**Supplementary data**

**Reactivation of hyperglycemia-induced hypocretin (*HCRT*) gene silencing by *N*-acetyl-D-mannosamine in the orexin neurons derived from human iPS cells**

**Koji Hayakawa1, Yasuharu Sakamoto2, Osamu Kanie3, 4, Atsuko Ohtake3, Shusaku Daikoku3, Yukishige Ito2, 3 and Kunio Shiota1, 5**

1 Laboratory of Cellular Biochemistry, Department of Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Tokyo 113-8657, Japan

2 Synthetic Cellular Chemistry Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

3 Japan Science and Technology Agency ERATO Glycotrilogy Project, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

4 Present address: Institute of Advanced Biosciences, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan

5 Waseda Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan

**Supplementary Figure S1-S3**

**Supplementary Table S1-S3**

****

**Figure S1. Activities of ManNAc derivatives to induce *Hcrt* gene expression in neural cells derived from mouse embryonic stem cells (mESCs)**

mESCs were cultured in SDIA+BMP4 medium for 10 days. ManNAc derivatives were added on day 4 at the indicated concentrations, and *Hcrt* mRNA expression was evaluated by RT-PCR. *Actb* was used as the internal control.



**Figure S2. Response of ManNAc-treated cells to metabolic signals**

(A-C) ManNAc-treated cells (10 μM) were incubated for 3 hours with various doses of ghrelin, leptin (A), GABA, Neuropeptide Y (NPY) (B), and glucose (C), and OREXIN-A secretion into the medium was evaluated using an ELISA. Non-treated cells were incubated with 100 nM ghrelin as control. Means ± S.D. (n = 3).



**Figure S3. *HCRT* gene expression and epigenetic status in hiONs under euglycemia and hyperglycemia *in vitro***

(A) Culture protocol to prepare hiONs or non-orexin neural cells. BMP4 (5 nM) was added on day 7, and the culture was continued until day 20, with (hiONs) or without (non-orexin neural cells) 1 μM ManNFAc.

(B) *HCRT* expression in hiONs in culture medium containing 5 mM glucose (euglycemia) or 25 mM glucose (hyperglycemia) for 12 days. *HCRT* expression was evaluated by RT-qPCR. Means ± SD (n = 3).

(C) Epigenetic status of the *HCRT* gene locus in non-orexin neural cells and hiONs. Levels of *O*-GlcNAcylation, histone acetylation and accumulation of epigenetic modulators (MGEA5, SIRT1, and OGT)at the *HCRT* gene locus in hiONs were measured by ChIP-qPCR. Regions 1 and 2 refer to Figure 5B. Values are means ± SD derived from three independent qPCR reactions and were normalized to input DNA.

(D) DNA methylation status of the *HCRT* gene locus by COBRA in hiONs. Means ± SD (n = 3).

**Table S1 List of ManNAc derivatives**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Name | Full  Name | Chemical formula | CAS No. | Molecular weight | Structure |
| ManNAc | *N*-acetyl-d-mannosamine | C8H15NO6 | 3615-17-6 | 221.21 |  |
| Ac4ManNAc | 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-d-mannopyranose | C16H23NO10 | 76375-61-6 | 389.35 |  |
| ManNFAc | 2-deoxy-2-fluoroacetamido-d-mannose | C8H14FNO6 | 78103-27-2 | 239.20 |  |
| Me-α-ManNAc | Methyl 2-acetamido-2-deoxy-α-d-mannopyranoside | C9H17NO6 | 32087-35-7 | 235.23 |  |
| SPh-αβ-ManNAc | Phenyl 2-acetamido-2-deoxy-1-thio-d-mannopyranoside | C14H19NO5S | 166516-70-7 | 313.37 |  |
| ManNAcF2 | 2-deoxy-2-difluoroacetamido-d-mannose | C8H13F2NO6 | 490037-89-3 | 257.19 |  |
| ManNAcF3 | 2-deoxy-2-trifluoroacetamido-d-mannose | C8H12F3NO6 | 86932-74-3 | 275.18 |  |
| 5S-ManNAc | 5-thio-*N*-acetyl-d-mannosamine | C8H15NO5S | 88043-82-7 | 237.27 |  |
| ManNGlyAcF | 2-deoxy-2-[*N*-(2-fluoroacetyl)glycylamino]-d-mannose | C10H17FN2O7 | New compound | 296.25 |  |
| ManNPrF | 2-deoxy-2-(3-fluoropropanamido)-d-mannose | C9H16FNO6 | New compound | 253.22 |  |
| 5S-ManNAcF | 2-deoxy-2-fluoroacetamido-5-thio-d-mannose | C8H14FNO5S | New compound | 255.26 |  |
| 1-Deoxy-ManNAc | 2-acetylamino-1,5-anhydro-2-deoxy-d-mannitol | C8H15NO5 | 10293-61-5 | 205.21 |  |
| ManNCOOMe | 2-deoxy-2-[(methoxycarbonyl)amino]-d-mannose | C8H15NO7 | 172950-45-7 | 237.21 |  |
| ManNCOOEt | 2-deoxy-2-[(ethoxycarbonyl)amino]-d-mannose | C9H17NO7 | New compound | 251.23 |  |

**Table S2 Primer list**

For RT-PCR

|  |  |  |  |
| --- | --- | --- | --- |
| Primer name | Forward | Reverse | Cycle of  Amplification |
| HCRT | CCAGACACCATGAACCTTCC | GCAAGTCTTTTGACGACAGC | 35 |
| ACTB | CCAACCGCGAGAAGATGA | CCAGAGGCGTACAGGGATAG | 20 |
| Hcrt\_mouse | CTCCAGGCACCATGAACTTT | AGTTCGTAGAGACGGCAGGA | 35 |
| Actb\_mouse | TTCTACAATGAGCTGCGTGTGG | ATGGCTGGGGTGTTGAAGGT | 20 |

For Bisulfite-PCR

|  |  |  |
| --- | --- | --- |
| Primer name | Forward | Reverse |
| HCRT\_upstrem\_1 | TGGTTTTGTTGTTTAGGTTGGA | TTCCAAACCAACCTAACCAA |
| HCRT\_upstrem\_2 | GGAATTTGGGAGTATAAAAGAGG | AATCACACACACACACACAAAAA |
| HCRT\_TSS\_1 | GTGGAAGGAAGGTTTATGGTGTT | CCTCTTTTATACTCCCAAATTCC |
| HCRT\_downstrem\_1 | GGAGTGTTTGTTTGTAGTAGAAGTTG | AACACCATAAACCTTCCTTCCAC |
| HCRT\_downstrem\_2 | GGTTTGGGTTAGGATAG | CAACTTCTACTACAAACAAACACTCC |
| HCRT\_for\_COBRA | TTTTGAATAGTTTTTGAATAGAATTATAGG | CACTCCAACCTAAACAACAAAAA |

For ChIP-PCR

|  |  |  |
| --- | --- | --- |
| Primer name | Forward | Reverse |
| HCRT\_Region1 | CTGATCACCCCCTTGTCTGT | TTTTGTGCTCCCAGATTCCT |
| HCRT\_Region2 | GCAGGGAAACACATGGAAAT | GCAGGAAACTGGTTCAAAGC |

**Table S3 Antibody list**

|  |  |  |  |
| --- | --- | --- | --- |
| Name | Company | Cat. No. | Applications1 (Final conc.) |
| OREXIN-A | Santa Cruz | sc-8070 | IF (2 μg/ml) |
| OREXIN-B | Santa Cruz | sc-8071 | IF (2 μg/ml) |
| NCAM | Millipore | AB5032 | IF (2 μg/ml) |
| TUBB3 (TUJ1) | COVANCE | MMS-435P | IF (2 μg/ml) |
| H3K9 acetylation | MAB Institute | MA305B | ChIP (30 μg/ml) |
| H3K14 acetylation | Millipore | 07-353 | ChIP (30 μg/ml) |
| H3K27 acetylation | MAB Institute | MA309B | ChIP (30 μg/ml) |
| H3K56 acetylation | Active Motif | 39281 | ChIP (30 μg/ml) |
| H4K8 acetylation | Active Motif | 61103 | ChIP (30 μg/ml) |
| H4K16 acetylation | Active Motif | 39167 | ChIP (30 μg/ml) |
| p300 | Santa Cruz | sc-584 | ChIP (30 μg/ml) |
| CBP | Santa Cruz | sc-369 | ChIP (30 μg/ml) |
| SIRT1 | Millipore | 04-1557 | ChIP (30 μg/ml) |
| SIN3A | Santa Cruz | sc-994 | ChIP (15 μg/ml) |
| EZH2 | Cell Signaling | 5246 | ChIP (30 μg/ml) |
| *O*-GlcNAc (RL2) | Abcam | ab2739 | ChIP (30 μg/ml) |
| OGT | Abcam | ab50273 | ChIP (30 μg/ml) |
| MGEA5 | Protein tech | MU392A-UC | ChIP (15 μg/ml) |
| Mouse normal IgG | Abcam | ab18413 | ChIP (30 μg/ml) |
| Rabbit normal IgG | Abcam | ab27478 | ChIP (30 μg/ml) |

1, IF, immunofluorescence assay; WB, western blotting; ChIP, chromatin immuno precipitation assay.