Hypocretin deficiency : neuronal loss and functional consequences

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The Number of Hypothalamic Hypocretin (Orexin) Neurons is Not Affected in Prader-Willi Syndrome

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CHAPTER 1

The Number of Hypothalamic Hypocretin (Orexin) Neurons Is Not Affected in Prader-Willi Syndrome

Context **Narcoleptic patients with cataplexy have a general loss of hypocretin (orexin) in the lateral hypothalamus, possibly due to an autoimmune-mediated degeneration of hypocretin neurons. In addition to excessive daytime sleepiness, Prader-Willi syndrome (PWS) patients may show narcolepsy-like symptoms, such as sleep onset rapid eye movement sleep and cataplexy, independent of obesity-related sleep disturbances, which suggests a disorder of the hypocretin neurons.**

Objective **We hypothesized that the narcolepsy-like symptoms in PWS are caused by a decline in the number of hypocretin neurons.**

Design **We estimated the number of hypocretin neurons in postmortem hypothalami using immunocytochemistry and an image analysis system.**

Setting **This study was conducted at the Netherlands Institute for Brain Research.**

Patients **Eight PWS adults, three PWS infants, and 11 controls were studied.**

Results **There was no significant difference in the total number of hypocretin-containing neurons among the seven PWS patients (in whom sufficient hypothalamic material was available to quantify total cell number) and seven age-matched controls, either in adults or in infants. A significant decline with age was found in adult PWS patients ($r = -0.9$; $P = 0.037$).**

Conclusion **We conclude that a decrease in the number of hypocretin neurons does not play a major role in the occurrence of narcolepsy-like symptoms in PWS.**

Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness (EDS), cataplexy, premature transitions to rapid eye movement (REM) sleep, known as sleep-onset REM periods, sleep paralysis, and hypnagogic hallucinations.[1] In addition, obesity is a common feature in narcoleptic patients.[2] Patients with cataplexy have lowered cerebrospinal fluid (CSF) levels of the neuropeptide hypocretin (orexin) as an indirect reflection of a loss of hypocretin neurons in the perifornical area of the hypothalamus, possibly due to an autoimmune process.[3,4] Prader-Willi syndrome (PWS), the most common syndromal cause of human obesity, is characterized by an insatiable hunger from childhood onward, mental retardation, hypogonadism and growth deficiency, whereas hypotonia, feeding problems, and failure to thrive are the predominant features in the neonatal period.[5] The molecular genetic cause is nonexpression of the paternal genes in the PWS region on chromosome 15q11-13.[6] EDS in PWS is a symptom that has only recently attracted attention because it was first thought to be due to sleep apnea

related to obesity.[7] There have been several reports, however, that PWS patients show EDS, sleep onset with REM, and in some cases even cataplexy, independent of obesity-related sleep disturbances.[8,9] Interestingly, there are preliminary studies reporting lower CSF levels of hypocretin in several patients, which suggests hypocretin neurons are affected in PWS.[10-12] We determined the number of hypocretin-containing cells in the postmortem lateral hypothalamus of PWS adults, infants, and matched controls using immunocytochemistry.

Patients and Methods

Hypothalamic material

Hypothalami from eight PWS adults and three PWS infants from different clinical centers were used. Eight adult controls and three control infants, matched for age, sex, postmortem delay (PMD), fixation time, and premortal illness duration, were obtained through The Netherlands Brain Bank. Clinicopathological details are given in Table 1. Permission was obtained for a brain autopsy and for the use of human material and clinical information for research purposes. Exclusion criteria for control subjects were: primary neurological or psychiatric disease, glucocorticoid therapy during premortal illness, and weight problems, such as excessive weight loss before death or tube feeding. An exception was control 91-009 (Table 1), who suffered from tetraplegia secondary to cervical birth trauma. The clinical histories of the PWS adults and infants have been described previously, except for 03-021, 00-028, and 02-074.[13-16] No direct mentioning of the occurrence of EDS, sleep onset REMs, or cataplexy could be found in the records of either the previously published or unpublished PWS medical histories. All PWS patients met Holmes clinical criteria, and six had genetically confirmed diagnoses (Table 1). Tissues were fixed in 10% PBS (pH 7.4) formalin at room temperature. Hypothalami were paraffin-embedded and serially sectioned at 6µm from rostral to caudal. Every 100th section was stained with thionin for orientation.

Hypocretin-1 immunocytochemistry

Every 100th section in the expected hypocretin-1 cell area, from the level where the fornix touches the paraventricular nucleus to the level where the fornix reaches the corpora mammillaria, was stained using a hypocretin-1 (orexin A) antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA; catalog no. H-003-30, batch no. R2626) and visualized according to the avidin-biotin complex method using diaminobenzidinenickel solution to finish the staining as described previously by Goldstone et al.[17] If these slices did not cover the whole hypocretin-1 area, extra sections were added at equal distances, both rostral and caudal, until no more hypocretin cells were present. Mean (± sd) number of sections added per subject was 1.75 ± 2.79.

Antibody specificity

To test the specificity of the antibody, a dot blot was performed, adding a dilution of 1:1250 antihypocretin onto 2% gelatin-coated nitrocellulose paper (0.1-µm pore size) containing different spots with 30 µl hypocretin-1, somatostatin (1–14), somatostatin (1–28), galanin, melanin-concentrating hormone-1 receptor, β-lipotropin, substance-P,

Table 1.1

NBB no.	Sex	Age (years)	PMD (hours)	Fixation time (days)	Brain weight (g)	Cause of death	Other clinical problems	Premorbid illness duration (days)	Hcrt-1 cell number (x 1000)
Prader-Willi syndrome (sufficient material available for quantification)									
98-168	F	6 Mo	9.75	60	772	Asphyxia	PW71B maternal methylation pattern, severe hypotonia	1.0	78.7
03-021	M	3	41.0	63	1360	Unknown (possible asphyxia)	Gastro-enteritis	1.0	52.7
96-034	F	25	35.1	26	1300	DIC post operation. Repair perforated gastric ulcer	BMI 24.6, ch 15q11-13 del	1.5	90.2
00-028	M	32	<48.0	59	1550	Sudden death following 2 days of fever, diarrhoea and vomiting	Weight 76 kg, ch 15q11-13 del	3	87.9
91-058	F	33	5.0	33	1223	Pneumonia	Congestive cardiac failure, BMI 27.1	4	82.5
02-074	M	49	-	50	-	-	Diabetes	-	42.7
90-111	F	64	20.0	14	1150	Respiratory failure	BMI 30.9	4	74.2
Mean		29.5	22.7	43.6	1226			2.4	72.7
Median		32.0	20.0	50.0	1262			2.3	78.7
SD		22.9	18.9	19.2	261			1.4	18.1
Controls (used in means between groups analysis)									
86-041	M	6 Mo	6.5	14	800	SIDS	-	1	88.8
88-050	M	9 Mo	41.0	164	940	SIDS	-	1	82.2
02-076	M	27	-	31	1520	Drowning	-	1	79.9
85-041	F	28	5.4	44	1365	Cardiogenic shock post myocardial infarction	Crohn's disease	1	77.3
91-009	F	36	71.5	61	1348	Faecal peritonitis from perforated peptic ulcers	Tetraplegia secondary to cervical birth trauma	1	111.9
94-035	M	49	7.7	40	1404	Cardiac arrhythmia	Hypertension	1	78.5
01-069	F	68	5.75	32	1153	Respiratory insufficiency	-	-	55.2
Mean		29.9	23.0	55.1	1219			1.0	82.0
Median		28.0	7.1	40.0	1348			1.0	79.9
SD		24.4	27.5	50.0	265			0.0	16.8

Table 1.1 (Continued)

NBB no.	Sex	Age (years)	PMD (hours)	Fixation time (days)	Brain weight (g)	Cause of death	Other clinical problems	Premorbid illness duration (days)	Hcrt-1 cell number (x 1000)
Prader-Willi Sydrome (insufficient material available for quantification)									
99-079	F	9 Mo	10.0	76	-	Cardiovascular failure after bronchopneumonia	Hypoglycemia, hypothermia, ch 15q11-13 del	2.0	ND*
83-011	F	30	4.5	365	1310	Sepsis post operation. Repair enterocutaneous fistula	Jejuno-ileal bypass and small bowel resection 6-10 y ago, BMI 42.2	35	ND*
93-056	M	38	45.0	385	1540	Diabetic ketoacidosis	BMI 38.5, ch 15q11-13 del	1	ND*
95-104	M	51	16.0	32	1570	Pneumonia	Hypertension, testicular seminoma 28y, BMI 33.8, ch15 UPD	7	ND*
Mean		29.9	18.8	214.5	1473			11.3	
Median		34.0	13.0	220.5	1540			4.5	
SD		21.3	18.0	186.4	2142			16.0	
Controls (used in regression analysis)									
97-153	F	7 Mo	20.4	39	760	SIDS	-	1	56.0*
92-037	F	32	30	45	1280	Bronchopneumonia/bronchitis	Hyperventilation	-	80.5*
99-071	M	39	16.50	130	1400	Myocardial infarction	Hypercholesterolemia	1	127.3*
94-118	M	49	22.3	33	1254	Faecal peritonitis post revision ileocolonic anastomosis	Adenocarcinoma	32	54.9*
Mean		30.2	22.3	61.8	1174			11.3	79.7
Median		35.5	21.4	42.0	1267			1.0	68.3
SD		20.9	5.7	45.8	283			17.9	33.9

BMI, Body Mass Index (in kg/m2); ch, chromosome; del, deletion; DIC, Disseminated Intravascular Coagulation; F, female; M, male; Mo, months, NBB no., Netherlands Brain Bank number; ND, not determined; PMD, post mortem delay; SIDS, Sudden Infant Death Syndrome; SD, standard deviation; UPD, uniparental disomy; -, Unknown. * Incomplete patient, or control matched with an incomplete patient

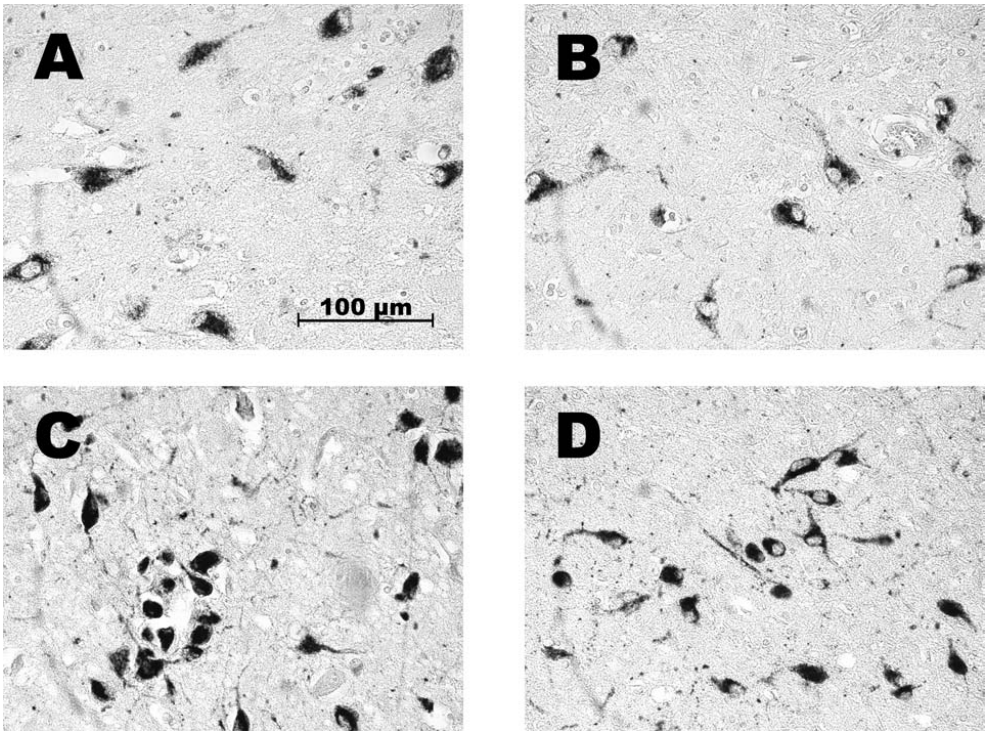

Figure 1.1 **Examples of staining of hypocretin-IR cell bodies**

Examples of staining of hypocretin-IR cell bodies in the lateral hypothalamus of an adult control subject #02-076 (A), an adult Prader-Willi patient #91-058 (B), a control infant #97-153 (C) and a Prader-Willi infant #99-079 (D). There was no significant difference in the intensity of staining and the distribution pattern. Note that the density of cell bodies is higher in the infant subjects, which is accompanied by a smaller volume of the hypothalamic area containing these neurons.

γ-melanocyte-stimulating hormone, LHRH, adrenocorticotropic hormone (1–39), neurotensin, oxytocin, CRH , agouti-related protein (83– 132), neuropeptide-Y, GHRH (1– 40), arginine-vasopressin, desacetylmelanocyte-stimulating hormone, neuropeptide EI, β-melanocyte-stimulating hormone, glycoprotein hormone receptor, cocaine- and amphetamine-regulated transcript, or melanin-concentrating hormone. The next day, the nitrocellulose sheet was incubated with secondary antibody, avidin-biotin peroxidase complex, and diaminobenzidinenickel solution to finish the staining. The only spot that showed staining was the one containing hypocretin-1. Specificity was further confirmed by the absence of staining in hypothalamic sections using antiserum preadsorbed with human hypocretin-1 peptide fixed overnight with 4% formaldehyde onto gelatin-coated nitrocellulose filter paper, 0.1 µm, and the presence of staining when preadsorbed with α-melanocyte-stimulating hormone peptide, which did not differ from unadsorbed serum.

Immunocytochemistry quantification

An estimate of the total number of hypocretin-1 immunoreactive (IR) cells was made using an image analysis system (ImagePro version 4.5, Media Cybernetics, Silver Spring) connected to a camera (JVC KY-F55 3CCD) and plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany). Randomly selected fields were counted in every section, covering in total 15% of a manually outlined area containing hypocretin-1 IR cells. This was done by one person while blinded for the diagnosis. Each positively stained profile containing a nucleolus was counted. Calculation of the total number of hypocretin-1 IR neurons was performed by a conversion program based upon multiplication of the neuronal counts by sample frequency of the sections, as was described previously by Goldstone et al.[17] Mean (±sd) number of sections quantified per subject was 13.9±3.5. The coefficient of variation (sd/mean x 100%) of this method was 7.6% (calculated by counting one complete patient five times). Reliability was further confirmed by graphically presenting the actual numbers of neurons counted in every section from rostral to caudal to review the distribution pattern (figures not shown due to space restrictions).

Statistics

Spearman's ρ correlation was performed to assess the effect of age, PMD, fixation time, and duration of premortal illness on hypocretin-1 IR cell number. Means between groups were tested by Mann-Whitney U test, considering $P < 0.05$ to be significant.

Results

Distribution of hypocretin-1-containing neurons

The location and intensity of the hypocretin-1 IR cell bodies was similar in controls, PWS adults, and infants. Hypocretin-1 IR neuronal cell bodies were restricted to the peri-fornical region in the lateral hypothalamus. On the level where the fornix crosses the paraventricular nucleus, some hypocretin-1 IR cell bodies started to appear in the supraoptic area. In subsequent levels, the fornix migrated to the corpora mammillaria while passing through an area with a high number of hypocretin-1 IR cell bodies. When the fornix reached the corpora mammillaria, there were still many hypocretin-1 IR cell bodies visible.

Hypocretin-1 cell number in PWS and controls

In four PWS patients (three adults, one infant), the area showing hypocretin-1 IR cell bodies was not completely present in the available hypothalamic material. Therefore, the total cell counts of these patients and their matched controls were not included in this final analysis. Extrapolation of the data obtained from the material that was available by comparing the distribution patterns of the incomplete patients with those of complete cases did not point to a different number of cells and would thus not have changed the final outcome. Controls 94-035 and 94-118 were an equal match to incomplete patient 95-104. Exclusion of either one of these controls did not influence the outcome. In the analysis presented here, control 94-118 was excluded. There were no significant differences among sex, PMD, fixation time, or premortal illness duration

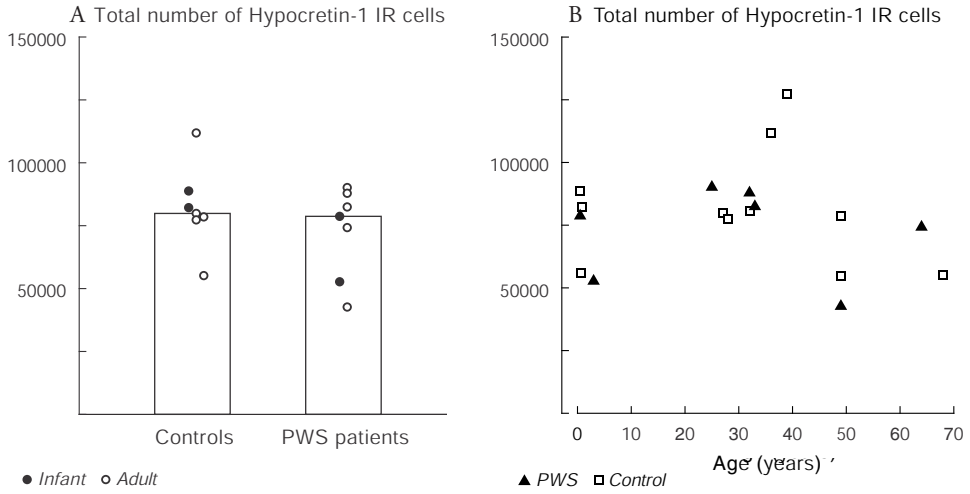

Figure 1.2 **Results**

(A, Left) Median number of hypocretin IR cells in Prader-Willi Syndrome patients (right bar) and controls (left bar). Open circles represent adults, filled circles represent infants. There is no significant difference between the two groups (Mann-Whitney U: n=14, p=0.56). (B, Right) Correlation between age and total number of hypocretin IR cells. Squares represent controls; triangles represent Prader-Willi Syndrome patients. The total number of hypocretin-1 neurons declines with age. In PWS adults, a correlation between age and total IR cell number was found (n=5, r=0.900, p=0.037).

between groups. Furthermore, there was no significant correlation of these variables with hypocretin-1 cell number in PWS, controls, or the combined group. The mean (±sd) number of cells found in controls was approximately 82,000±16,800. There was no significant difference in hypocretin-1 IR cell number in PWS adults or infants compared with controls (n = 14; P = 0.56; Figs. 1 and 2A).

Effects of age on cell number

The total number of hypocretin-1 IR neurons declines with age (Figure 1.2B). In PWS adults, a negative correlation between age and total hypocretin-1 IR cell number was found (n = 5, r = -0.900, P = 0.037). In controls (all eight adults included), this was not the case (n = 8, r = -0.395, P = 0.333), whereas after pooling of all adult subjects, a trend remained present (n = 13, r = -0.537, P = 0.059).

Discussion

In this study, the number of hypocretin-1 IR neurons in postmortem material in PWS patients was not different from that in controls. A significant decrease in hypocretin-1 IR neurons with age was found in PWS adults but not in controls. This lack of significance is caused by two control cases with a remarkably high number of hypocretin-1 IR neurons (91-009 and 99-071). Excluding these two controls leads to a significant correlation with age in the combined adult group (n = 11, r = -0.699, P = 0.017). The decrease in hypocretin-1 IR neurons with age and its functional implications in relation to sleep

homeostasis, endocrine changes, and the autonomic nervous system need further study. An effect of age on hypocretin gene expression and brain content has been found in rats,[18] but in a study by Kanbayashi et al.,[19] human lumbar CSF hypocretin-1 levels did not seem to be related to age. The estimated total number of some 80,000 neurons is similar to the number reported by Thannickal et al.[20] using immunocytochemistry on paraffin-embedded material. A lower total number of hypocretin-expressing cells (15,000–20,000) was found by Peyron et al.[3] using in situ hybridization on frozen material.

Although we found relatively low numbers of hypocretin-1 IR neurons (45,000 –55,000) in one PWS adult (02-074) and one PWS infant (03-021), similar numbers were also found in two control adults (94-118 and 01-069) and one control infant (97-153). This lower number of hypocretin-1 IR cells is not likely to cause any narcolepsy-like symptoms because narcoleptic patients have a 90–95% reduction of hypocretin-1 IR cells, and we found the same low numbers in controls. In agreement with the main findings of this paper, we recently measured a normal level of hypocretin-1 in the CSF of one PWS patient (Lammers, G. J., unpublished data). Because it is still unclear to what extend CSF levels reflect the total number of hypocretin-1 neurons in the brain, the lowered levels of hypocretin-1 in the CSF of PWS patients measured by Mignot et al., Nevsimalova et al., and Arii et al.[10-12] could be caused by other, unknown factors. No CSF samples were available for the PWS patients and control subjects in this study. Furthermore, it is not known whether the PWS subjects had narcoleptic features. The clinical records available to us were either incomplete in this respect, and the appropriate investigations (e.g. electrophysiology, sleep studies) were not performed. It is still possible that individual PWS subjects with clear narcoleptic features may turn out to have a disturbed hypothalamic hypocretin system, reflected in a lower number of hypocretin IR neurons.

In conclusion, although the determination of hypocretin-1 mRNA and receptors may give additional information in the future, neither the hypocretin cell number nor the intensity of staining was different in PWS patients tested. It is not conclusive whether a decrease in hypocretin neurotransmission explains the occurrence of the narcolepsy-like symptoms associated with some patients afflicted with PWS.

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Visualizing the Hypocretin Receptor in Prader-Willi Syndrome

Fronczek R, Wong K, Unmehopa UA, Lammers GJ, Swaab DF. Unpublished

CHAPTER 1A

Visualizing the Hypocretin Receptor in Prader-Willi Syndrome

Introduction

In the study described in chapter 2 we found no lowered number of hypocretin neurons in PWS patients. There have been several reports, however, that the hypocretin receptors may be involved in narcolepsy-like symptoms.[1,2] We hypothesized, therefore, that the number of hypocretin-receptor-1 and/or -2 expressing cells may be decreased in the hypothalamus of PWS patients. The aim of this study was to quantify hypocretin-receptor-1 and -2 expressing cells in PWS patients and controls. Therefore, we had to determine the optimal antibody for the hypocretin-receptors in human hypothalami. We tested several antibodies on hypothalami and pituitaries of healthy controls obtained from the Netherlands Brain Bank.

Methods

Post-mortem material and Antibodies

Hypothalami from four control subjects (Table 1b.1) and pituitaries from three control subjects (Table 1b.2) were used to test 7 different antibodies against both hypocretin receptor 1 and 2 (Table 1b.3).

Staining procedure

Sections were stained using the different antibodies at the manufacturer's recommended dilution and 1:500, and visualized according to the ABC-method using DAB-nickel solution to finish the staining as described previously.[5]

Antigen retrieval techniques (used before incubation with the first antibody)

Proteinase K treatment at 10µg/ml at 37°C
Citrate Buffer pH 6.0, 10 min boiling
pH 4.0 (citrate buffer), pH 7.0 (TBS) and pH 9.0 (0.05 M Tris-HCl buffer)
Microwave treatment at 900W for 2x5 min.

Blocking steps (added to pre-incubation rinsing and incubation solution)

1%, 3%, 5% milk (ELK, Melkunie, The Netherlands)
0.1% Bovine Serum Albumin (BSA) + 1.5% Normal Goat Serum (NGS)
TRIS-pH 9.0 high salt 0.05%

Table 1b.1 Overview of the hypothalami used in the experiments

NBB Number	Age (yrs)	Sex	Brain-weight (g)	pH	Post-Mortem Delay (hours)	Diagnosis
93-025	68	M	1157	-	<41:00	Respiratory Insufficiency
97-088	78	F	1351	6.20	4:15	Braak 1
91-207	75	F	920	-	5:35	-
96-081	61	F	1311	-	5:15	Space Occupying Process

Table 1b.2 Overview of the pituitaries used in the experiments

NBB Number	Age (yrs)	Sex	Brain-weight (g)	pH	Post-Mortem Delay (hours)	Diagnosis
93-025	68	M	1157	-	<41:00	Respiratory Insufficiency
96-013	68	F	1122	6.80	10:30	-
96-075	76	M	1449	7.31	5:45	Dementia with Senile Involutive Cortical Canges

Table 1b.3 Overview of the antibodies used in the experiments

Company	Code	Target	Publication
Lifespan Biosciences	LS-A6638	hypocretin receptor 1	-
	LS-A6641	hypocretin receptor 1	
	LS-A6677	hypocretin receptor 1	
Chemicon	AB3092	hypocretin receptor 1	Suzuki et al. 2002[3]
	AB3094	hypocretin receptor 1	
Alpha Diagnotics	OX1R11-A	hypocretin receptor 1	Blanco et al. 2001[4]
	OX2R22-A	hypocretin receptor 2	

Results

Both the Chemicon and Alpha Diagnostic antibodies did not produce any staining in the hypothalamus. The hypocretin receptor 2 antibody of both companies did produce a very weak staining in a few pituitaries (Figure 1b.1). Antigen retrieval or blocking steps did not lead to any improvement.

The Lifespan antibodies did not produce any stained cell bodies in the hypothalamus or the pituitary. However, what could be seen in some cases was a dense network of 'boutons' in the striatum, especially using Lifespan 1 (Figure 1b.2). However, this staining was not seen in all hypothalami. Antigen retrieval or blocking steps did not lead to any improvement.

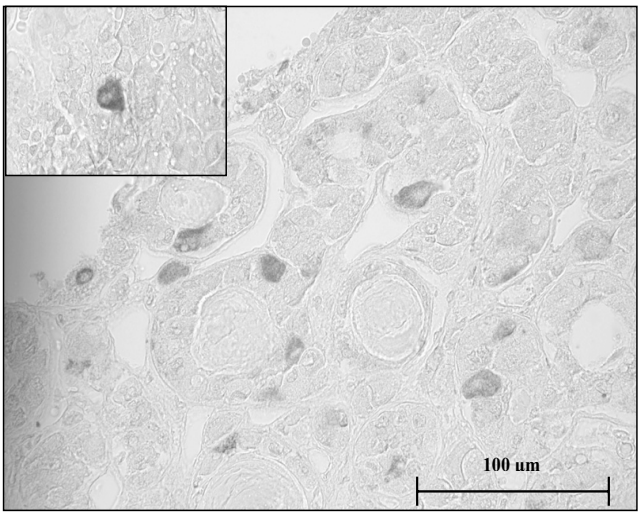

Figure 1b.1
Example of staining
Alpha Diagnostics OX2R22-A (receptor 2) 1:500 40x: Pituitary 95-207 (large) and Pituitary 96-013 (inset).

Discussion

In this study, none of the antibodies produced a reliable staining. Two antibodies directed against hypocretin receptor 2 (Chemicon and Alpha Diagnostics) did produce a weak signal in some pituitaries, but this signal could not be improved. Furthermore, the three different Lifespan antibodies produced a dense network of stained boutons in the striatum of some hypothalami.

In contrast, previous studies have reported results using some of the antibodies we studied: Suzuki et al. identified hypocretin receptor 1 immunoreactivity in chemically identified target neurons in the rat (not human) hypothalamus using the Chemicon antibody.[6] These hypocretin-containing neurons induced a phospoholipase C-mediated release of Ca^{2+} from intracellular stores. Blanco et al. studied the cellular localization of hypocretin receptors in the human pituitary, using the Alpha Diagnostic antibodies.[7] The results demonstrated that hypocretin receptors 1 and 2 were expressed by somatotrope and corticotrope cells, respectively in the human pituitary. The fact that we only found a weak and vague hypocretin receptor 2 signal in the pituitaries we studied, could be due to the type of material we used (older subjects, longer fixation times), but non-specific staining in the Blanco study cannot be excluded, since only preabsorbtion of the primary antibodies with hypocretin-1 and 2 before incubation was used as a test of specificity in that study.[8] Suzuki et al not only used preabsorbtion with the antigen to determine specificity, but performed a western blot analysis as well, which showed that the Chemicon antibodies recognize a hypothalamic rat polypeptide of 50 kDa, the expected molecular mass of orexin receptors given the amino acid sequence of orexin receptors in the rat brain.[9] Note that all these results were obtained from rat material.

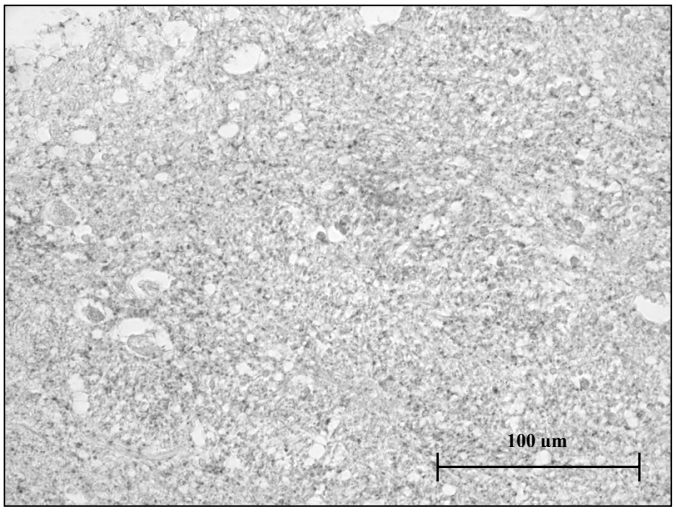

Figure 1b.2
Example of staining
Lifespan Biosciences Ls-A6638: 9713/14AP3-1 (receptor 1) 1:500 40x: NBB 93-025 (M68).

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