Supplementary Information for:

Use of metabolic glycoengineering and pharmacological inhibitors to assess lipid and protein sialylation on cells

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Running title: Separate labeling of sialoproteins and sialolipids in cells
Key words: metabolic glycoengineering, sialic acid, polysialic acid, neural cell adhesion molecule, ganglioside, neuron, confocal imaging

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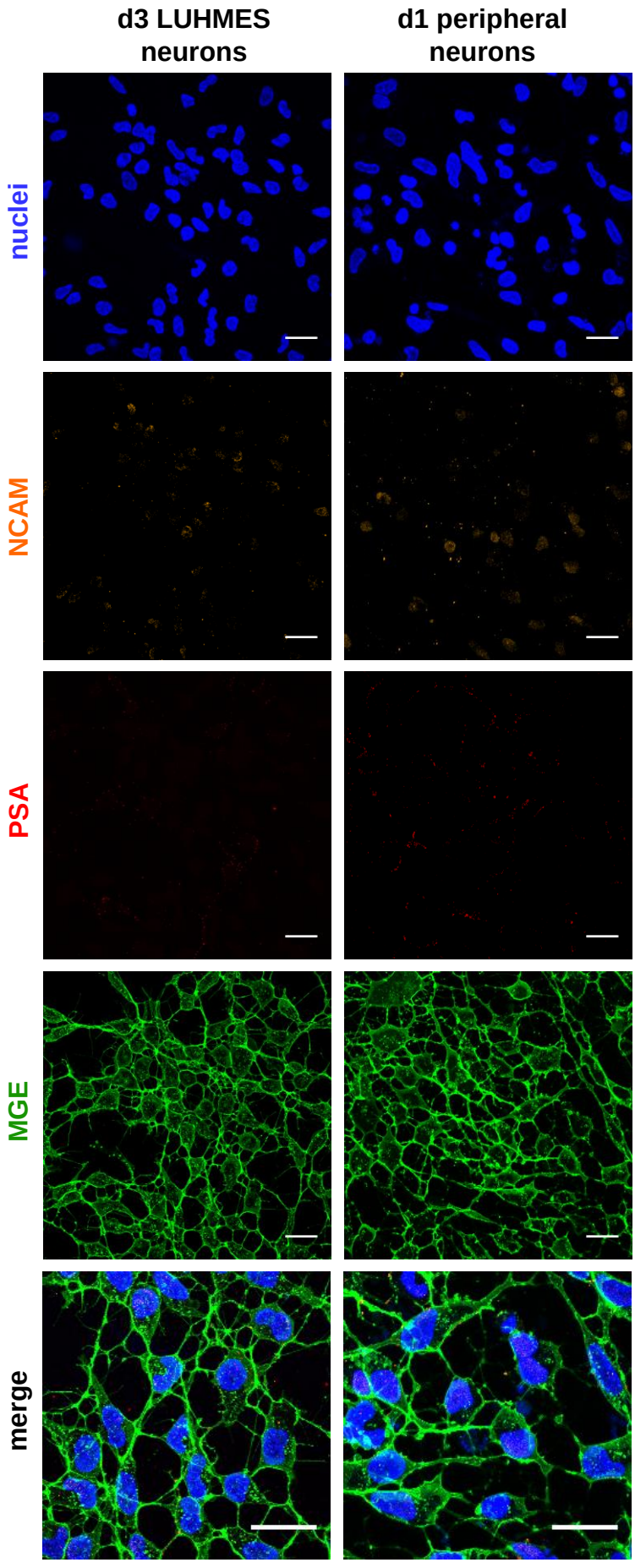

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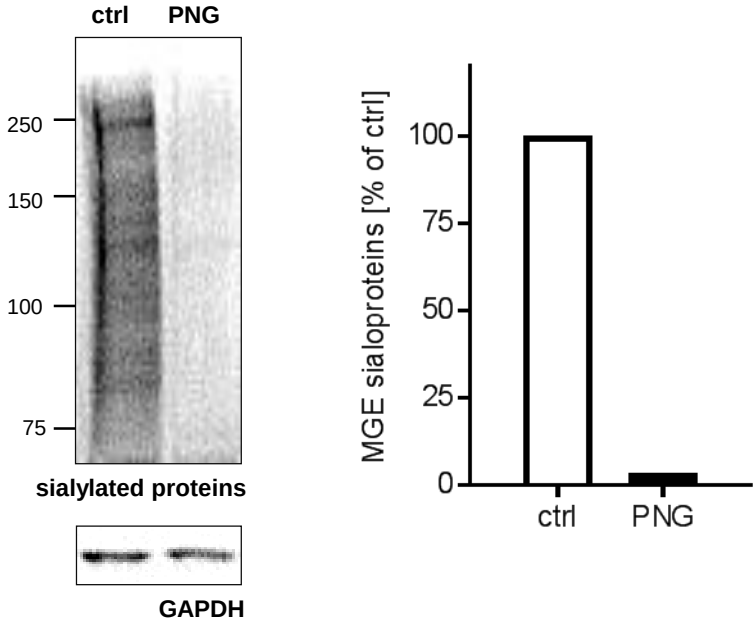
Suppl. Fig. S1: Expression of polysialic acid (PSA) and neural cell adhesion molecule (NCAM) in developing immature neurons.

LUHMES neurons (d2) and peripheral neurons (d0) were treated with 10 µM Ac_4ManNAz and cell surface sialoglycans were stained 24 h later (on d3 for LUHMES and d1 for peripheral neurons), after their coupling to biotin. After the live metabolic glycoengineering (MGE) staining, cells were fixed, and anti-PSA antibody, anti-NCAM antibody, and H-33342 were used to further label PSA, NCAM and nuclei. Confocal fluorescent representative images of the MGE and immunostains are shown. Scale bar = 25 µm.



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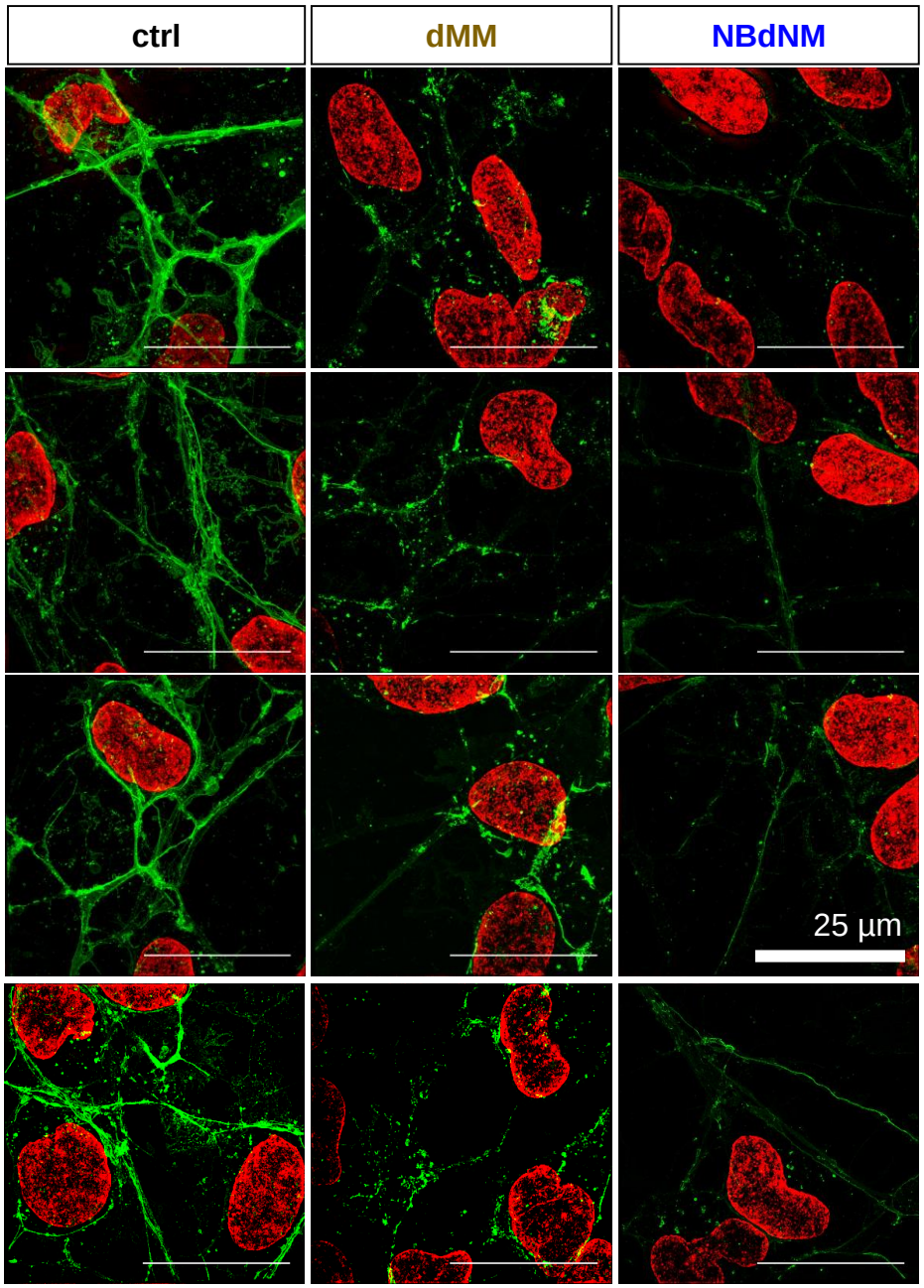
Suppl. Fig. S2: Effect of protein N-glycosidase F (PNG) on metabolic glycoengineering (MGE) sialoglycoproteins.

LUHMES cells (d5) were treated with Ac_4ManNAz [10 µM] for 24 h. PNG (2500 U) was added for 2 h before the MGE ligation. Cells were then washed. MGE sialic acid on the surface of live cells were ligated to dibenzocyclooctyne (DBCO)-biotin [100 µM] for 20 min. Afterwards, cell lysates were prepared and resolved by SDS-PAGE. Western blot against biotin was performed using anti-biotin antibody to detect the labeled sialylated proteins. Left: Representative membrane image. For orientation, the position of molecular weight markers (in kDa) is indicated on the left-hand side. Right: Quantification of MGE sialoproteins by densitometric analysis after PNG treatment presented as percentage of enzyme-untreated control (ctrl).

High resolution MGE imaging



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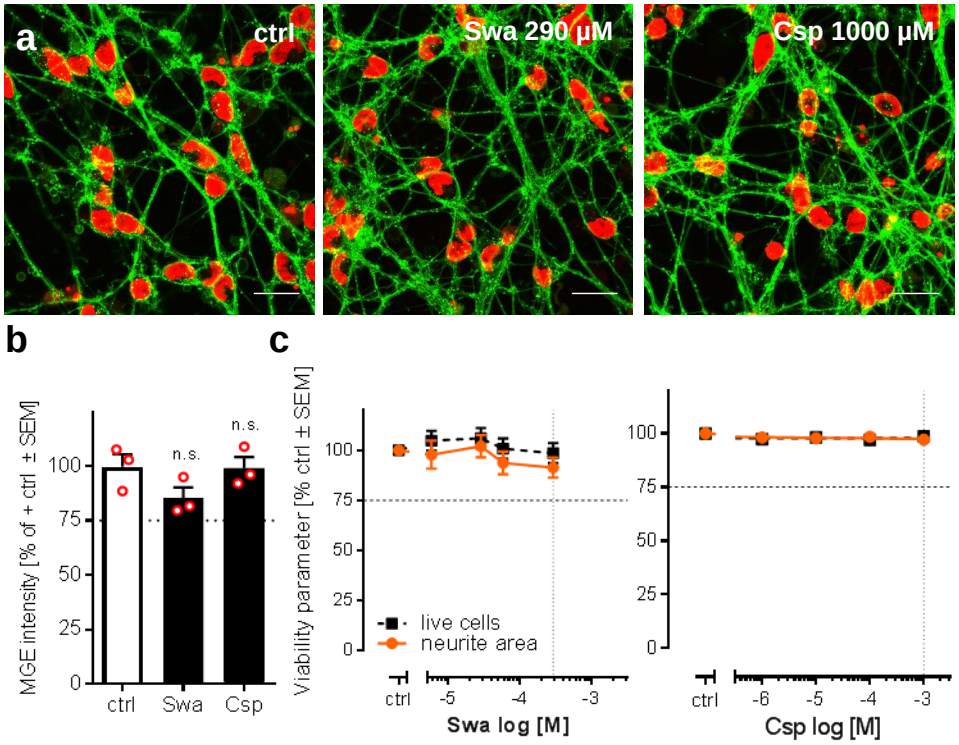
Suppl. Fig. S3: Morphological presentation of sialolipids and sialoproteins.

LUHMES cells (d5) were co-treated with Ac_4ManNAz and either deoxymannojirimycin (dMM, 1 mM) or *N*-butyl deoxynojirimycin (NBdNM, 1 mM) for 24 h. MGE sialoglycans were then ligated with dibenzocyclooctyne (DBCO)-biotin, followed by streptavidin-Alexa Fluor 488 and H-33342. Cells were fixed and imaged using the Deltavision OMX Blazev4 super resolution microscope. Representative images show MGE sialoglycans (green) and nuclei (red).



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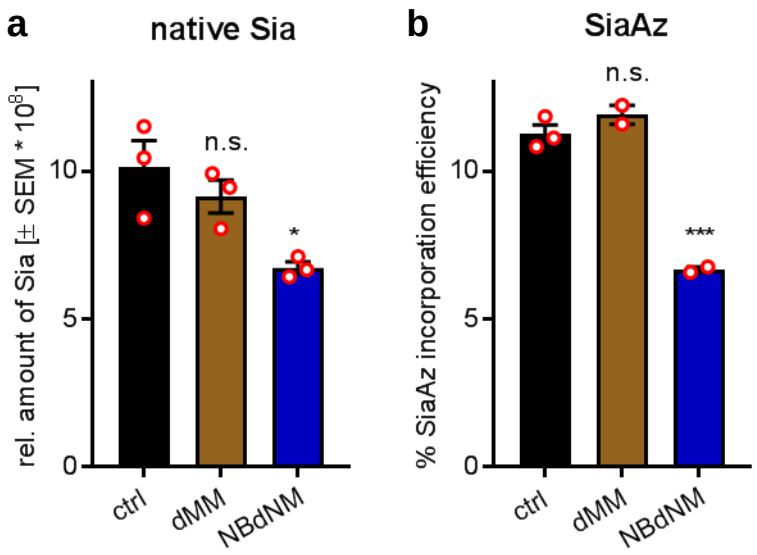
Suppl. Fig. S4: Effect of protein glycosidase inhibitors on sialoglycan synthesis.

a. Representative confocal fluorescent images of metabolic glycoengineering (MGE) sialic acid (Sia) (green) and nuclei (red) of d6 LUHMES cells treated with indicated concentrations of swainsonine (Swa) and castanospermine (Csp). **b.** Quantification of the level of MGE Sia presented as the percentage of untreated control (ctrl). **c.** LUHMES cells (d5) were treated with Swa (5-290 µM) or Csp (1-1000 µM) for 24 h and analyzed by high content imaging using H-33342/calcein-AM staining. Concentration-response curves indicate the percentage of live cells (dashed line) and of the neurite area (solid line), relative to the untreated cells. The horizontal line is drawn at 75% (toxicity threshold). The vertical dotted line indicates the concentration selected for MGE Sia labeling in (a). Data are means ± SEM from three independent cell preparations (= three biological replicates). Statistics: one-way ANOVA followed by Dunnett's test; (treatments vs. ctrl), n.s. = not significant.



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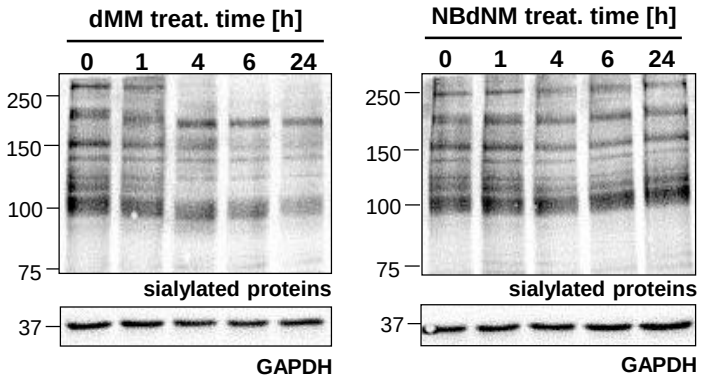
Suppl. Fig. S5: Incorporation efficiency of sialic acids after selective sialoprotein and sialolipid inhibition.

LUHMES cells (d5) were treated with 10 µM Ac_4ManNAz (or with DMSO only as solvent control) for 6 h to introduce the azide tag into the sialic acids (Sia). Cells were then washed, scraped off the culture surface and pelleted. Glycans were hydrolysed by acetic acid [3 M] treatment at 80 °C for 90 min to release monomeric sialic acids. This was subsequently labeled by 1,2-diamino-4,5-methylenedioxybenzene (DMB) for 2.5 h at 56 °C in the dark. The samples were analyzed by RP-HPLC with a fluorescence detector; 10 -20% acetonitrile with 0,1 % formic acid in water with 0,1 % formic acid in 40 minutes using a Kinetex 2.6 µm, C18, 100 Å, LC column 150 x 4.6 mm from phenomenex. **a.** Absolute Sia amount after the treatment with deoxymannojirimycin (dMM, 1 mM) or *N*-butyl deoxynojirimycin (NBdNM, 1 mM) presented as a percentage of untreated control (ctrl). **b**. Incorporation efficiency of Az-tagged Sia after the treatment with deoxymannojirimycin (dMM, 1 mM) or *N*-butyl deoxynojirimycin (NBdNM, 1 mM) presented as a percentage of total Sia. Under control conditions, about 10% of all Sia of cells was the added Az-modified variant. Data are means ± SEM from two to three biological replicates (= independent cell preparations). n.s. = not significant, *$p < 0.05$, ***$p < 0.001$ (inhibitors vs. solvent control (ctrl)).



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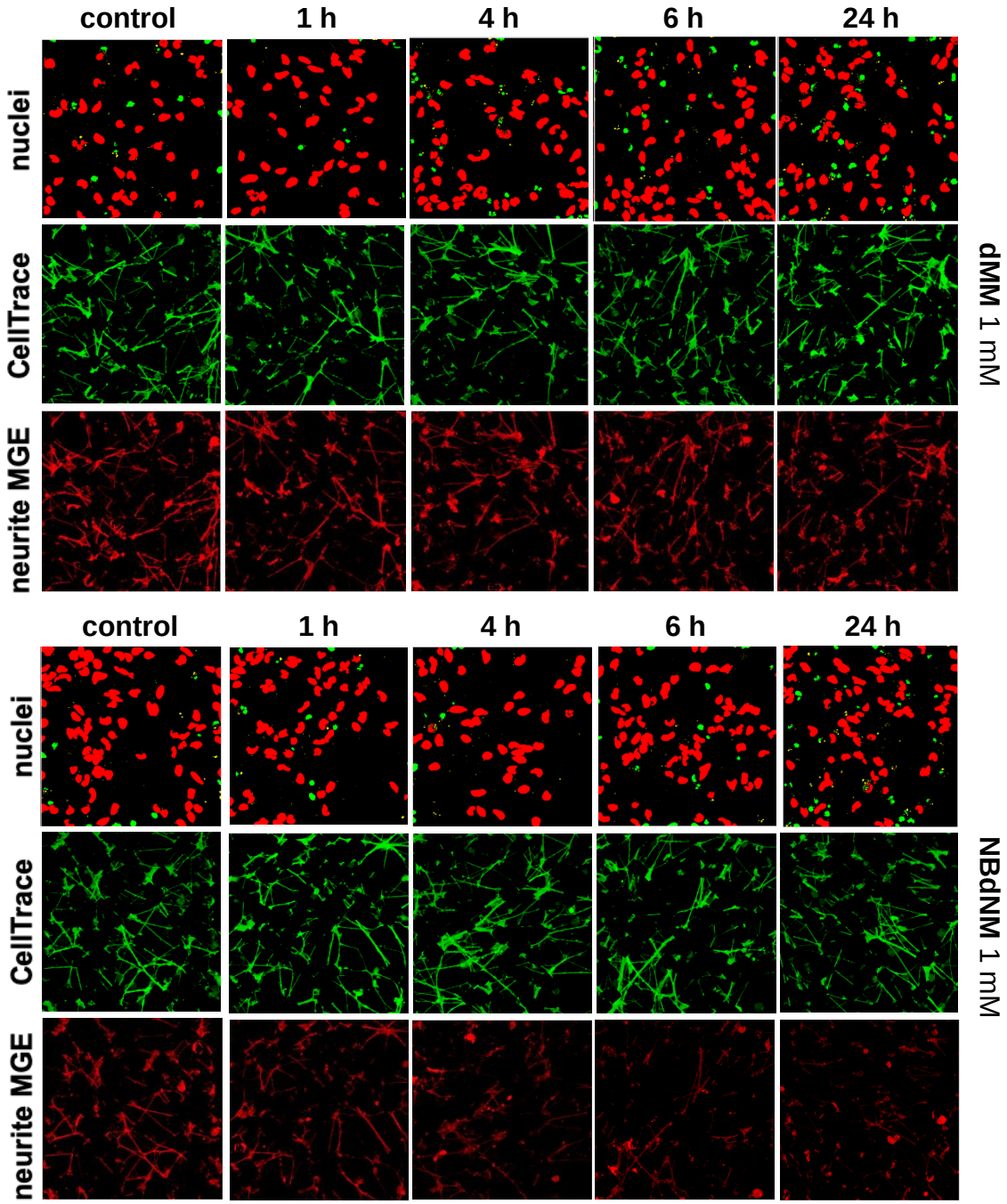
Suppl. Fig. S6: Temporal resolution of the analysis of sialoproteins by selective MGE inhibition.

As detailed in Fig. 6, LUHMES cells (d5) were treated with deoxymannojirimycin (dMM, 1 mM) or *N*-butyl deoxynojirimycin (NBdNM, 1 mM) for a duration of 1, 4, 6 and 24 h. Ac_4ManNAz [10 µM] was added for the last 6 h. Cells were then washed and ligated to dibenzocyclooctyne (DBCO)-biotin [100 µM] for 20 min on the intact cells. Afterwards, cell lysates were prepared and resolved by SDS-PAGE. Western blot against biotin was performed using anti-biotin antibody to detect the labeled MGE sialylated proteins or GAPDH as a loading control. Representative membrane images. For orientation, the position of molecular weight markers (in kDa) is indicated on the left hand side.



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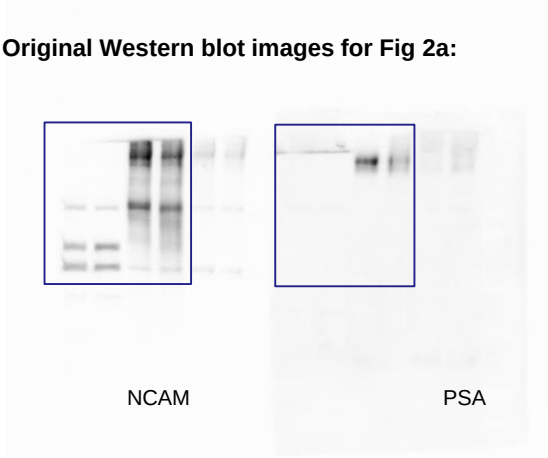
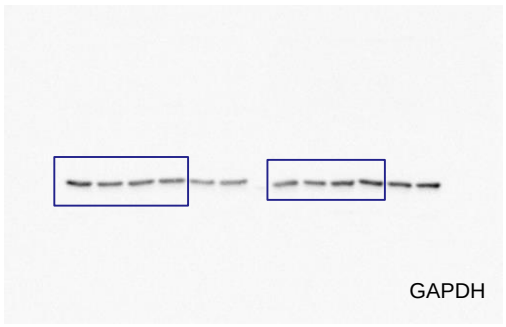
Suppl. Fig. S7: Representative images of deoxymannojirimycin (dMM) and *N*-butyl deoxynojirimycin (NBdNM)-treated LUHMES, as used for the quantification algorithm.

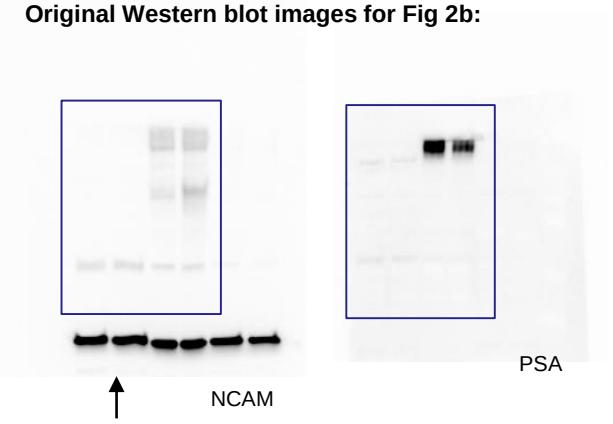
LUHMES cells (d5) were treated with either dMM or NBdNM (1 mM) for a duration of 1, 4, 6 and 24 h, as described in Fig. 6. Solvent-treated cells served as a control. Ac_4ManNAz [10 µM] was added for the last 6 h. Cells were then washed and ligated to dibenzocyclooctyne (DBCO)-biotin [100 µM] for 20 min on the intact cells and subsequently stained with a mixture of the nuclear dye H-33342, the vital cytosolic marker CellTrace and fluorescent streptavidin (Strep-AF488) for 30 min. Cells were then fixed and imaged with a confocal microscope. The representative images show the images after processing by the detection algorithm of the SUIKER program. The top row shows live cell bodies and their shape/size (red), dead cells (green) or debris (yellow). The middle row shows the structures that the program detects as neurites. The bottom row shows the MGE sialoglycans that the software detected on neurites.

Original Western blot images for Fig 2a:

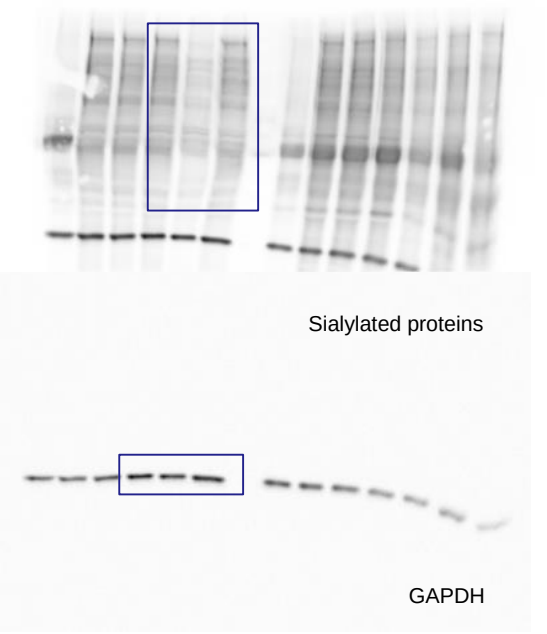




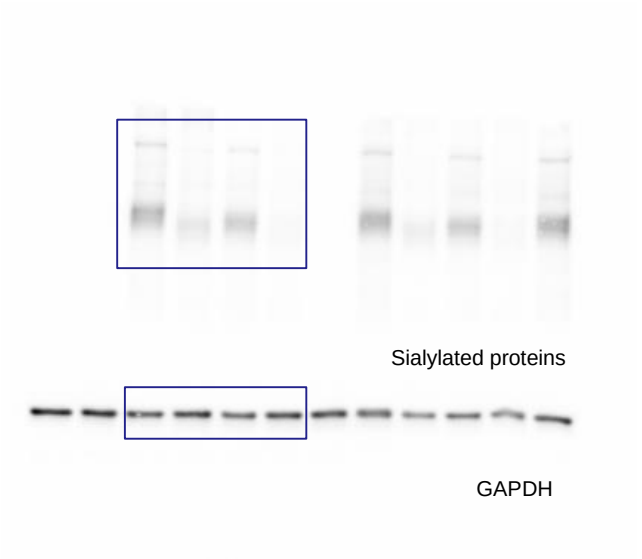
Original Western blot images for Fig 2b:



Original Western blot images for Fig 4d:



Original Western blot images for Fig 5a:



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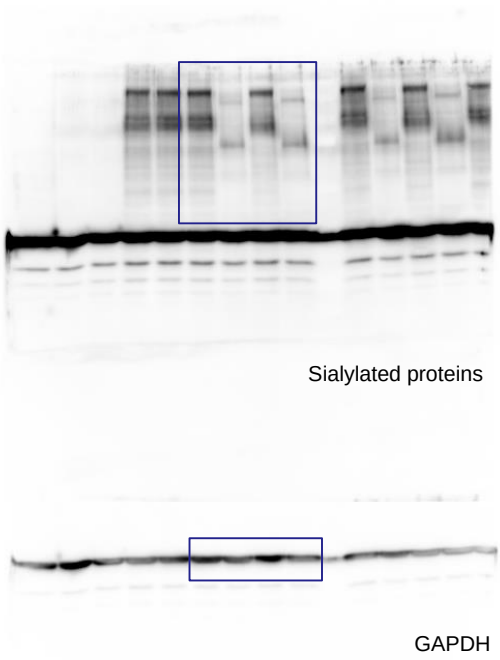
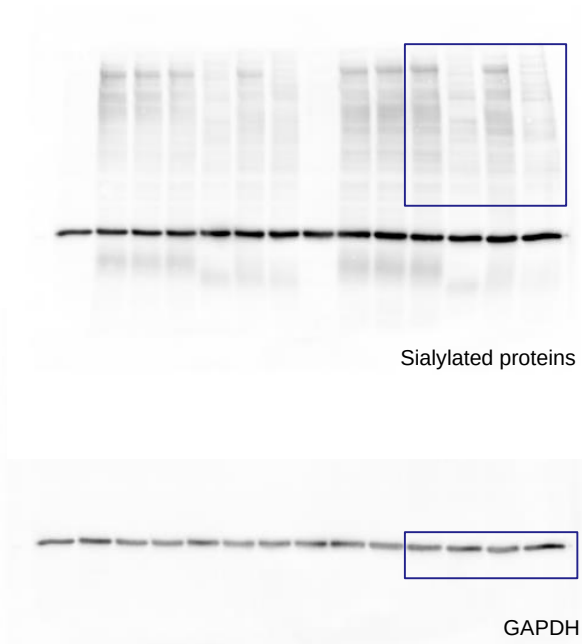
Suppl. Fig. S8: Original Western blot images

Original Western blot images for Fig 7a:



Original Western blot images for Fig 8a:



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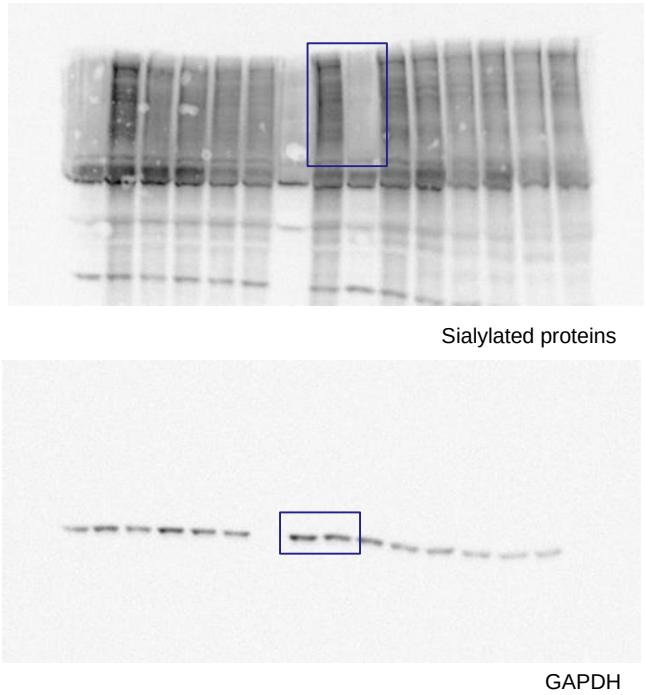

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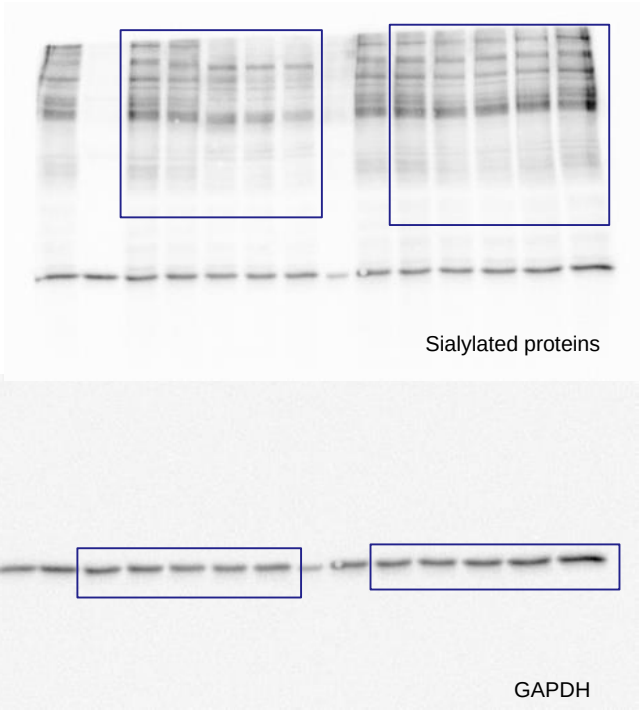

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Suppl. Fig. S8: Original Western blot images

Figure	Statistical test	Groups tested	Degrees of freedom	F value	P value
Fig 3b	One-way ANOVA		11	48.38	<0.0001
	Dunnett's multiple comparisons	- ctrl vs. + ctrl - ctrl vs. PNG - ctrl vs. EndoN	8 8 8		<0.0001 0.6615 0.9999
Fig 3d	One-way ANOVA		11	23.06	0.0003
	Dunnett's multiple comparisons	- ctrl vs. + ctrl - ctrl vs. PNG - ctrl vs. EndoN	8 8 8		0.0001 0.0026 0.0061
Fig 4d	One-way ANOVA		11	17.01	0.0009
	Dunnett's multiple comparisons	dMM vs. ctrl dMM vs. NBdNM	9 9		0.0009 0.0020
Fig 4f	One-way ANOVA		17	138.7	<0.0001
	Dunnett's multiple comparisons	- ctrl vs. siaV - ctrl vs. dMM - ctrl vs. NBdNM - ctrl vs. dMM+NBdNM	12 12 12 12		0.9998 <0.0001 <0.0001 0.9885
Fig 5a	One-way ANOVA		11	170.6	<0.0001
	Dunnett's multiple comparisons	dMM vs. + ctrl dMM vs. NBdNM dMM vs. dMM+NBdNM	8 8 8		<0.0001 <0.0001 0.9584
Fig 5b	One-way ANOVA		14	45.86	<0.0001
	Dunnett's multiple comparisons	- ctrl vs. dMM - ctrl vs. NBdNM - ctrl vs. dMM+NBdNM	10 10 10		0.0123 0.5624 0.9053
Fig 6b left	One-way ANOVA		14	8.128	0.0035
	Dunnett's multiple comparisons	0 vs. 1 0 vs. 4 0 vs. 6 0 vs. 24	10 10 10 10		0.6699 0.0100 0.0081 0.0039
Fig 6b right	One-way ANOVA		14	4.757	0.0208
	Dunnett's multiple comparisons	0 vs. 1 0 vs. 4 0 vs. 6 0 vs. 24	10 10 10 10		0.2029 0.1530 >0.9999 0.8011
Fig 6c left	One-way ANOVA		14	7.921	0.0038
	Dunnett's multiple comparisons	0 vs 1 0 vs 4 0 vs 6 0 vs 24	10 10 10 10		0.8606 0.0472 0.0112 0.0703
Fig 6c right	One-way ANOVA		14	60.34	<0.0001
	Dunnett's multiple comparisons	0 vs. 1 0 vs. 4 0 vs. 6 0 vs. 24	10 10 10 10		0.0017 <0.0001 <0.0001 <0.0001

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Suppl. Fig. S9: Table of statistical data values.

Fig 7a	One-way ANOVA		11	59.09	<0.0001
	Dunnett's multiple comparisons	+ ctrl vs. dMM + ctrl vs. NBdNM + ctrl vs. dMM+NBdNM	8 8 8		<0.0001 0.3919 <0.0001
Fig 7b	One-way ANOVA		14	24.45	<0.0001
	Dunnett's multiple comparisons	- ctrl vs. dMM - ctrl vs. NBdNM - ctrl vs. dMM+NBdNM	10 10 10		0.0308 0.0001 0.7493
Fig 8a	One-way ANOVA		11	10.52	0.0011
	Dunnett's multiple comparisons	+ ctrl vs. dMM + ctrl vs. NBdNM + ctrl vs. dMM+NBdNM	12 12 12		0.0016 0.1995 0.0013
Fig 8b	One-way ANOVA		14	35.99	<0.0001
	Dunnett's multiple comparisons	- ctrl vs. dMM - ctrl vs. NBdNM - ctrl vs. dMM+NBdNM	10 10 10		0.0370 <0.0001 0.2719
Fig S4b	One-way ANOVA		17	58.48	<0.0001
	Dunnett's multiple comparisons	ctrl vs. Swa ctrl vs. Csp	12 12		0.1888 0.9999
Fig S5a	One-way ANOVA		8	7.734	0.0218
	Dunnett's multiple comparisons	ctrl vs. dMM ctrl vs. NBdNM	6 6		0.4793 0.0155
Fig S5b	One-way ANOVA		6	88.75	0.0005
	Dunnett's multiple comparisons	ctrl vs. dMM ctrl vs. NBdNM	4 4		0.2906 0.0006

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Suppl. Fig. S9: Table of statistical data values.

Parameters used for sample size and power calculation:

Type I error rate α: 5% (0.05); Power 1-β: 80% (0.8); Mean under H_0: 43 (value from our pilot experiment for MGE signal reduction after glycoinhibition); Standard deviation: 14.6 (standard deviation of the mean under H_0); MDE (= minimum detectable effect): 75% (we assume here that our tested compound must reduce the MGE signal/membranal sialylation by a minimum of 25% in order to see an effect, therefore a detection of 75% MGE signal or below).

The output for sample size for each group based on these parameters was 3. This is in good agreement with our approach to repeat each experiment three times (= three biological replicates). The three biological replicates correspond to three independent cell preparations. The independence in this case means that the cells were thawed, treated, and measured separately (three times for each experiment) as the source of variability are indeed the different batches of cells which may react differently, pipetting errors which may occur during the treatments or the endpoint of the assay measured.

The power based on the above parameters was > 99%.

Sample size was calculated based on the online tool: "Sample Size Calculator“ (https://bit.ly/3Wu0Q9w)

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Suppl. Fig. S10: Sample size calculation for the study.