Decreas O-Linked GlcNAcylation Protects from Cytotoxicity Mediated by Huntingtin Exon1 Protein Fragment*

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Background: Earlier reports indicate that O-GlcNAcylation might be protective in neurodegenerative disorders.

Results: Suppressing O-GlcNAcylation modulates autophagy to enhance the viability of neuronal cells expressing cytotoxic mutant huntingtin exon 1 protein (mHtt).

Conclusion: O-GlcNAcylation regulates the clearance of mHtt by modulating the fusion of autophagosomes with lysosomes.

Significance: This regulatory mechanism emerges as a novel therapeutic strategy for Huntington disease.

O-GlcNAcylation is an important post-translational modification of proteins and is known to regulate a number of pathways involved in cellular homeostasis. This involves dynamic and reversible modification of serine/threonine residues of different cellular proteins catalyzed by O-linked N-acetylglucosaminyltransferase and O-linked N-acetylglucosaminidase in an antagonistic manner. We report here that decreasing O-GlcNAcylation enhances the viability of neuronal cells expressing polyglutamine-expanded huntingtin exon 1 protein fragment (mHtt). We further show that O-GlcNAcylation regulates the basal autophagic process and that suppression of O-GlcNAcylation significantly increases autophagic flux by enhancing the fusion of autophagosomes with lysosomes. This regulation considerably reduces toxic mHtt aggregates in eye imaginal discs and partially restores rhabdomere morphology and vision in a fly model for Huntington disease. This study is significant in unraveling O-GlcNAcylation-dependent regulation of an autophagic process in mediating mHtt toxicity. Therefore, targeting the autophagic process through the suppression of O-GlcNAcylation may prove to be an important therapeutic approach in Huntington disease.

O-GlcNAcylation is a glucose-dependent post-translational modification. When glucose enters the cell, ~5% of it enters into the hexosamine biosynthetic pathway through a series of metabolic transformations and finally gets trans-
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functional protein, whereas expansion of CAG repeats coding for >40 glutamines as a repeat track results in a dominant mutation, a consequence of which is that the mutant Htt loses its proper folding state, tends to aggregate, and becomes cytotoxic (12, 13). The wild-type huntingtin protein plays important roles in normal functioning of the brain such as vesicular transport, neuronal gene transcription, and BDNF production (14) and may also function as an anti-apoptotic protein (15). The mHtt aggregates interfere with normal synaptic transmission (16), impair axonal transport of mitochondria (17), sequester crucial transcription factors (18), and hamper their functioning.

O-GlcNAcylation is a nutrient-sensitive protein modification. With the emerging understanding of the important roles of O-GlcNAcylation in various neurodegenerative disorders along with reports about glucose-dependent regulation of protein clearance machineries (19, 20) and that of protein aggregation-mediated toxicity (21, 22), we aimed to explore the role of this glucose-dependent post-translational modification in the regulation of mHtt-mediated toxicity and its clearance. We report here that suppression of O-GlcNAcylation increases basal autophagy flux by enhancing autophagosome-lysosome fusion and helps in the clearance of toxic aggregates of mutant huntingtin exon 1-coded protein, thereby increasing survival and suppressing the degenerative phenotypes in cellular and Huntington fly models, respectively.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Cell culture media and drugs (aza-serine, glucosamine, 3-methyladenine, and bafilomycin A1) were purchased from Sigma. Polyfect transfection reagent was from Qiagen India Pvt. Ltd., India. Antibodies were procured from following sources: anti-LC3 and anti-Myc for immunostaining (Cell Signaling Technology); anti-p62 (Enzo Life Sciences); anti-GFP and anti-Myc for immunoblot (Roche Applied Science); anti-HA as used in experiments done in Neuro2A cells and anti-O-GlcNAc and anti-γ-tubulin (Sigma). Rabbit polyclonal anti-hemagglutinin (used in experiments with Drosophila) was from Santa Cruz Biotechnology, and secondary antibodies were procured from Jackson ImmunoResearch Inc., except anti-rabbit conjugated with Cy3 (Sigma) and Alexa-Fluor 488 were from Molecular Probes.

Expression Constructs—Mammalian expression constructs were obtained from the following sources. The GFP-tagged truncated Huntingtonin Q97 expression vector was generously provided by Dr. Lawrence Marsh (University of California at Irvine). Plasmid coding for OGA (Myc-tagged) was a kind gift from Dr. John A. Hannover (NIDDK, National Institutes of Health); the HA-tagged OGT was gifted by Dr. Gerald W. Hart (The Johns Hopkins University School of Medicine, Baltimore, MD); the mRFP-GFP-LC3 construct was gifted by Dr. T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan), and construct coding for the mutant form of α-synuclein as GFP fusion was a gift from Dr. Peter Lansbury (Harvard Medical School).

Cell Culture, Treatment, and Transfection—The experiments were conducted in the murine neuroblastoma cell line Neuro2A under normal glucose conditions (25 mM). Neuro2A cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were treated with 40 μM asazaserine (inhibitor of O-GlcNAcylation), 10 mM glucosamine (inducer of O-GlcNAcylation), 100 nM bafilomycin A1 (inhibitor of the fusion of autophagosomes with lysosomes), and 10 mM 3-methyladenine (3MA; an inhibitor of autophagosome formation). The cells were transiently transfected with expression constructs at around 50% of confluence using PolyFect transfection reagent (Qiagen) as recommended by the manufacturer. Under these conditions, the transfection efficiency was consistent and around 70% as assessed by microscopic observation of the fluorescence positive cells transiently expressing the GFP-tagged protein. In all the experiments, the cells were harvested at 36 h post-transfection, and wherever required, treatment with the pharmacological agents was given for the last 12 h unless stated otherwise.

Fly Stocks and Rearing Condition—All fly stocks were maintained under uncrowded conditions at 24 ± 1°C. For each experiment, regular or asazaserine (250 μg/ml)-supplemented food was prepared from the same batch. Using the w1118; UAS-httex1p Q93/CyO (20) and w1118; UAS-httex1p Q93/CyO (21) fly stocks, appropriate genetic crosses were set to obtain w1118; UAS-httex1p Q93/CyO (20) and w1118; UAS-httex1p Q93/GMR-GAL4 (21) fly stocks for the experiments. The GMR-GAL4 driver targets expression of the UAS-httex1p Q93 transgene in developing eye discs (23) and thereby induce retinal neurodegeneration (24). In some cases, Oregon R+ stock was used as wild type. Freshly hatched larvae for a given experiment were derived from a common pool of eggs of the desired genotype and reared in parallel on regular or asazaserine-supplemented food.

We also reared larvae on food supplemented with glucosamine (1, 10, or 25 mg/ml). However, in each case, all the larvae died before reaching third instar stage, and therefore, no further studies on the effect of glucosamine on polyglutamine (polyQ) degeneration in the fly model could be carried out.

Immunostaining—Cells on coverslips were fixed with 4% paraformaldehyde in 1 × PBS for 20 min followed by permeabilization for 5 min in 1 × PBS with 0.05% Triton X-100. The expression of Myc-OGA and HA-OGT was checked by probing with anti-Myc or anti-HA antibodies followed by FITC- or TRITC-conjugated secondary antibodies, respectively. Nuclei were counterstained with 10 μM 4’,6-diamidino-2-phenylindole (DAPI). Images were obtained with a Nikon (Japan) Eclipse 80i fluorescence microscope using a ×10 or ×40 objective lens.

Eye discs from wandering late third instar GMR-GAL4 > UAS-httex1p Q93 larvae reared on normal or asazaserine-supplemented food were dissected and immunostained as described previously (25) with anti-HA (1:80 dilution, Santa Cruz Biotechnology). Chromatin was counterstained with DAPI. Immunofluorescence-stained eye discs were examined with a Zeiss LSM 510 meta confocal microscope using appropriate lasers, dichroics, and filters.

Cell Death Assay—For the MTT assay, cells were treated with asazaserine or glucosamine for 12 h or transfected for 36 h, and thereafter, cells were incubated with 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT) (Sigma) and chased for 2 h. After removal of the medium, cells were incubated with DMSO...
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(100%) for 10 min to dissolve formazan crystals. The change in optical density was recorded through spectrophotometer at $\lambda_{570\ nm}$ against background reading at $\lambda_{650\ nm}$. Alternatively, treated or transfected cells were fixed, permeabilized, and stained with DAPI as mentioned for immunostaining, and the apoptotic nuclei were scored in a blinded fashion as reported earlier (26).

Quantification of LC3-positive Cytoplasmic Puncta—Cells transiently expressing the tandem mRFP-GFP-LC3 construct were fixed, and the fluorescence images of about 50 cells for each set were examined using a Zeiss AxioImager 2 microscope outfitted with an ApoTome accessory. The green, red, and yellow puncta in the captured images were quantified using the co-localization macro in ImageJ software, as described (27).

Immunoblotting—Protein samples were resolved on 6–12% SDS-PAGE as required and transferred to nitrocellulose membrane (MDI, India). Thereafter, the membranes were blocked with either 5% nonfat dry milk powder or 5% BSA in 1× TBST and probed sequentially with the desired primary and secondary antibodies at their recommended dilutions followed by detection with a chemiluminescent detection kit (Supersignal West Pico, Pierce).

Filter Trap Assay—The filter trap assay was carried out essentially as described by Juenemann et al. (28). Briefly, the pellet fraction of the cell lysate was suspended in the benzonase buffer (1 mM MgCl$_2$, 50 mM Tris/HCl, pH 8.0) and treated with an RNase/DNase mixture (50 units each; Fermentas) and incubated at 37 °C. The reaction was arrested with the addition of 2× termination buffer (40 mM EDTA, 4% SDS, 100 mM DTT), and 50 µg of the sample was mixed in 2% SDS buffer (2% SDS, 150 mM NaCl, 10 mM Tris/HCl, pH 8.0) and filtered through a 0.2-µm pore size cellulose acetate membrane (GE Healthcare) using a slot blot apparatus (Bio-Rad). The filter membrane was used for immunodetection as described for the immunoblot.

Proteasome Activity Assays—Cells that were either transfected or treated with the indicated drugs (12 h) were harvested in lysis buffer (1% PBS, 0.1% Triton X-100, 0.5% Nonidet P-40), and the cleared lysate was used for the proteasome activity assay using a fluorogenic proteasome substrate (N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; Calbiochem). Briefly, 10 µg of protein for each sample was incubated in a reaction buffer, and the generation of fluorescent signal was measured using a spectrofluorometer (PerkinElmer Life Sciences) as recommended by the manufacturer. Reactions in the presence of the proteasomal blocker, MG132, served as control.

Pseudopupil Analysis—Heads of 1-day-old GMR-GAL4 > UAS-htxex1p Q93 flies, reared because the first instar larval stage on normal or azaserine-supplemented food, were decapitated, and the arrangement of photoreceptor rhabdomeres in the ommatidia of compound eyes was visualized by the pseudopupil technique (29) using $\times 63$ (NA = 1.4) oil objective on a Nikon E800 microscope, and the images were recorded with a Nikon DXM 1200 digital camera. The total number of flies observed for each group was 50.

Phototaxis Assay—Phototaxis of adult flies was assayed using a Y maze consisting of a Y-shaped glass tube of 12-mm internal diameter and 30-cm length of each arm. Twenty replicates, each with 10 flies, were carried out for each feeding regime and age of flies. Wild-type Oregon $R^+$ flies were used as positive control. The same sets of flies were used for phototaxis assay on days 0, 5, 10, and 15.

Statistical Analysis—Sigma Plot 11.0 software was used for statistical analysis. For cell biology assays, data were analyzed by two-tailed, unpaired Student’s $t$ test. For assays involving flies, one-way analysis of variance was performed for comparison between the control and formulation-fed samples. Pooled data are expressed as mean ± S.E. of means of the different replicates of the experiment.

RESULTS

Global Suppression of O-Linked Glycosylation Reduces the Aggregation Propensity and Cytotoxicity of Mutant Huntingtin in a Cellular Model—Based on previous findings (21, 22), we were interested in exploring the role of O-GlcNAcylation in suppressing the cytotoxicity caused by aggregate-prone proteins. For this, we used a mammalian expression construct that codes for the OGT or OGA, the two proteins that work antagonistically to regulate the O-linked protein glycosylation. Transient expression of OGT in the murine neuroblastoma cell line Neuro2A resulted in increased global O-GlcNAcylation, although overexpression of OGA led to a reduction in global O-GlcNAcylation (Fig. 1A). To check if O-GlcNAcylation could alter the aggregate-forming propensity of mutant huntingtin, we co-expressed OGA or OGT with an expression construct coding for the amino-terminal huntingtin protein having 97 polyglutamine repeats tagged with green fluorescence protein (mHtt-Q97-GFP) in Neuro2A. Cells that expressed only the mHtt-Q97-GFP served as control (co-transfected with the empty vector pcDNA). As shown in Fig. 1, B and D, co-expression of OGA resulted in a significant reduction in the number of transfected cells showing the mHtt-Q97-GFP-positive aggregates as compared with the control cells that were co-transfected with the empty vector, pcDNA. Conversely, OGT co-expression resulted in a higher proportion of cells with the mHtt-Q97-GFP aggregates (Fig. 1, B and D). Co-expression of OGA or OGT did not affect the transfection efficiency of the mHtt-Q97-GFP coding construct (see the insets, Fig. 1D). Similarly, there was no significant difference in cell survival when OGA or OGT was expressed alone as compared with cells that were transfected with an empty vector (Fig. 1C). To test further whether the reduction in the global O-GlcNAcylation helps the cell to reduce the aggregation of mHtt-Q97-GFP, we evaluated the total and SDS-insoluble forms of mHtt-Q97-GFP by immunoblot and the filter trap assay, respectively (Fig. 2A). Consistent with our observations on mHtt aggregates in situ, co-expression of OGA led to a significant reduction in the level of SDS-insoluble forms of mHtt-Q97-GFP when compared with that in cells co-expressing OGT or only the mHtt-Q97-GFP (pcDNA control; Fig. 2A). Co-expression of OGA or OGT did not show significant change in the total level of mHtt-Q97-GFP in the immunoblots (Fig. 2A). We also found that co-expression of OGA, but not of OGT, resulted in a significant reduction in mHtt-Q97-GFP-mediated cell death, as measured by the MTT assay (Fig. 2B), and also by scoring apoptotic nuclei (Fig. 2D). To check whether the protective effect of OGA was limited to
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mtHtt-Q97-GFP or whether OGA can ameliorate the toxicity of other disease-associated cytotoxic proteins, we expressed Parkinson disease-associated α-synuclein mutant A30P protein (26) either alone or along with OGA or OGT and measured the cell viability by MTT assays as well as by counting the apoptotic nuclei. As shown in Fig. 2, C and E, OGA, but not OGT, was able to confer protection against the toxicity of the A30P mutant, suggesting that the OGA-mediated protective response could be a generic effect of O-GlcNAcylation and is not specific to mtHtt-Q97-GFP. Taken together, our results suggest a causal role for O-GlcNAcylation in modulating the level of insoluble, aggregated mutant huntingtin and its cytotoxicity.

Inhibition of O-GlcNAcylation Enhances Autophagy—Our next aim was to identify the mechanism by which suppression of O-GlcNAcylation reduces the level of the cytotoxic and insoluble form of mtHtt-Q97-GFP. Because the autophagic process is known to clear the aggregated proteins (30, 31), we were interested in testing the impact of O-GlcNAcylation in the basal autophagic process. For this, Neuro2a cells were transfected with the expression construct coding for OGT or OGA or with an empty vector (pcDNA control). At 36 h post-transfection, the cells were harvested, and the levels of two autophagic marker proteins, LC3 and p62, were evaluated. As shown in Fig. 3A, transient overexpression of OGA led to a reduction in the level of both p62 and LC3II, suggesting that suppression of O-GlcNAcylation resulted in an enhanced autophagic flux. To further confirm that the observed effect is indeed because of the changes in global O-GlcNAcylation, we examined the autophagic process after treating the cells with azaserine, which inhibits glutamine fructose-6-phosphate amidotransferase, one of the key enzymes of the hexosamine bio-synthesis pathway and thereby inhibits O-GlcNAcylation (32, 33). As shown in Fig. 3B, treatment of Neuro2A cells with azaserine for 12 h resulted in a significant reduction in global O-GlcNAcylation levels. As was observed for OGA expression, azaserine also led to a reduction in the level of the autophagic markers LC3II and p62 (Fig. 3C), confirming that a reduction in the cellular O-GlcNAcylation level correlates with increased levels of basal autophagic flux. To further confirm that the observed effect of azaserine on the autophagic process is indeed through O-GlcNAcylation process, we treated cells both with azaserine and glucosamine and looked at the level of LC3II and p62. Glucosamine is known to rescue the effect of azaserine on the O-GlcNAcylation process; hence, the double treatment should rescue the effect of azaserine on the autophagic process (Fig. 3B). As shown in Fig. 3C, azaserine-glucosamine treatment increased the level of LC3II and p62 as compared with only azaserine treatment, confirming that the level of autophagic induction inversely correlates with the O-GlcNAcylation level.

Our next aim was to identify the key step through which the O-GlcNAcylation regulates the autophagic process. The reduction in the level of the autophagy marker LC3II upon depletion of glycosylation may be because the autophagosome formation is inhibited (inhibition of autophagy initiation) or due to the enhanced degradation of LC3II (increased autophagic flux) via lysosome because LC3 itself is an autophagy substrate (34). Our observation that the cellular level of another autophagic substrate, p62, was also at lower levels upon OGA overexpression suggests that the second possibility is more likely. Therefore, we checked whether inhibition of fusion of the autophagosome with the lysosome would rescue the level of LC3II and p62. For
this, the cells were transfected with the expression construct coding for OGA or OGT and then were treated with bafilomycin A1 (BafA1), an inhibitor of autophagosome lysosome fusion (34), for 12 h, and then the cellular levels of LC3II and p62 were evaluated. As shown in Fig. 4A, we found that the BafA1-mediated inhibition of the autophagosome-lysosome fusion led to an increase in the level of both LC3II and p62 even in those cells that overexpressed OGA or OGT. Very similar observations were made when the glycosylation was inhibited by azaserine treatment (Fig. 4B). To further confirm that suppression of O-GlcNAcylation indeed increases the autophagy flux, we utilized the tandem mRFP-GFP-LC3 expression construct whose expression product is known to show differences in pH sensitivity and has been widely used to monitor the autophagic process (35). For this, the Neuro2A cells were transiently transfected with the mRFP-GFP-LC3 tandem construct and empty vector (pcDNA), or along with the expression vector coding for OGA or OGT, and scored the co-localization of green and red signals in the cytoplasmic LC3-positive puncta and also the number of green and red puncta. Here, autophagosomes are visible as yellow puncta and autophagolysosomes (post-lysosomal fusion) as red puncta (35). As shown in Fig. 5, co-expression of OGA led to a significant increase in the fraction of red/green-positive LC3 puncta, although no such difference was noted for OGT. Similarly, there was a significant increase in the LC3 puncta that were positive only for red fluorescence (Fig. 5), suggesting that suppression of O-GlcNAcylation did enhance the autophagy flux.

Next, we tested whether the reduction in the level of the insoluble fraction of mutant huntingtin seen in the O-GlcNAcylation-deprived condition is due to an enhanced autophagy flux. As shown in Fig. 6A, BafA1 treatment led to an increase in the level of insoluble fraction of the mutant huntingtin even in the OGA-overexpressing cells, suggesting that decreased O-GlcNAcylation promotes the clearance of the aggregate-prone protein by enhancing autophagosome-lysosome fusion. Finally, to demonstrate that autophagy is the mechanism through which O-GlcNAcase is able to protect cells from huntingtin aggregates, we treated cells that co-express mtHtt-Q97-GFP and OGA with an autophagy inhibitor, 3-MA. As shown in Fig. 6B, 3-MA treatment led to a significant increase in the insoluble form of mtHtt-Q97-GFP even when OGA was co-expressed, suggesting that the protective effect conferred by OGA is indeed through the autophagic process.

Having shown an indirect correlation between O-GlcNAcylation and autophagic flux, we checked the possible effect of O-GlcNAcylation on proteasomal activity. For this, the cells that transiently expressed OGA or OGT or that were

FIGURE 2. O-GlcNAcylation inhibition reduces mHtt-Q97-mediated cytotoxicity. A, Western blot images of the insoluble, aggregated form of mHtt-Q97-GFP in filter trap assay using a slot-blot apparatus (top) or its total form resolved by immunoblotting (bottom) when expressed with either pcDNA (empty vector control), OGT, or OGA, as indicated in the middle. Expression of OGT and OGA was established by probing them with anti-HA and anti-Myc antibodies, respectively. The bar diagram above shows the fold changes in signal intensities, based on densitometric analysis of the SDS-insoluble and -aggregated form of mHtt-Q97-GFP (normalized to total level detected in the immunoblot; n = 3; ***, p < 0.001; *, p < 0.1). B and C, bar diagrams representing the fold change in the viability of cells expressing mHtt-Q97-GFP (B) or the α-synuclein mutant A40P (C) as measured by an MTT assay. Cells transfected with the indicated constructs were processed for the measurement, and in each set the value obtained for the GFP-transfected cells was considered as 1, and the relative values obtained for indicated combinations were plotted. D and E, bar diagram showing the percentage of cells expressing mHtt-Q97-GFP (D) or the α-synuclein mutant A40P (E) with abnormal (apoptotic) nuclei (as shown in F) as compared with cells that expressed GFP (control) when co-transfected with OGA or OGT coding constructs (in B–E, n = 3; ***, p < 0.05; ***, p < 0.005 on Student’s t test). F, representative images showing a normal (left) and an abnormal (apoptotic; right) nuclei as judged by DAPI staining (scale, 5 μm).
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A

B

C

FIGURE 3. O-GlcNAcylation modulates autophagy. A, immunoblots (bottom panel) of Neuro2A cells, transiently expressing pcDNA empty vector alone or OGA-Myc or OGT-HA for 36 h, to show levels of the autophagic markers LC3II and p62. Probing with anti-γ-tubulin served as loading control. Note the change in the level of LC3II band (identified by an arrow) in cells that expressed OGA. Co-expression of OGT did not show such an effect. Bar diagrams above show the fold changes in signal intensities of the LC3II and p62 (both normalized to γ-tubulin signal) bands when compared with the control (pcDNA transfected cells). B, Neuro2A cells were grown in a medium with or without azaserine and/or glucosamine for 12 h as indicated, and the changes in the global glycosylation level were evaluated. The bar diagrams above show the fold changes in the glycosylation levels compared with cells that were fed with glucose. C, samples shown in B were tested for the level of autophagy markers LC3 and p62 as indicated. Note the reduction in the intensity of the band for LC3II (identified by an arrow) and p62 in the azaserine-treated cells and their restoration in the azaserine/glucosamine double-treated cells. Bar diagrams above represent the fold changes in the signal intensities for LC3II and p62 (both normalized to γ-tubulin signal) bands compared with the control (glucose-fed cells) (in A–C, n = 3; *, p < 0.5; **, p < 0.05; ***, p < 0.005 on Student’s t test).

A, B, or C, samples shown above show the fold changes in signal intensities for LC3II and p62 (both normalized to γ-tubulin signal) bands compared with the control (glucose-fed cells) (in A–C, n = 3; *, p < 0.5; **, p < 0.05; ***, p < 0.005 on Student’s t test).

Azaserine Feeding Partially Restores the Rhabdomere Morphology and Suppresses the Progressive Loss of Vision in GMR-GAL4 > UAS-httex1p Q93-expressing Flies—The external eye morphology and vision of freshly eclosed GMR-GAL4 > UAS-httex1p Q93 flies are near normal. However, these flies show a progressive age-dependent degeneration, becoming almost completely blind by 10 days (36–38). As known from earlier studies (36–38), the eye surface of GMR-GAL4 > UAS-httex1p Q93 flies did not show any appreciable change with age in any of the feeding regimes (data not shown). However, as also reported earlier (36, 38), the pseudopupil images of rhabdomeres of 1-day-old GMR-GAL4 > UAS-httex1p Q93-expressing flies fed on a normal diet showed severely degenerated rhabdomeres so that, unlike the stereotyped pattern of rhabdomeres in pseudopupil image of eyes of wild-type flies (Fig. 9A), no distinct rhabdomeres were seen in their eyes (Fig. 8B). Interestingly, GMR-GAL4 > UAS-httex1p Q93 flies reared on the azaserine-supplemented food displayed at least some organized rhabdomere-like structures in ~60% flies (Fig. 8C).

Expression of UAS-httex1p Q93 in eye cells with the GMR-GAL4 driver causes progressive neuronal degeneration of the photoreceptor neurons so that the flies lose their vision as they age (36, 38). To examine whether the azaserine-mediated restoration of the rhabdomeric organization improved the vision of flies, we tested the functionality of vision in 5-, 10-, and 15-day-old flies (wild type and GMR-GAL4 > UAS-httex1p Q93) by the phototaxis behavioral assay, which examines the choice of flies to move between illuminated and dark chambers. Although nearly all wild-type flies of different ages moved to the
illuminated chamber (positive phototaxis), the GMR-GAL4 > httex1p Q93 flies reared on normal food progressively lost their vision so that the proportion of flies selecting the lighted chamber declined with age (Fig. 9D). By day 10, these flies became nearly blind because they moved randomly between the dark and light chambers (Fig. 9D). Significantly, a greater proportion of GAL4 > httex1p Q93 flies reared on azaserine-supplemented food continued to move to the illuminated chamber even on day 15 (Fig. 9D). Thus, azaserine feeding partially restored the vision in GAL4 > httex1p Q93 flies so that the proportion of flies selecting the illuminated chamber was significantly higher on each day of phototaxis assay than in those grown on normal food (Fig. 9D).

**FIGURE 4.** Suppression of O-GlcNAcylation increases autophagy flux. A, Neuro2A cells at 24 h post-transient transfection with an empty vector (pcDNA) or with a construct coding for OGA or OGT were either left untreated or treated with BafA1 for 12 h as indicated, and the levels of autophagy markers LC3 and p62 were evaluated by immunoblotting. Note the increase in the signal intensities of LC3II (arrow) and p62 in all samples treated with BafA1. The blot was probed with anti-Myc and anti-HA antibodies to show the expression of OGA and OGT, respectively; probing with anti-γ-tubulin served as the loading control. B, immunoblot to show levels of LC3II (arrow) and p62 in Neuro2A cells, as in A, untreated or treated with azaserine, alone or in combination with BafA1 as indicated; γ-Tubulin served as the loading control. Bar diagrams above represent the fold changes in the signal intensities for LC3II and p62 (both normalized to γ-tubulin signal) bands compared with the control (n = 3; ***, p < 0.005 on Student’s t test).

**FIGURE 5.** Suppression of O-GlcNAcylation increases autophagy flux. 
A, representative images of cells showing LC3-positive puncta in cells that were transiently transfected with mRFP-GFP-LC3 expression construct along with an empty vector (pcDNA) or an expression construct coding for OGA or OGT as indicated. Puncta that are positive both for red and green fluorescence represent autophagosomes, although those positive only for red fluorescence represent autolysosomes (bar, 10 μm). B, bar diagram showing the fraction of puncta positive for both RFP and GFP (yellow) or only the RFP (red) in transiently transfected cells co-expressing mRFP-GFP-LC3 and pcDNA or OGA or OGT, as indicated. n = 3; **, p < 0.1; ***, p < 0.01; ****, p < 0.001 on Student’s t test.

**FIGURE 6.** Suppression of O-GlcNAcylation increases autophagy flux. Western blots showing changes in the levels of insoluble, aggregated form of mHtt-Q97-GFP (filter trap assay; top) or its total form (immunoblotting; bottom) when expressed with OGA and treated or not treated with BafA1 (A) or 3-MA (B) as indicated. The bar diagrams, shown above, represent fold changes in the signal intensity of the SDS-insoluble, aggregated form of mHtt-Q97-GFP (normalized to total level detected in the immunoblot) as measured by densitometric analysis (n = 3; *, p < 0.1; **, p < 0.01; ***, p < 0.005 on Student’s t test).
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DISCUSSION

Dynamic modification of Ser/Thr residues of proteins by O-linked N-acetylglucosamine (O-GlcNAc) is an important post-translational modification for cellular signaling (1, 3, 39). More than 500 proteins involved in diverse cellular functions, including the transcription, translation, metabolism, and stress response, have been identified to undergo this modification (1, 3, 39). It is significant that about 270 of these proteins are known in the brain tissue alone (40). Therefore, it is not surprising that aberrant O-GlcNAcylation is associated with various disorders, including the neurodegenerative disorders (3–11).

A common pathological feature of many neurodegenerative diseases, including Alzheimer, Parkinson, and Huntington diseases, is the accumulation/aggregation of one or more proteins in different regions of the brain, which is believed to underlie neurodegeneration (12, 37, 41). These proteotoxic aggregates are cleared by coordinated action of the cellular proteolysis system (ubiquitin-proteasome system and autophagy-lysosomal pathways) and molecular chaperones (12, 30). Although the regulation of UPS by O-GlcNAcylation is fairly well understood, there are contrasting reports about a protective role of O-GlcNAcylation in neurodegeneration. For example, it is shown that O-GlcNAcylation of ubiquitin-activating enzyme E1 promotes ubiquitination (42) and is thus expected to enhance protein degradation, the same modification in the Rpt2 ATPase subunit of the proteasome inhibits its ATPase activity and suppresses proteasome function (43), which would lead to the accumulation of ubiquitinated proteins. Interestingly, it is shown that elevated O-GlcNAcylation in brain inhibits proteasome function and promotes neuronal apoptosis (44).

We find that overexpression of either OGT or OGA led to significant reduction in the proteasome activity in our cellular model, and this corroborates well with a recent report on proteasomal function in Caenorhabditis elegans mutants for OGT or OGA (45). However, we did not find any difference in the proteasome activity when the cells were treated with azaserine or glucosamine for the duration and concentration used, suggesting that the level and/or activity of OGT and OGA, rather than flux through the hexosamine biosynthetic pathway alone, is/are more critical in modulating the activity of the proteasome. In view of these observations, and existing reports that accumulation of protein aggregates blocks proteasome function (46, 47), it appears that proteasome alone might not be

FIGURE 7. Effect of O-GlcNAcylation on proteasomal activity. Bar diagram showing fold change in the proteasomal activity in cells transiently transfected with a construct coding for OGA, OGT, or an empty vector (pcDNA) (A) or with the drug azaserine or glucosamine (B) in the presence or absence proteasomal blocker MG132, as indicated (n = 3; *, p < 0.5; ***, p < 0.05 on Student’s t test).

FIGURE 8. Azaserine feeding reduces accumulation of mutant Huntington protein in fly model. A–D, confocal projection images (projections of four consecutive optical sections which show the morphogenetic furrow) of eye imaginal discs of late third instar GMR-GAL4 > UAS-httex1p Q93 Drosophila larvae, reared from the first instar stage onward to normal (A and B) or azaserine-supplemented food (C and D), immunostained for HA-tagged mutant Htt (green, A–D, identified as “PolyQ”); nuclei are counterstained with DAPI (blue, B and D). The insets in A and C are higher magnification images of a part of the eye discs in A and C, respectively, to more clearly show the polyQ aggregates, which are very abundant in A but nearly absent in C. Arrows in B and D indicate position of the morphogenetic furrow. Scale bar in A represents 20 μm and applies to A–D. E, immunoblot of total proteins from heads of 1-day-old GMR-GAL4/UAS-httex1p Q93 flies, reared on normal (−) or azaserine-supplemented (+) food because the first instar stage, probed with anti-HA antibody, was used to detect Htt-Q93 protein. F, histograms show mean relative levels of HA-tagged polyQ protein (mean ratios of Htt-Q93 and γ-tubulin densities) determined from triplicate immunoblots as in E; the mean ratio of HttQ93 and γ-tubulin densities in Aza-food was taken as 1. (*, p < 0.001.)
Decreasing O-Linked GlcNAcylation Increases Autophagic Flux

**Figure 9.** Azaserine feeding suppresses mHttQ93-induced neurodegeneration in adult *Drosophila* eyes and reduces the age-dependent loss of vision. A, pseudopupil images of eyes of 1-day-old wild type, or GMR-GAL4 > UAS-httex1p Q93 flies grown on control (B), or on azaserine-containing food (C). Arrow in C indicates the presence of two distinct rhabdomeres in one of the ommatidial units; these are not seen in any ommatidial unit in control flies. Scale bar in A indicates 20 μm and applies to A–C. D, histograms showing phototaxis (percent flies moving to illuminated chamber, y axis) of wild type and GMR-GAL4 > UAS-httex1p Q93 flies reared on control or azaserine-supplemented food on different days (x axis) after emergence. Each value in the bar diagram is the mean of 20 replicates with 10 flies in each set. The * in bar diagrams indicates the p value to be <0.05 when comparing the mean phototaxis of GMR-GAL4 > UAS-httex1p Q93 flies reared on control (Cont) and azaserine (Azo)-supplemented food, respectively, on days 5, 10, and 15.

sufficient to clear these cytotoxic aggregates. This notion is strengthened with the emerging understanding of the role of autophagy in degradation of such aggregates in cell and animal models (12, 27, 48) and that the identification of novel regulators of autophagy that help in the clearance of toxic protein aggregates is important. Considering the established fact that O-GlcNAcylation acts as a nutrient sensor (3, 49) and the role of nutrients (serum amino acid, glucose) in regulation of the autophagic process (50, 51), we hypothesized that changes in the O-GlcNAcylation level might modulate the autophagic process. Interestingly, we found here that inhibition of O-GlcNAcylation, either by overexpressing OGA or by azaserine treatment, decreased the polyQ aggregation by enhancing the autophagic process is in agreement with a recent finding that cardiac O-GlcNAcylation regulates autophagic signaling in the rat model of type II diabetes (53). Interestingly, a recent report, which appeared while this manuscript was in preparation, by Wang et al. (45), in the C. elegans model of human neurodegenerative diseases also indicates that the suppression of O-GlcNAcylation decreases neurodegeneration. Taken together, our *in vitro* and *in vivo* findings indicate that inhibition of O-GlcNAcylation stimulates autophagy and thereby reduces the load of proteotoxic huntingtin aggregates and provides protection from neurodegeneration.

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