFC013-expressing live SH-SY5Y cells. Note the reticular distribution of the fluorescence in the GFP-InsP₃R constructs versus the uniform fluorescence of GFP alone. *D*, SH-SY5Y cells transfected with pBFC016. Panels show anti-GFP, anti-type I InsP₃R, and merged signals. There is significant overlap of the two signals in transfected cell, whereas the GFP signal is absent in non-transfected cells. *E*, SH-SY5Y cells transfected with pBFC016. Panels show anti-GFP, anti-SERCA2, and merged signals. There is a reticular pattern of expression, with significant overlap of the SERCA and GFP signals.

Fig. 3. InsP₃R-dependent calcium signaling is intact in GFP-InsP₃R-expressing cells. *A*, pBFC016-transfected and non-transfected cells loaded with rhod-2. *B*, (Ca^{2+})_i measured over time in a pBFC016-expressing (solid green line) and non-transfected cell (solid red line) from the same culture. 1 μ M ATP was applied where indicated. GFP fluorescence from the transfected cell is stable over the period of stimulation (dashed green line). Expressed as $\Delta F/F_0$. *C*, Amplitude of the rhod-2 response to 1 μ M ATP in transfected vs. non-transfected cells.

Fig. 4.

GFP-InsP₃R can undergo FRAP. *A*, pBFC016-transfected SH-SY5Y cell before and just after bleaching, and after recovery of fluorescence has occurred. Circles indicate the bleached regions. Bar represents 5 μ m. *B, C* Time course of fluorescence recovery for *B*, pBFC016 or *C*, GFP alone. (○) represents fluorescence of an unbleached region; (●) represents fluorescence of a bleached region from the same cell as (○). Data were background subtracted and normalized to baseline fluorescence. The solid line represents a fit of the data to a curve of exponential rise to max.

Fig. 5.

GlcNAc can inhibit fluorescent recovery of bleached GFP-InsP3R. Fluorescent images of *A*, pBFC016- or *B*, pBFC013-transfected cells treated for 72h with 8mM GlcNAc before and just after bleaching, and after a period of time equal to the recovery from Fig. 4. Circles indicate the bleached regions in each cell. Bars represent 5 μ m. *C,D*, Summary of the percentage recovery as determined by the amount of fluorescent recovery for *C*, pBFC016- or *D*, pBFC013-transfected SH-SY5Y cells without (upper) or with (lower) prior GlcNAc treatment. Solid line represents the mean for each group. Note that only the GlcNAc treated cells show a number of cells which do not recover.