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Transcriptional regulation of the hypocretin/orexin gene by NR6A1

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Abstract

The hypocretin (also known as orexin) neuropeptide system coordinates the regulation of various physiological processes. A reduction in *Nr6a1* expression was observed in hypocretin neuron-ablated transgenic mice. To show that prepro-hypocretin transcription is functionally modulated by NR6A1, we performed chromatin immunoprecipitation (ChIP) analysis, double-immunostaining, a luciferase reporter assay, and an *in utero* electroporation study. ChIP analysis showed that endogenous NR6A1 binds to a putative NR6A1-binding site. Double-immunostaining indicated almost all hypocretin neurons were positive for NR6A1 immunoreactivity. NR6A1 overexpression in SH-SY5Y cells modulated hypocretin promoter activity, an effect that was countered by lacking a putative NR6A1-binding site. Electroporation with *Nr6a1* in the foetal hypothalamus promoted hypocretin transcription as compared to *GFP*-electroporation. These experiments confirmed that NR6A1 works as a regulator for hypocretin transcription.

Keywords

NR6A1/GCNF; hypocretin/orexin; transcriptional regulation

Introduction

Hypocretin (also known as orexin) is a hypothalamic neuropeptide involved in the regulation of various physiological processes [1,2]. Prepro-hypocretin expression is localised exclusively to the perifornical area of the lateral hypothalamus [3]. Two conserved promoter regions—OE1 and OE2—were identified within a 3.2-kb fragment located upstream of the prepro-hypocretin gene. These regions were found to target specific expression within the lateral hypothalamus [4]. Although several feedback loops have been

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reported to regulate the hypocretin system, little is known about the specific transcriptional units that regulate OE1, OE2 and prepro-hypocretin transcription.

In our previous study, we identified downregulated-genes in the orexin/ataxin-3 hemizygous transgenic mice (animals that lack most hypocretin neurons) [5]. This list comprised *Nr6a1* and several other genes known to be colocalised with hypocretin neurons. NR6A1 binds to a direct repeat of the DR0 element (AGGTCA) and functions as a transcriptional repressor [6–9]. The suppression of *Pou5f1* by NR6A1 has been shown to facilitate the transition from primitive to definitive neural stem cells [10], indicating that NR6A1 fosters a favourable environment for neuronal differentiation.

To identify novel *cis*-acting regulatory sequences for the human prepro-hypocretin gene, an *in silico* search was performed. The DR0 element was identified at position -1350/-1345 and NurRE, a DR0-like motif (AGGTCC), was identified at position -544/-537, relative to the translation start site of the prepro-hypocretin gene (Figure 1A). The 18-bp motif (-548/-530) including the NurRE deviated significantly from the random expectation analysis (0.09 matches per 1000 bp) employed in the Genomatix Software GmbH (http://www.genomatix.de). The 18-bp motif was also well conserved between the human and the mouse (Figure 1B). In this study, we report NR6A1 modulates hypocretin transcription.

Materials and Methods

Ethics Statement

All animal experiments were performed according to the procedures of Animal Experiments approved by Ethics Committee of the Tokyo Institute of Psychiatry (approval ID: 22-27) and the Tokyo Metropolitan Institute for Neuroscience (approval ID: 21-27).

Expression vectors and reporter plasmids

Human *NR6A1* cDNA was obtained from human hypothalamic poly-A RNA (Takara Bio Company, Kyoto, Japan) and cloned into a pCMVTNT vector (Promega, Madison, WI, USA) (NR6A1/TNT). A CAG promoter was cloned into a pcDNA3 vector (Invitrogen, CA, USA) (pCAGGS). Full-length murine *Nr6a1* was purchased (OMM4760, Open Biosystems, AL, USA) and cloned into pCAGGS (pCAG-mNr6a1). The pCAG-EGFP vector, which contains *EGFP* cDNA, was prepared as an experimental control for *in utero* electroporation.

The reporter plasmids pGL3-basic, pGL3-Control, pGL4.74 [hRluc/TK] (Promega), and pTAL-luc (Takara Bio Company) were purchased. The pGL4.74 [hRluc/TK] plasmid encoding Renilla luciferase was used for an internal control of transfection efficiency. Schemes of the reporter plasmids are illustrated in Figure 2. The 3.2 kb-basic, 2.0 kb-basic, and ΔDR0/0.7 kb-basic plasmids were constructed by inserting the human prepro-hypocretin promoter sequence from position -3278/+87 [3], -2023/+87, or -634/+87 into the upstream region of a firefly luciferase gene in the pGL3-basic plasmid. These respective prepro-hypocretin 5'-flanking fragments were obtained by PCR using the HCRT_+87Rv primer and HCRT_-3278Fw, HCRT_-2023Fw, or HCRT_-634Fw primers (Table). The deletion mutant ΔNurRE/0.7kb-basic, which contains an internal deletion at -548/-530 within the ΔDR0/0.7 kb-basic plasmid, was generated by using a site-directed mutagenesis and the HCRT_-548Rv_P and HCRT_-530Fw_P phosphorylated-primers (Table). The NurREx1_sense, NurREx1_anti-sense, NurREx3_sense, and NurREx3_anti-sense oligonucleotides (Table) were used to obtain the double-stranded DNA of the NurRE . Overhanging ends were used to insert the oligonucleotides directly into the pGL3-Control and pTAL-luc reporter plasmids.

Cell culture

SH-SY5Y (derived from human neuroblastoma), HeLa (human cervical carcinoma), HEK293T (human embryonic kidney), SF126 (human glioblastoma), and Becker (human astrocytoma) cells were obtained from ATCC (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (GIBCO) at 37°C and 5% CO_2.

ChIP-PCR

SH-SY5Y cells were cross-linked with 1% formaldehyde and then blocked with 0.125M glycine. After wash, a nuclear extract was prepared according to a previously described method [11]. Digested genomic DNA ranging in size from 150 to 900 bp was obtained by incubating the nuclear extract with Micrococcal Nuclease (Cell Signaling Technology, Inc., Tokyo, Japan). The sizes of the DNA fragments were confirmed by gel electrophoresis. The lysate was sonicated and centrifuged. The supernatant was then diluted in ChIP buffer (Cell Signaling Technology, Inc.) and incubated with 10 μg mouse anti-GCNF monoclonal antibody (PP-H7921-00, Perseus Proteomics, Japan), which was used to capture the NR6A1 protein. The samples were then incubated with Protein-G agarose beads. The NR6A1-antibody-Protein-G complexes were washed and DNA templates for PCR were purified. ChIP–PCR analysis was carried out using the HCRT_-611Fw, HCRT_-467Rv, HCRT_-633Fw, and HCRT_-431Rv primers (Table).

Double immunohistochemical staining

Six-week-old male C57/Black6J mice were perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Whole brains were removed, post-fixed, and cryosectioned into 30-μm coronal sections. The sections were incubated with a rabbit anti-HCRT-1 antiserum (1:5000) [12] and a mouse anti-GCNF monoclonal antibody (1:500). Next, they were incubated with a goat Alexa594-labelled anti-mouse IgG and a goat Alexa488-labelled anti-rabbit IgG (1:2000) (Molecular Probes, Paisley, UK). The resulting hypocretin and NR6A1 signals were visualised under a fluorescence microscope equipped with a digital camera (Olympus BX51; DP70, Tokyo, Japan).

Luciferase reporter assay

Cells were seeded at a density of 2.5×10^5 cells per well in 24-well cell culture plates. The luciferase plasmids and an expression vector were cotransfected with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. The amounts of cotransfected plasmids and vectors per well were as follows: 200 ng of firefly luciferase-encoding reporter plasmid (pGL3 or pTAL), 20 ng of Renilla luciferase-encoding internal control plasmid (pGL4.74), and 200 ng of pCMVTNT or NR6A1/TNT vector. The luciferase activities were measured sequentially and in duplicate using dual luciferase reporter assay reagents (Promega) according to the manufacturer's protocol. The firefly luciferase luminescence (FLU) activities from the pGL3 or pTAL plasmid and the Renilla luciferase luminescence (RLU) activity from the pGL4.74 plasmid were measured independently. The relative luciferase activity was determined by dividing the FLUs by the RLUs. The relative luciferase activities were standardised by the value of the corresponding control conditions, which were either the cotransfection of a pGL3-basic plasmid or a pTAL-luc plasmid with a pCMVTNT vector. Data for the standardised activities were expressed as the mean of at least eight independent experiments ± SE. Changes in each reporter activity with/without the NR6A1/TNT vector were compared using Student's t-test. $P < 0.05$ was considered to be statistically significant.

In utero electroporation

In utero electroporation was performed according to a previously reported method [13] with some modifications. The pCAG-mNr6a1 vector was mixed with Fast Green and injected into the third ventricle of each C57/Black6J mouse embryo (embryonic day 12) by free-hand injections with a glass capillary pipette through the uterine wall. Electrodes (CUY650P3, BEX, Tokyo Japan) were placed on both sides near the head of the embryo, and electroporation was performed using a square-pulse electroporator (CUY21 EDIT In vivo Electroporator, BEX), which resulted in gene transfer on one side of the hypothalamus (Figure S1). Control mice were electroporated with pCAG-EGFP. Gene-electroporated mice were collected on postnatal day 1. Total RNA was isolated from each side of the hypothalamus independently (gene-electroporated side or control side). The expression level of each murine gene was determined by quantitative RT-PCR as described in Figure S3 using the primers listed in Table. The relative expression level of each gene was expressed as the relative expression levels of the gene-electroporated side divided by the relative expression levels of the control side. Changes in each transcription were compared using Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

NR6A1 binds to DNA containing a NurRE

ChIP-PCR studies were performed to show that endogenous NR6A1 binds to the prepro-hypocretin promoter. DNA containing a putative NR6A1-binding site (NurRE) was amplified with two primer pairs from purified DNA immunoprecipitated with anti-GCNF (NR6A1) antibodies (Figure 1C). No amplification was found from purified DNA incubated with normal rabbit serum or water without DNA template. Quantitative RT-PCR analysis in SH-SY5Y cells transfected with the NR6A1/TNT vector showed a 50-fold increase in expression of *NR6A1* as compared with untransfected cells. This transient overexpression of *NR6A1* induced increased NR6A1 binding to the proximal region including the NurRE (data not shown), suggesting that the binding capacity of NR6A1 to the NurRE is relatively low.

NR6A1 colocalises with hypocretin neurons

NR6A1 colocalises with almost all murine hypocretin cells (Figure 1G) and is also expressed in various cells that lack hypocretin expression. NR6A1 immunoreactivity was clearly colocalised with DAPI staining, indicating that NR6A1 is localised in the nucleus in the murine hypothalamus (Figure 1G).

NR6A1 overexpression suppresses hypocretin promoter activity in SH-SY5Y cells

To ascertain if NR6A1 affects prepro-hypocretin gene transcription, prepro-hypocretin promoter activity was studied in human NR6A1 expression in various cells using an *in vitro* luciferase assay. Cotransfection of a pGL3-basic plasmid with the pCMVTNT or the NR6A1/TNT vector showed similar luciferase expressions in all the cells, indicating an absence of confounding endogenous transcriptional regulatory elements in the pGL3-basic plasmid (Figure 2A, Figure S2). Cotransfection of the prepro-hypocretin promoter -3278/+87 region (3.2 kb-basic) with the pCMVTNT vector significantly increased the luciferase activity around fivefold in the SH-SY5Y cells (Figure 2A) and around twofold in the HEK293T cells. No change was observed in any of the other cells tested (Figure S2). The observed prepro-hypocretin promoter activities were suppressed by cotransfection with the NR6A1/TNT vector in SH-SY5Y cells (Figure 2A).

Further experiments were performed only in the SH-SY5Y cell, because the 3.2 kb-basic showed weak or no activity in the other cells. Then, we generated a series of deletion mutants to search for the *cis*-acting sequence regulating prepro-hypocretin transcription.

Under conditions with the pCMVTNT vector, all the deletion mutants--the construct lacking the -3278/-2023 region and the OE2 sequence (2.0 kb-basic), the construct lacking the -3278/-634 region (ΔDR0/0.7 kb-basic), and the construct lacking the -3278/-634 region and the -548/-530 region (ΔNurRE/0.7kb-basic)—led to significant increases in relative luciferase activity in the SH-SY5Y cells (Figure 2A). These results indicate that the OE2, five *Alu* repeats including the DR0 element, and the 18-bp region including the NurRE are not critical for basal transcription in SH-SY5Y cells. Additionally, these results suggest that endogenous NR6A1 levels in SH-SY5Y cells have no effect on hypocretin transcription. Although no response to NR6A1 was found for the condition with the ΔNurRE/0.7 kb-basic plasmid, all of the other deletion mutants, including the ΔDR0/0.7 kb-basic, showed responsiveness against NR6A1. In other words, the transcriptional activities of the prepro-hypocretin promoter sequence were suppressed by NR6A1 through the 18-bp region in the SH-SY5Y cell (Figure 2A).

NR6A1 modulates the sequence activity including NurRE

Next, we investigated whether the 18-bp region itself had transcriptional modulator activity (enhancer, silencer, or insulator). Since it was suggested that NR6A1 directly binds to the SV40-related sequence within the pGL3-Control plasmid (data not shown), we cloned the 18-bp into the pTAL-luc plasmid, which had no responsiveness against NR6A1 in the preliminary experiments using SH-SY5Y cells. We found luciferase activation both in a single copy and the three copies of the 18-bp sequence (Figure 2B). In the condition with the NR6A1/TNT vector, the three copies was activated, even though a single copy was repressed.

NR6A1 affects murine hypothalamic hypocretin transcription

To confirm the physiological relevance of NR6A1 in hypothalamic hypocretin transcription, *Nr6a1* overexpression in the murine hypothalamus was generated using *in utero* electroporation. Electroporated GFP expression was found only on one side of the hypothalamus and some hypocretin neurons were colocalised with GFP (Figure S1, arrow) on embryonic day 12, thus showing the efficiency of our *in utero* electroporation procedure at embryonic day 12. The expression levels were elevated on the electroporated-side as compared with the control side (*Nr6a1*; mean + SE: 4.74 + 1.37, ranging from 2.1 to 12.6 times, n = 7) (*GFP*; 5.86 + 1.60, ranging from 2.3 to 13.1 times, n = 6) (Figure 3). Nr6a1 showed specific effects against hypocretin transcription compared with GFP ($p < 0.05$, Figure 3), but there were no specific effects on *Nptx2* and *Pdyn* transcriptions (genes concurrently expressed in hypocretin neurons [14] [15]), or *Pmch* transcription (found in cells near hypocretin neurons) (Figure 3).

Discussion

We have identified an 18-bp sequence including the NurRE (AGGTCC) in the proximal region of the hypocretin gene as a putative NR6A1 binding site with a luciferase reporter assay using deletion mutants. The random expectation probability was low (0.16 matches per 1000 bp) for this NurRE sequence, increasing the likelihood of functional effects on regulating prepro-hypocretin promoter activity. This 18-bp region was also well conserved between the human and mouse. ChIP-PCR studies showed endogenous NR6A1 binding to the proximal region containing the NurRE. Transient NR6A1 overexpression in SH-SY5Y cells induced increased NR6A1 binding to proximal region containing the NurRE (data not shown) with ChIP-PCR study. Double immunohistochemical staining with hypocretin and NR6A1 revealed that almost all hypocretin cells expressed the NR6A1. These results suggest a functional connection between NR6A1 and the 18-bp sequence containing the NurRE in regulating human prepro-hypocretin transcription.

Two conserved regions—OE1 and OE2—have been identified in the promoter region of the human prepro-hypocretin gene. They are required in recapitulating proper hypothalamic expression in transgenic mice [4]. Considering its proximity, our hypothesis was that the 18-bp sequence (-548/-530) containing the NurRE would need the proximal OE1 promoter (-291/-235) to function in concert with this regulatory region. The construct lacking the 18-bp sequence with the NurRE showed no responsiveness against NR6A1 in SH-SY5Y cells, although the construct lacking the DR0 element included in one of the five *Alu* repeats remained responsiveness. The suppression of NR6A1 was replicated in the experiment using a NurREx1-pTAL reporter plasmid in the SH-SY5Y cells, suggesting that the 18-bp sequence in the prepro-hypocretin upstream region has functional modulatory activity against the proximal promoter.

Although it was suggested that NR6A1 leads to deacetylation of core histone molecules [16] to repress target gene expressions, no transcriptional repression was observed in our HEK293 cells (Figure S2) differed from in the SH-SY5Y cells (Figure 2). It has been reported that cotransfection with CREMtau and NR6A1 promoted reporter activities as compared to basal activities [16], because NR6A1 cannot completely disturb the CREMtau activations. Therefore, our NR6A1 effect in each cell might be characterised by distinct sets of endogenous corroborating nuclear factors induced by NR6A1 itself. Interestingly, a construct with three copies of the NurRE had the opposite effect on the proximal promoter. A chimeric binding site is supposed to have been generated by connecting each NurRE to other motifs.

To further demonstrate the physiological relationships between NR6A1 and hypocretin neurons, we generated mice overexpressing *Nr6a1* in one side of brain by *in utero* electroporation. Hypothalamic hypocretin transcription was activated by *Nr6a1* overexpression but not by *GFP* overexpression. The number of hypocretin cells did not change between the electroporated-side and the control side in GFP electroporated-mice (approximately 1000 cells, data not shown), suggesting that our electroporation procedure had low cytotoxicity. The expression of *Nptx2* and *Pdyn*, which are genes concurrently expressed in hypocretin neurons [14] [15], was not altered. Overexpression of *Nr6a1* in other types of cells in which prepro-hypocretin transcription might be affected indirectly must be taken into account in our method. Considering the proven function of NR6A1 in neuronal differentiation [10], a variety of cell types other than hypocretin neurons were expected to be affected by *Nr6a1* overexpression. Nevertheless, *Pmch* expression was not affected by *Nr6a1* overexpression. Our data indicated that NR6A1 is a relatively specific regulator for hypocretin expression in the murine lateral hypothalamus. Interestingly, these murine genes have DR0 elements (AGGTCA) (*Nptx2*: -919/-913, -881/-875, -10/-4; *Pmch*: -1162/-1156) and a NurRE (AGGTCC) (*Pdyn*: -1121/-1115) in their upstream regions, even though murine hypocretin has no DR0 element or NurRE. The importance of direct repeats and their proximity to the genes are suggested. In addition, other binding motifs might be present in the 18-bp sequence or another sequence of the upstream region of the murine hypocretin gene. The opposite results between human cells and murine hypothalamus might be explained by the presence of another NR6A1 regulatory element within the upstream region of the murine hypocretin gene.

Furthermore, we found different hypocretin cell counts in the murine hypothalamus on postnatal day 1 (approximately 1000 cells) as compared with the adult (approximately 3500 cells, [5]), suggesting that there are individual regulatory mechanisms affecting hypocretin transcription before and after maturation. NR6A1 expression resulted in the inhibition of hypocretin promoter activity in adult neural cells, while it increased hypocretin transcription in the foetal hypothalamus. It is possible that this discrepancy results from the switch effect of NR6A1 during the developmental stage. It has been reported that some mRNA

transcriptions are under differential controls by the same DNA-binding factors during developmental stage. For instance, TRAX, a DNA-binding factor, downregulates *GAP43* on postnatal day 30, whereas TRAX upregulates or maintains *GAP43* before postnatal day 16 [17]. These data suggest that “on-on” or “off-off” control between TRAX and *GAP43* have been switched to a “on-off” control during the developmental stage. A similar switching mode is supposed to occur between hypocretin and NR6A1. As speculation for the mechanism of such a switching, some constituent factors within the NR6A1 regulatory complex might be modified or renewed-chromatin modifications within the prepro-hypocretin promoter region might be occurred during the developmental stage.

A lack of diurnal variation in *Nr6a1* expression (Figure S3) suggests that NR6A1 has a very small contribution to the diurnal rhythm formation of prepro-hypocretin transcription [18]. A recent study showed that FOXA2 translocates into the nucleus, binds to the hypocretin promoter, and stimulates prepro-hypocretin expression in the lateral hypothalamus during fasting [19]. NR6A1 might mediate signals other than diurnal rhythms, such as FOXA2 activity.

GFP does not exist naturally. Therefore a huge difference may exist between the electroporated side and the control side of the murine brain. Because the electroporated brains were dissected into mid-sagittal sections by free-hand cutting, sampling errors could have affected our data. Unlike some of the electroporated genes, hypocretin cells are not located close to the midline, sampling errors were not critical. Leakage of expression vectors into the control side without detectable fluorescence levels could have also occurred in our system.

Conclusion

We found that NR6A1 is a novel modulator of hypocretin expression *in vitro* and *in vivo*. To clarify whether NR6A1 directly or indirectly affected hypocretin expression, further *in vivo* studies using cross-hybridisation with Nr6a1tm3Coo mice [20] and hypocretin promoter-Cre mice [21] will need to be completed.

Research highlights

NR6A1 colocalises with hypocretin neurons.

NR6A1 binds to putative NR6A1-binding site within a hypocretin promoter.

NR6A1 regulates hypocretin expression *in vitro* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NR6A1	nuclear receptor subfamily 6, group A, member 1
OE	orexin response element
POU5f1	POU class 5 homeobox 1
NurRE	nuclear receptor response element
GCNF	germ cell nuclear factor
GFP	green fluorescent protein
Nptx2	neural pentaxin 2
Pdyn	prodynorphin
Pmch	pro-melanin-concentrating hormone
CREMtau	cAMP response element modulator tau
TRAX	translin-associated factor X
GAP43	growth-associated protein–43
FOXA2	forkhead box A2

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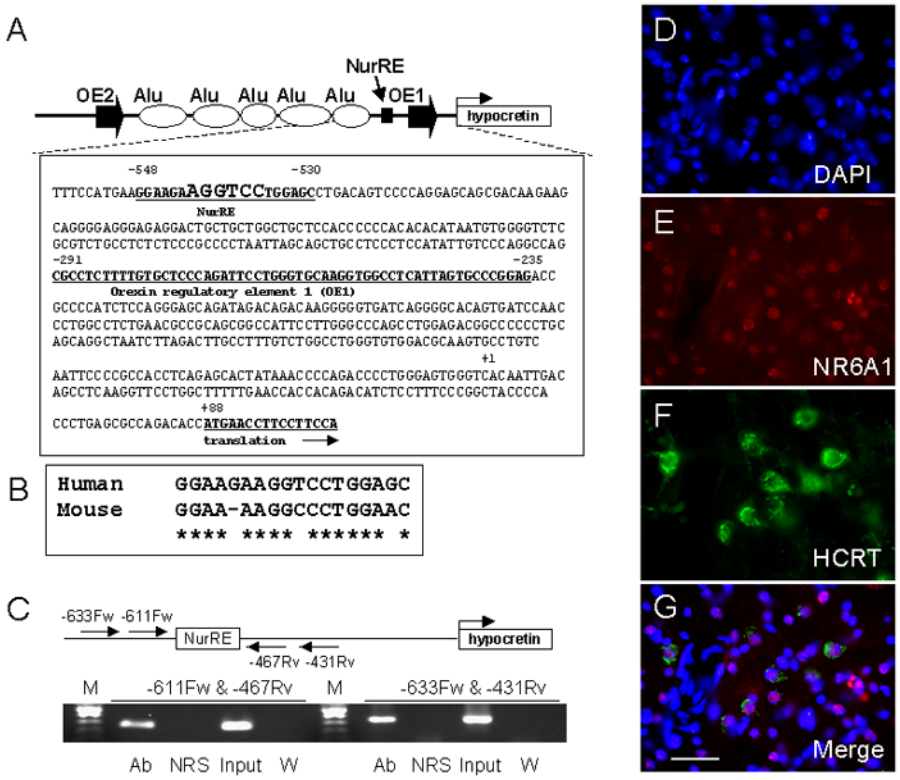

Figure 1. Schematic representation of the human prepro-hypocretin gene regulatory region, chromatin immunoprecipitation (ChIP)-PCR, and colocalisation of NR6A1 in the murine hypocretin neurons

(A) Schematic representation of the human prepro-hypocretin gene regulatory region. Two highly conserved regions—OE1 and OE2 (black arrows)—and five *Alu* repeats (ovals) have been previously reported [3]. The first residue of the transcription start site and the translation start site are marked as +1 and +88. The 18-bp region of the potential NR6A1 binding site, including the NurRE identified by the Genomatix Software GmbH (http://www.genomatix.de), is shown in bold and underlined.

(B) The 18-bp region of the potential NR6A1 binding site. Asterisks show conserved nucleic acids between the human and the mouse.

(C) ChIP-PCR analysis for endogenous NR6A1 binding to the proximal region of the prepro-hypocretin gene in SH-SY5Y cells. Two primer pairs, -611Fw/-467Rv and -633Fw/-431Rv, were used to amplify the DNA containing the putative NR6A1-binding site. A schematic representation of the primer binding sites is shown in the upper panel. The results of the ChIP-PCR analysis are shown in the lower panel. Ab: samples immunoprecipitated with the anti-GCNF antibody; NRS: with normal rabbit serum; W: water (negative control).

(D–G) Murine lateral hypothalamic sections. (D) DAPI-labelled nuclei (blue). (E) Alexa-594 visualised murine NR6A1 (red). (F) Alexa-488 visualised murine hypocretin (green). (G) Merged image of (D), (E), and (F) immunofluorescence. Scale bar represents 20 μm.

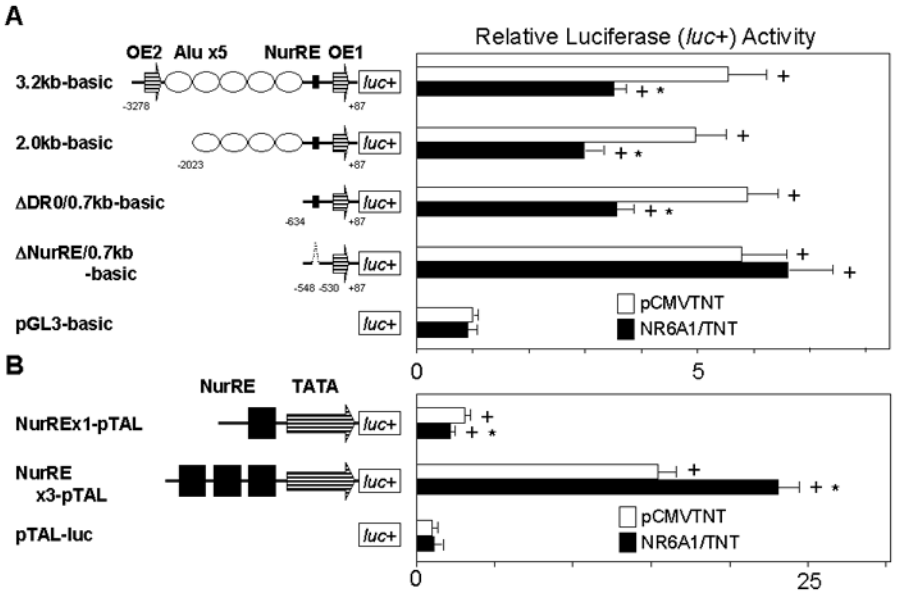

Figure 2. Transcriptional activities of the prepro-hypocretin promoter and its deletion mutants, and activity of the NurRE on the TATA-like promoter sequence in SH-SY5Y cells

(A) SH-SY5Y cells were cotransfected with an NR6A1/TNT vector and reporter plasmids with/without the partial deletions shown in the schematic representation. The results are shown as the mean of at least eight independent experiments ± SE. +: $p < 0.05$ versus pGL3-basic with the pCMVTNT vector. *: $p < 0.05$ versus the same reporter plasmid with the pCMVTNT vector.

(B) Transcriptional activities of the NurREx1-pTAL and the NurREx3-pTAL plasmids in SH-SY5Y cells. The results are shown as the mean of at least eight independent experiments ± standard error. +: $p < 0.05$ versus pTAL-luc with pCMVTNT vector. *: $p < 0.05$ versus the same reporter plasmid with the pCMVTNT vector.

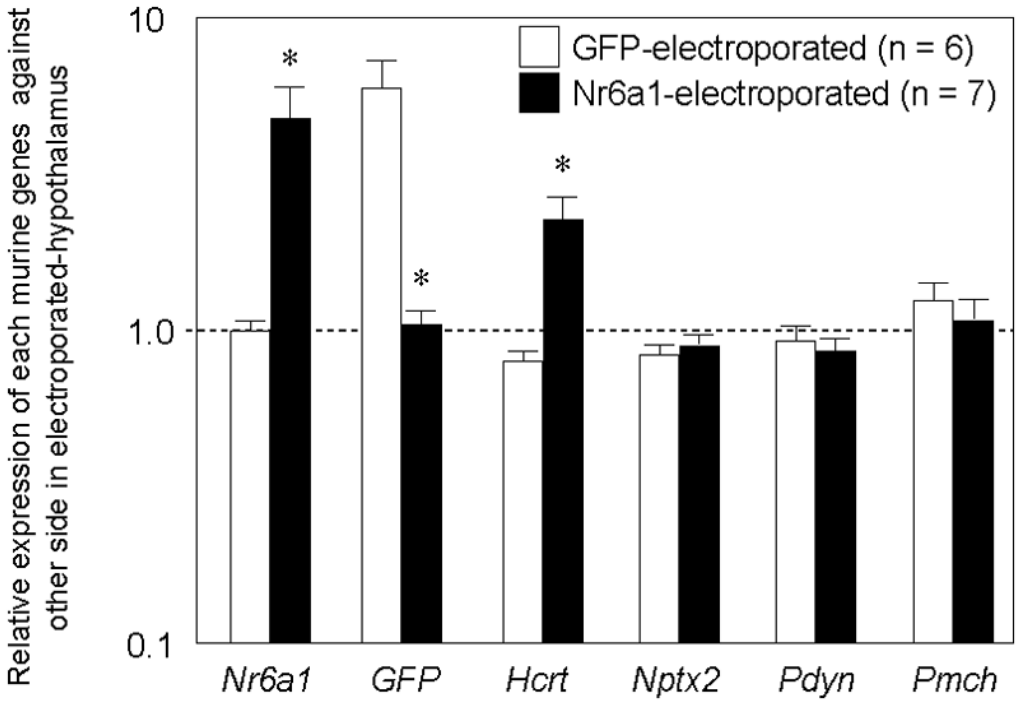

Figure 3. The relative expression levels of hypothalamic genes after *in utero* electroporation

In utero electroporation was performed to evaluate the effect of NR6A1 on hypocretin transcription. Control mice were electroporated with pCAG-EGFP. The relative expression level of each gene was expressed as the relative expression levels of the gene-electroporated side divided by the relative expression levels of the control side (GFP, n = 6; NR6A1, n = 7). Changes in transcription levels were compared using Student's t-test. *: $p < 0.05$ versus GFP electroporated-mice.

Table

Primers and oligonucleotides

Primers for hypocretin promoter sequence			
HCRT_+87Rv	5'-CCGCTCGAGGGTGTCTGGCGCTCAGGGTG-3'	The first exon sequence just before the translation initiator ATG in the human prepro-hypocretin gene (*Xho* I site is underlined)	
HCRT_-3278Fw	5'-CGACGCGTGGATCCAGATGCCTCTGAATAG-3'	The sequence starting from -3278 (*Mlu* I sites is underlined)	
HCRT_-2023Fw	5'-CGACGCGTCACCTGTAATCCTAGCAGTTTG-3'	The sequence starting from -2023 (*Mlu* I sites is underlined)	
HCRT_-634Fw	5'-CGACGCGTGGTTTTCCATCTCAGAGGCTAG-3'	The sequence starting from -634 (*Mlu* I sites is underlined)	
HCRT_-548Rv_P	5'-TTCATGGAAAGGCTCCTTAGCTG-3'	The sequence just before the NurRE.	
HCRT_-530Fw_P	5'-TGACAGTCCCCAGGAGCAGCGACAA-3'.	The sequence just after the NurRE.	
Oligonucleotides for double strand DNA			
NurREx1_sense	5'-CGCGTGGAAGAAGGTCCTGGAGCCC-3'	The NurRE sequence containing *Mlu* I overhanging end	
NurREx1_anti-sense	5'-TCGAGGGCTCCAGGACCTTCTTCCA-3'	The NurRE sequence containing *Xho* I overhanging end	
NurREx3_sense	5'-CGCGTGGAAGAAGGTCCTGGAGCCGGAAGA AGGTCCTGGAGCCGGAAGAAGGTCCTGGAGCCC-3'	The 3 copies of NurRE sequence containing *Mlu* I overhanging end	
NurREx3_anti-sense	5'-TCGAGGGCTCCAGGACCTTCTTCCGGCTCCAG GACCTTCTTCCGGCTCCAGGACCTTCTTCCA-3'	The 3 copies of NurRE sequence containing *Xho* I overhanging end	
Primers for ChIP-PCR analysis			
HCRT_-611Fw	5'-GGTTTTCCATCTCAGAGGCTAGT-3'		
HCRT_-467Rv	5'-AGCTGCCTCCCTCCATATTGTCCCAGGCCAG-3'		
HCRT_-633Fw	5'-CAGCTAAGGAGCCTTTCCATGAA-3'		
HCRT_-431Rv	5'-CTCTCTCCCGCCCCTAATTAG-3'		
Primers for the murine mRNA quantification			
Hcrt_Fw	5'-CTCCAGGCACCATGAACTTT-3'	Hcrt_Rv	5'-GGGATGTGGCTCTAGCTCTG-3'
Nr6a1_Fw	5'-CATCAGCTGAAGGTCAGCAA-3'	Nr6a1_Rv	5'-TGAAATCCTGACAAATGTACCA-3'
GFP_Fw	5'-ACCACTACCTGAGCACCCAGT-3'	GFP_Rv	5'-GTCCATGCCGAGAGTGATCC-3'
Nptx2_Fw	5'-GGCGATGTTGATGATCTCCT-3'	Nptx2_Rv	5'-CACATCTGCATCACCTGGAC-3'
Pdyn_Fw	5'-TGTAGCCTTCTTCCAAAGCAA-3'	Pdyn_Rv	5'-TCTAATGTTATGGCGGACTGC-3'
Pmch_Fw	5'-GCCAACATGGTCGGTAGACT-3'	Pmch_Rv	5'-CGCTCTGAAAGGATCCGTAG-3'