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N-Linked Deglycosylated Melanopsin Retains Its Responsiveness to Light[†]

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ABSTRACT: Melanopsin is an opsin expressed in the plasma membrane of retinal ganglion cells that mainly project to the circadian clock and thus is important for nonvisual responses to light. Rat melanopsin contains two potential sites (Asn31 and Asn35) for N-linked glycosylation in the N-terminal extracellular part. To investigate if melanopsin is N-linked glycosylated and whether N-bound glycans influence the response of melanopsin to light as evidenced by *Fos* mRNA induction, we transfected PC12 cells to stably express rat wild-type melanopsin or mutant melanopsin lacking both N-linked glycosylation sites. Immunoblotting for membrane-bound melanopsin from the PC12 cells transfected to express wild-type melanopsin disclosed two immunoreactive bands of 62 and 49 kDa. Removal of N-linked glycosylation by tunicamycin or PNGase F changed the 62 kDa band to a 55 kDa band, while the 49 kDa band corresponding to the core melanopsin protein was unaffected. Likewise, mutation of the two extracellular N-linked glycosylation sites gave a melanopsin size comparable to that of PNGase F or tunicamycin treatment (55 kDa). Further in vitro O-linked deglycosylation of wild-type or mutant melanopsin with O-glycosidase and neuraminidase converted the 55 kDa band to a 49 kDa band. Finally, neither in vivo N-linked deglycosylation nor mutations of the two N-linked glycosylation sites significantly affected melanopsin function measured by Fos induction after light stimulation. In conclusion, we have shown that heterologously expressed rat melanopsin is both N-linked and O-linked glycosylated and that N-linked glycosylation is not crucial for the melanopsin response to light.

The mammalian eye is involved in two quite different sensory tasks. In addition to the conventional visual function mediated by the classical photoreceptors, rods and cones, the eye also performs nonvisual photoresponses, including circadian entrainment, constriction of the pupil, acute suppression of activity (masking) and modulation of pineal melatonin synthesis and release (*1*). These nonvisual responses are primarily driven by a subpopulation of retinal ganglion cells, which are intrinsically photosensitive (*2*). The dendrites of these cells form an extensive photosensitive network in the entire inner retina (*2*), while their axons project to the master circadian clock located in the hypothalamic suprachiasmatic nucleus and to other brain areas known to participate in nonvisual responses to light (*3*−*6*). The properties of the intrinsically photosensitive ganglion cells differ from those of rods and cones in many respects. Thus, the ganglion cells are much less sensitive to light than rods and cones, and in contrast to the fast hyperpolarization of the membrane potential in rods and cones, light stimulation causes a sluggish depolarization of the membrane potential in the ganglion cells, exhibiting a maximum at 484 nm (*7*). Furthermore, in the intrinsically photosensitive retinal ganglion cells, bright continuous illumination evokes a long-lasting depolarization that faithfully encodes stimulus energy (*7*) and causes a sustained expression of the immediate early response gene *Fos* (*8*).

The photosensitivity of these retinal ganglion cells is dependent on the novel photopigment melanopsin (*9*−*14*), and it has been shown that heterologous expression of mouse or human melanopsin confers photosensitivity to nonphotosensitive cell lines (*15*−*17*). Melanopsin is an opsin-like protein, which was first identified in dermal melanophores of *Xenopus laevis*, a non-neural cell type that redistributes its pigment in direct response to light (*18*). Subsequently, mammalian melanopsins were cloned, and the occurrence in a small subset of retinal ganglion cells, the only cells known to express melanopsin in mammals, was established. Mammalian melanopsins resemble invertebrate opsins more than vertebrate opsins in structure (*19*, *20*) and in their ability to use light both to initiate the signaling cascade and for reisomerism of the retinal chromophore (*16*, *17*, *20*, *21*) . The deduced amino acid sequence of melanopsin is consistent with the photopigment being a member of the seven-transmembrane receptors which signal through GTP[1] binding proteins (*22*). By immunostaining at the ultrastructural level, Belenky et al.(*23*) showed melanopsin was localized almost entirely in the plasma

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[1]Abbreviations: GTP, guanosine triphosphate; PNGase F, peptide N-glycosidase F; RT-PCR, reverse transcriptase polymerase chain reaction.

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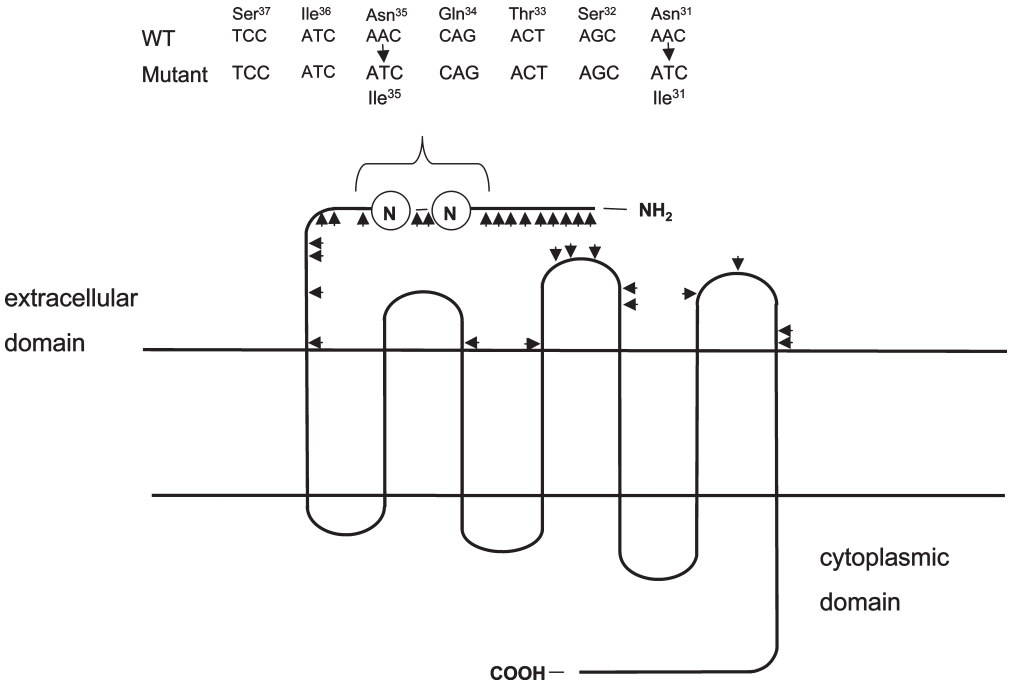

FIGURE 1: Schematic representation of rat melanopsin illustrating the two concensus N-linked glycosylation sites (Asn-X-Ser/Thr) at the amino-terminal extracellular domain. The top panel shows the partial peptide and cDNA sequences of the wild type (WT) and the mutant melanopsin (N31/35I) in which the two asparagines (Asn31 and Asn35) were replaced with isoleucine (Ile). Arrowheads indicate potential extracellular O-linked glycosylation sites.

membrane of the retinal ganglion cells. Immunoblotting of membrane proteins from rat retina and cells heterologously expressing rat melanopsin has revealed a dominant band with an apparent electrophoretic mobility equivalent to a molecular mass of 62 kDa (*24*), although the calculated mass of melanopsin from its amino acid sequence would be approximately 49 kDa. This difference could be due to glycosylation as the N-terminal extracellular part of rat melanopsin contains two potential sites for N-linked glycosylation (Figure 1), i.e., the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (*25*). In addition, a number of potential extracellular O-linked glycosylation sites are also present in the melanopsin sequence (Figure 1).

To investigate if melanopsin is N-linked glycosylated and whether N-bound glycans affect the response of melanopsin to light, we used a clone of rat PC12 cells (PC12-rMel) stably expressing rat melanopsin. First, the electrophoretic mobility of untreated and in vitro and in vivo deglycosylated melanopsin was compared with that of genuine melanopsin. Second, we studied the importance of N-linked glycosylation for the light-induced activation, as evidenced by *Fos* mRNA induction, of both in vivo deglycosylated PC12-rMel cells and a cell clone (mutant N31/35I) expressing melanopsin that lacked both N-linked glycosylation sites.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Rat retina cDNA was prepared using SuperScript (Invitrogen, Carlsbad, CA) and random primers, with total retina RNA as the template. The cDNA was used as a template for amplification of melanopsin cDNA using Platinum *Pfx* DNA polymerase (Invitrogen) and primers flanking the coding region. The entire coding region of melanopsin (196−1623, NCBI accession no. AY072689) was inserted in the KpnI and EcoRV sites of pcDNA3 (Invitrogen). The resulting plasmid, which was verified by sequencing (MWG Biotech, Ebersberg, Germany), was used for expression in PC12 cells.

Site-Directed Mutagenesis of N-Linked Glycosylation Sites. The plasmid described above was used as a template for mutation of the two consensus N-linked glycosylation sites present in rat melanopsin (Figure 1) by substituting the asparagine (AAC) residues Asn31 and Asn35 with isoleucine residues (ATC). Isoleucine was chosen because of its nonreactive and nonpolar side chain, which is consequently not expected to alter the secondary structure. In addition, similar substitutions have been used in studies to eliminate N-linked glycosylation sites in rhodopsin (*26*, *27*). The asparagine to isoleucine residue substitutions were introduced using the mutagenic primers 5′-CAAGGCATTTGGATCAGCACTCAGATCATCTCCGTCAGAG-3′ (forward) and 5′-CTCTGACGGAGATGATCTGAGTGCTGATCCAAATGCCTTG-3′ (reverse) and the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The final construct verified by sequencing was used to make the PC12-rMel 86C1 cell clone expressing melanopsin deficient in N-linked glycosylation, designated mutant melanopsin (N31/35I).

Cell Culture and Transfection. The rat pheochromocytoma PC12 cells obtained from American type Culture Collection (Manassas, VA) were cultured on collagen-coated dishes at 37 °C in a humidified 5% CO_2 atmosphere in RPM1640 medium (Biological Industries, Haemek, Israel) supplemented as previously described (*28*). Lipofectamine2000 (Invitrogen) was used to transfect the cells with either of the two plasmids described above. Stably transfected cell clones were obtained by diluting the cells and selection with 0.5 mg/mL Geneticin (G418) (Invitrogen). The expression of melanopsin was verified by Western blotting, and two cell clones were selected for further studies: PC12-rMel(E8) having strong expression of native rat melanopsin and PC12-rMel-86C1 expressing melanopsin with mutated N-linked glycosylation sites (mutant N31/35I).

Cells for immunocytochemistry were seeded on LAB-TEK Permanox chamber slides (Nalge Nunc, Naperville, IL). The cells were fixed for 15 min in Stefanini's fixative [2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2)], washed three times in PBS, and stored at −20 °C until use.

Light Activation of Melanopsin. PC12-rMel and mutant N31/35I cells were grown to 70% confluence, and on the day of the experiments, the medium was replaced with medium containing a mixture of retinal isomers (10 μM), which was obtained by illuminating *all-trans*-retinal (Sigma-Aldrich, St. Louis, MO). The retinal-containing medium was added to the cells in dim red light, and cells were kept in darkness until light stimulation or harvest as dark controls. Light stimulation was performed using bright white light (> 300 lx). In the majority of the experiments, the cells were exposed to light for 30 min as initial experiments had shown that the peak in *Fos* mRNA expression appeared 30 min after the onset of light stimulation. Illumination for 60 min was used in experiments where stated. To evaluate the retinal dependency of the light activation of melanopsin, induction of *Fos* mRNA after illumination was evaluated both in the absence and in the presence of added retinal.

RNA Preparation, cDNA, and Real-Time RT-PCR Analysis. The cells were placed on ice and harvested by removal of the growth medium, scraped off in ice-cold phosphate-buffered saline, and centrifuged at 420*g* for 5 min at 4 °C, and the pellets were frozen on dry ice. The cell pellets were kept at −80 °C until RNA isolation. Total RNA was prepared by the acid guanidinium thiocyanate/phenol/chloroform extraction method (*29*), and cDNA synthesis of 0.5 μg of RNA was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) in reaction volumes of 50 μL.

Real-time RT-PCR was performed using an ABI 7000 instrument and TaqMan chemistry (Applied Biosystems). Primers and probe for the rat *Fos* assay were designed using Primer Express (Applied Biosystems). Each reaction mixture contained 200 nM probe (ACGCTCCAAGCGGAGACAGATCAACTT) having a 5′-6-FAM reporter and a 3′-TAMRA quencher, 400 nM forward primer (CGGAGGAGGGAGCTGACA), and 600 nM reverse primer (GCAACGCAGACTTCTCGTCCT). The quantity of β2-microglobulin (β_2-m) of the samples was determined as described previously (*30*) and used to normalize the *Fos* mRNA data for variation in template added to the reaction mixtures. cDNA used for standard curves was prepared from 50 μg of total RNA from nerve growth factor-stimulated PC12 cells, which was reverse transcribed in reaction mixtures containing 50 ng/μL RNA. The cDNA was pooled, serially diluted in five 5-fold dilutions, and given arbitrary values. The PCR was performed as previously described except the reaction volumes were 20 μL instead of 25 μL (*30*). No change in the expression of the reference gene β_2-m was observed after light stimulation.

Melanopsin Western Blotting. Melanopsin was detected in crude membrane fractions extracted from stably transfected PC12 cells according to the method of Laabich and Cooper (*31*) with our minor modifications (*24*). Membrane samples from approximately 5×10^5 cells were applied to each well separated on 4 to 12% Bis-Tris NuPAGE gels (Invitrogen) and transferred by semidry blotting to 0.2 μm polyvinylidine difluoride membranes (Invitrogen). The membranes were blocked in 3% nonfat dry milk in PBS with 0.1% Tween 20 for 1 h at room temperature before overnight incubation at 4 °C with rabbit anti-melanopsin antibody (diluted 1:10000, code PA1-781, Affinity Bioreagents Inc., Golden, CO) raised against the C-terminal part of the protein. Visualization was performed using polyclonal swine horseradish peroxidase-conjugated anti-rabbit antibodies (diluted 1:3000, code P0399, Dako, Glostrup, Denmark) followed by ECL Plus chemiluminescence detection (Amersham, Piscataway, NJ).

Melanopsin Immunocytochemistry. A previously characterized homemade anti-melanopsin antibody (41K9, diluted 1:20000) (*10*) was used for the immunocytochemistry. For visualization, biotin-conjugated donkey anti-rabbit serum (711-065-152, diluted 1:800, Jackson Immunoresearch, West Grove, PA), the Tyramide Signal Amplification Biotin System (Perkin-Elmer, Boston, MA), and Cy2-conjugated streptavidin were used (Jackson Immunoresearch).

In Vitro Deglycosylation of Melanopsin. The crude membrane protein from stably transfected PC12 cells was prepared as described above and subjected to in vitro enzymatic N-linked deglycosylation with PNGase F or O-linked deglycosylation with O-glycosidase and neuraminidase. Deglycosylation was conducted under denaturing conditions according to the manufacturer's instructions (E-DEGLY, Sigma-Aldrich) with the following minor adjustments. In brief, 5 μL of prepared membrane proteins was added to 5 μL of denaturation buffer, 20 μL of 5× reaction buffer (both supplied with the kit), and 63 μL of distilled H_2O and the mixture heated at 80 °C for 5 min. After the mixture had cooled to room temperature, 5 μL of 15% Triton X-100 was added followed by addition of 2 μL of each respective enzyme. Deglycosylation was performed at 37 °C for 3 h. Longer incubation (up to 24 h) did not result in further deglycosylation of melanopsin.

Inhibition of N-Linked Glycosylation in Vivo. PC12-rMel cells were grown for 48 h in the presence of 3 μg/mL tunicamycin (Sigma-Aldrich) prior to light stimulation experiments. Tunicamycin inhibits endogenous N-linked glycosylation by blocking the transfer of *N*-acetylglucosamine 1-phosphate to dolichol monophosphate in the endoplasmic reticulum. Preliminary incubation of cells with 1−10 μg/mL tunicamycin for 1−3 days revealed that 3 μg/mL tunicamycin for 48 h resulted in maximum deglycosylation of melanopsin with no visible toxic side effects.

In Vivo N-Linked Deglycosylation. Removal of N-linked glycans displayed on the surface of live cells prior to experiments was performed essentially as described previously (*32*). Cells were grown in six-well plates to confluence, washed twice with Hanks buffer (Invitrogen), and then incubated for 1 h at 37 °C with 1 mL of Hanks buffer containing 100 μL of 10× G7 buffer and 20000 units of PNGase F (glycerol free, 500000 units/mL, catalog no. P0704, New England BioLabs). Cells were washed three times with Hanks buffer prior to addition of 10 μM retinal and experimental light stimulation as described above.

Statistical Analysis. Quantitative data are represented as means ± the standard error of the mean. Differences were tested by the Mann−Whitney U-test using GraphPad (San Diego, CA) Prism (version 4.0). $p < 0.05$ was considered statistically significant.

RESULTS

In Vitro Deglycosylation. A stable melanopsin-expressing PC12 cell line, PC12-rMel, was generated by transfecting PC12 cells with rat melanopsin cDNA. The melanopsin expression rendered the cells sensitive to light as evaluated by *Fos* mRNA induction upon light stimulation.

Immunoblotting of membrane protein preparations from the PC12-rMel cells showed two bands immunoreactive for melanopsin (Figure 2, lane 1). The larger and more intense band had an apparent molecular mass of 62 kDa, while the smaller band had a molecular mass comparable to that predicted from the amino acid sequence encoded by the cDNA (49 kDa). To clarify whether the 62 kDa band represented glycosylated melanopsin core protein, membrane proteins from PC12-rMel cells were subjected to enzymatic N-linked and O-linked deglycosylation in vitro, and the melanopsin-immunoreactive bands were compared with those obtained in mutant melanopsin (N31/N35I) in which the N-linked glycosylation sites have been mutated.

Digestion of membrane proteins from PC12-rMel cells with PNGase F, which cleaves the linkage between asparagine residues and almost every type of N-linked oligosaccharide chain, caused the disappearance of the 62 kDa band and the appearance of melanopsin immunoreactivity at 55 kDa (Figure 2, lane 2). In accordance, immunoblotting for melanopsin from membrane proteins extracted from the mutant N31/35I cells, which express melanopsin devoid of possible N-linked glycosylation residues, showed melanopsin immunoreactivity at 55 and 49 kDa (Figure 2, lane 3). Additional O-linked deglycosylation by in vitro treatment with O-glycosidase and neuraminidase abolished the 55 kDa melanopsin band in both wild-type and mutant N31/35I melanopsin-expressing cells and caused stronger intensity of the 49 kDa core melanopsin protein (Figure 2, lanes 4 and 5).

In Vivo N-Deglycosylation. PNGase F removes virtually all N-linked oligosaccharides from glycoproteins (*33*), and thus, when added to live cells, N-linked glycans of intact cells facing the extracellular space are expected to be digested by PNGase F. As shown in immunoblots with anti-melanopsin antibody (Figure 3), addition of PNGase F to the intact PC12-rMel cells led to a shift in the apparent molecular mass from 62 to 55 kDa, similar to that of the in vitro N-deglycosylated protein from purified membranes shown in Figure 2. The 49 kDa melanopsin immunoreactive band was unaffected.

Tunicamycin inhibits endogenous N-linked glycosylation by targeting the enzyme acetylglucosamine transferase, thus preventing the first synthetic step of the core oligosaccharide. The results of immunoblotting membrane proteins from PC12-rMel cells grown in the presence of tunicamycin were comparable to those obtained with PNGase F-mediated deglycosylation, thus showing replacement of the 62 kDa band with a band of approximately 55 kDa (Figure 3).

Role of N-Glycosylation in the Melanopsin Light Response. Melanopsin is activated by light, which leads to membrane depolarization and induction of the immediately early response gene, *Fos*, a well-established marker for neuronal activation (*34*). We found in initial experiments an additional 3-fold increase in the level of *Fos* mRNA upon light exposure by addition of 10 μM retinal, which was highly significant ($p < 0.001$) (data not shown). Experiments evaluating the light activation of melanopsin were therefore conducted in the presence of added retinal. To clarify if N-glycosylation is important for melanopsin function, we examined the *Fos* response to light in PC12-rMel cells grown in the absence or presence of tunicamycin or following PNGase F treatment of live cells. When compared to the response in untreated PC12-rMel cells, removal of N-linked oligosaccharides by PNGase F did not influence the induction of *Fos* mRNA provoked by light stimulation (Figure 4). Likewise, no significant change in the light-induced *Fos* response was observed after inhibition of endogenous N-linked glycosylation by tunicamycin treatment.

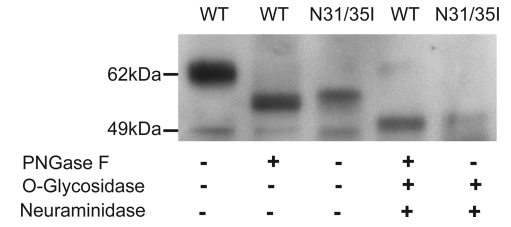

FIGURE 2: In vitro N- and O-linked deglycosylation of melanopsin. Membrane proteins extracted from PC12-rMel cells (WT) and mutant N31/N35I were submitted to N-linked (PNGase F) and/or O-linked (O-glycosidase and neuraminidase) treatment, and the Western blots were probed with anti-melanopsin antibody. Similar results were obtained in three independent experiments.

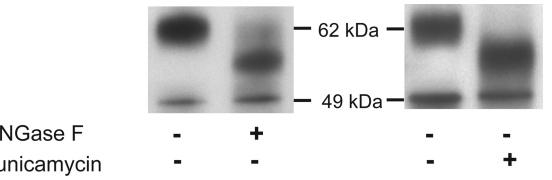

FIGURE 3: In vivo N-linked deglycosylation of melanopsin. Live PC12-rMel cells were treated with PNGase F or tunicamycin to remove the N-glycans or to inhibit asparagine-linked glycosylation, respectively. The Western blots were probed with anti-melanopsin antibody. Similar results were obtained in four independent experiments.

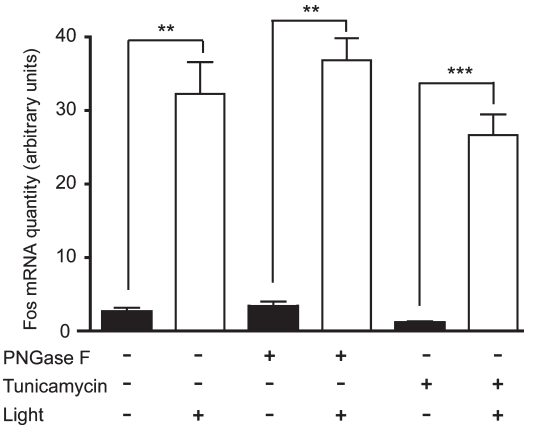

FIGURE 4: Pharmacological inhibition of N-linked glycosylation does not influence the photoresponse in melanopsin-expressing PC12 cells. Effect of 30 min light stimulation (> 300 lx of white light) on *Fos* mRNA expression in PC12-rMel cells and in the cells treated with PNGase F or tunicamycin. Control cells were kept in darkness. The *Fos* mRNA quantity was normalized with the β2-microglobulin mRNA quantity in the same sample, both determined by real-time RT-PCR. Values are given as means $\pm$ the standard error of the mean ($n = 5-8$). $p < 0.01$ (two asterisks); $p < 0.001$ (three asterisks).

We next examined the *Fos* response to light in the mutant N31/35I cells, which lack the two extracellular N-glycosylation sites of melanopsin. As shown in Figure 5A, the light-induced expression and time course of *Fos* mRNA in the mutant were similar to the response in the wild-type melanopsin. The absolute *Fos* mRNA values in the mutant were, however, somewhat lower which is explained by the lower abundance of melanopsin in the membrane preparations as illustrated in Figure 5B. Figure 5C shows melanopsin immunostaining of cells expressing wild-type (left panel) and mutant N31/35I melanopsin (right panel). A clear appearance of the nucleus and an intense staining of the cellular

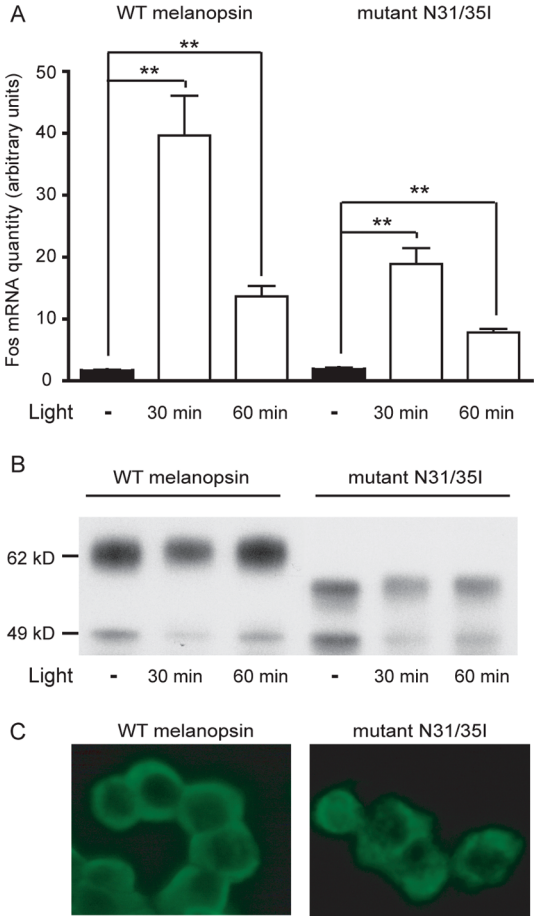

FIGURE 5: Photoresponse in melanopsin-expressing PC12 cells and in cells expressing melanopsin with mutated N-linked glycosylation sites. (A) Effect of 30 min light stimulation (> 300 lx of white light) on *Fos* mRNA expression in PC12-rMel cells (WT) and in cells in which the extracellular N-linked glycosylation sites have been mutated (mutant N31/35I). Control cells were kept in darkness. The *Fos* mRNA quantity was normalized with the β2-microglobulin mRNA quantity in the same sample, both determined by real-time RT-PCR. Values are given as means $\pm$ the standard error of the mean ($n = 6$). $p < 0.01$ (asterisks). (B) Representative Western blot for melanopsin on membrane proteins extracted from melanopsin-expressing PC12-rMel cells (WT) and the N31/35I mutant during light stimulation. (C) Microphotographs of melanopsin immunostaining of PC12-rMel cells (WT) and cells expressing mutant melanopsin (N31/35I).

membrane are seen in cells expressing wild-type melanopsin as opposed to cells expressing mutated melanopsin, where, besides membrane staining, a more granular immunostaining throughout the cells is seen.

DISCUSSION

The existence of a consensus sequence for N-glycosylation (Asn-X-Ser/Thr) in the amino acid sequence does not necessarily result in a glycosylated protein. It was therefore important to clarify if the canonical sites for N-linked glycosylation of the G-protein-coupled receptor, melanopsin, are actually utilized by the oligosaccharyl transferase.

By immunoblotting for melanopsin, we analyzed membrane proteins from PC12 cells stably transfected with rat melanopsin (PC12-rMel) for the extent of receptor glycosylation. We found a minor band running close to the melanopsin predicted molecular mass of approximately 49 kDa and a more prominent band at 62 kDa representing fully glycosylated melanopsin. In a pharmacological approach, PC12-rMel cells were incubated with the strong N-glycosylation inhibitor tunicamycin, which effectively prevented melanopsin glycosylation. Thus, tunicamycin treatment caused a change in the electrophoretic mobility of the 62 kDa fully glycosylated band to a band of approximately 55 kDa. This mobility shift was similar to the results obtained by both in vivo and in vitro enzymatic digestion with PNGase F, which removes virtually all N-linked oligosaccharides from glycoproteins.

Disruption of the glycosylation sites in the N-terminal extracellular domain by mutagenesis led to a melanopsin size comparable with the one obtained by PNGase treatment of wild-type melanopsin, showing that these sites are indeed glycosylated. In this study, the two asparagine residues at positions 31 and 35 were both mutated, and we are thus unable to tell if N-glycosylation occurs at individual or both sites. Besides the demonstration of N-linked glycosylation of melanopsin, we have also provided evidence of O-glycosylation of melanopsin. When PNGase F-treated proteins extracted from PC12-rMel or mutant cells were subjected to a further enzymatic deglycosylation with O-glycosidase and neuraminidase, the 55 kDa band underwent a change in electrophoretic mobility to a molecular mass corresponding to the melanopsin protein core at 49 kDa.

Sequence analysis has revealed that the presence of N-glycosylation sites in the N-terminal extracellular domain is a common characteristic of G-protein-coupled receptors, but for many of these receptors, it has not been determined experimentally whether the putative sites are actually utilized.

Furthermore, the role of oligosaccharides in G-protein-coupled receptor function, which could involve stability, activation state, folding, trafficking, membrane expression, ligand binding, and signal transduction, has only been established for a limited number of receptors. For some receptors, e.g., rhodopsin (*35*), β2-adrenergic receptors (*36*), somatostatin receptors (*37*, *38*), parathyroid hormone receptor (*39*), and the gastrin-releasing peptide receptor (*40*), glycosylation has been shown to be important in maintaining high-affinity binding and/or receptor coupling. On the other hand, glycosylation could also be important for receptor expression and/or stability as shown, for example, for the receptors for vasoactive intestinal polypeptide (*41*), luteinizing hormone (*42*), thyroid stimulating hormone (*43*), follicle stimulating hormone (*44*), gonadotropin releasing hormone (*45*), δ-opiod (*46*), and neurotensin (*47*).

Having shown that melanopsin is both N- and O-glycosylated, we next examined if the absence of N-linked glycosylation affected melanopsin function. There is compelling evidence that melanopsin is the photopigment rendering a subset of inner retinal neurons that are light responsive. These intrinsically photosensitive ganglion cells are important for non-image-forming visual functions such as circadian photoentrainment and the pupillary light reflex. Light triggers a depolarization of the membrane potential in the melanopsin-containing retinal ganglion cells and an expression of the immediate early gene, *Fos*. Experiments using PC12 cells stably expressing rat melanopsin revealed dependency of retinal as shown by a significantly enhanced *Fos* mRNA induction upon light exposure by prior addition of retinal. The cells were further used to examine the importance of N-bound glycans for the light-induced *Fos* expression by (i) in vivo inhibition of endogenous N-linked glycosylation by tunicamycin treatment, (ii) in vivo PNGase F-mediated removal of N-linked glycans exposed on the surface on live cells, and (iii) site-directed mutagenesis of the two possible N-linked glycosylation sites in melanopsin by mutation of the

asparagine residues located at positions 31 and 35 to isoleucine residues. All three approaches revealed that light is still able to induce *Fos* gene expression in N-deglycosylated PC12-rMel cells.

Asn31 and Asn35 are residues of the peptide backbone forming the N-terminal extracellular domain of melanopsin. The crystal structure of melanopsin is still to be resolved; however, computational modeling of the three-dimensional structure of hamster melanopsin has been published (*48*). The light sensitivity of melanopsin is due to the binding of 11-*cis*-retinal which upon illumination captures photons leading to isomerization to the *all-trans* isoform (*49*, *50*). A number of highly conserved residues in melanopsin between species taken together with analogue residues in rhodopsin identify putative amino acid residues participating in retinal binding and isomerization, i.e., the lysine residue located at position 336 in the seventh transmembrane domain that binds the 11-*cis*-retinal chromophore through Schiff base formation and the plausible counterions formed by tyrosine 45 and serine 219 (*48*, *51*). This has been experimentally verified as site-directed mutagenesis of lysine 336 in human melanopsin or lysine 337 in mouse melanopsin to alanine residues renders melanopsin unresponsive to light (*22*, *52*). These residues putatively involved in retinal binding are all located near the second extracellular loop of melanopsin and slightly embedded in the transmembrane domains (*48*, *51*). Thus, Asn31 and Asn35 are presumably not directly involved in retinal binding and isomerization, and this study supports another role of N-linked glycans for melanopsin function than light sensitivity. The mutant N31/35I cells stably expressing melanopsin devoid of N-linked glycans, however, exhibited a weakened light-induced *cFos* expression. As the abundance of immunoreactive melanopsin in the membrane proteins extracted from these mutant melanopsin-expressing cells was lower versus that of wild-type melanopsin-expressing cells, this could explain the attenuated *Fos* mRNA expression in these cells upon light exposure. Accordingly, immunostaining of the cells indicated that mutation of the N-linked glycosylation sites may partly impair proper transport of melanopsin to the cell membrane, thus suggesting that N-linked glycosylation affects the cellular trafficking or stable expression of melanopsin in the plasma membrane.

In conclusion, this study demonstrates for the first time that rat melanopsin heterologously expressed in PC12 cells is a glycan-rich receptor, which is both N-linked and O-linked glycosylated, and that N-linked glycosylation is not crucial for the melanopsin response to light.

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