CHAPTER 3

Decreased hypothalamic glucagon-like peptide-1 receptor expression in type 2 diabetes patients

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Submitted
ABSTRACT

Glucagon-like peptide-1 (GLP-1) and GLP-1 receptor agonist treatment in type 2 diabetes (T2DM) reduce blood glucose and food intake. It has been suggested that these effects are partly mediated through central GLP-1 receptors (GLP-1R). The rodent and nonhuman primate hypothalamus show clear GLP-1R expression. However, a detailed description of GLP-1R expression in the human hypothalamus is lacking, and it is unknown whether GLP-1R expression is altered in T2DM patients. We therefore investigated GLP-1R distribution in the human post-mortem hypothalamus by means of in situ hybridization.

We found that GLP-1R mRNA was expressed in a number of hypothalamic nuclei including the paraventricular nucleus (PVN) and the infundibular nucleus (IFN), both involved in the regulation of energy metabolism. A subset of the GLP-1R positive neurons in the IFN showed co-localization with the orexigenic neuropeptide Y (NPY), agouti related peptide (AGRP), or pro-opiomelanocortin (POMC) transcripts. Comparison of GLP-1R mRNA in the PVN and IFN between T2DM patients and control subjects revealed a decreased expression in T2DM patients.

Our studies show that GLP-1R is widely expressed throughout the human hypothalamus. The decreased expression of GLP-1R in the PVN and IFN of T2DM patients may be related to dysregulation of feeding behaviour and glucose homeostasis in T2DM.
INTRODUCTION

Glucagon-like peptide-1 receptor agonists (GLP-1RA) lower blood glucose via stimulating insulin secretion and inhibiting glucagon release, and are therefore used for treatment of type 2 diabetes mellitus (T2DM) (1). Interestingly, in addition to the glucose lowering properties, T2DM patients taking GLP-1RA report reduced appetite and food intake contributing to weight loss (2-4).

GLP-1RA are derivatives of the naturally occurring peptide hormone GLP-1, which is secreted from intestinal enteroendocrine L-cells in response to nutrient ingestion. A large number of studies in humans investigated peripheral effects of GLP-1 and GLP-1RA on pancreatic islet cells, liver and stomach (5;6). However, from rodent and non-human primate studies it is clear that GLP-1 is also centrally produced in the nucleus of the solitary tract (NTS) in the brainstem. These GLP-1 producing neurons show projections throughout the brain including the hypothalamus, an area of particular importance for the regulation of both feeding behaviour and glucose metabolism (7;8). Within the rodent hypothalamus GLP-1R expression has been demonstrated in a number of nuclei that play a role in the regulation of energy metabolism. These areas include the infundibular nucleus (IFN) (or arcuate nucleus (ARC) as it is called in rodents) and the paraventricular nucleus (PVN) (9). This observation suggests that, in addition to GLP-1 effects on metabolism and feeding behaviour via receptors in peripheral organs, there may be central effects of this peptide. Indeed, animal experiments support central effects as well (7;9-11). GLP-1 administration in the ARC of rats improves glucose homeostasis by regulating hepatic glucose production (12) and GLP-1 administration into the PVN of rats reduces food intake (13).

Less is known about the central effects of GLP-1 and the expression of the GLP-1R in the human brain. Given the increasing interest and use of GLP-1 based therapies and the emerging evidence that the brain is involved in glucose control, information on GLP-1R expression in the human hypothalamus of control subjects and patients suffering from T2DM is also of clinical interest. GLP-1 binding and expression of GLP-1R mRNA has been reported in the human hypothalamus (14). This study did, however, not provide a detailed description of the distribution within the hypothalamus. In the present study, we therefore investigated the distribution of GLP-1R mRNA in the human post-mortem hypothalamus using in situ hybridization. In view of the role of the PVN and ARC in the regulation of glucose metabolism and feeding behaviour in rodents, we performed quantifications of GLP-1R mRNA expression in the PVN and IFN, comparing patients with T2DM and control subjects. GLP-1R mRNA expressing cells of the IFN were further characterised using double labelling with the orexigenic Neuropeptide Y (NPY) and agouti-related peptide (AgRP), as well as the anorexigenic proopiomelanocortin (POMC).
MATERIALS AND METHODS

Subjects
In experiment 1 we studied the distribution of the GLP-1R using systematic sampling covering the entire rostro-caudal axis of the hypothalamus in human post-mortem tissue. We analysed 6 subjects (4 male) without clinical neurological or neuropsychiatric diseases or T2DM, ranging in age between 60 and 81 years. Clinico-pathological data of subjects in experiment 1 are presented in Table 1. In experiment 2 we quantified the expression of the GLP-1R in the PVN and IFN of the hypothalamus. Eight subjects with T2DM and 8 age and sex matched control subjects were analysed, ranging in age between 65 and 89 years (Table 1). All brain material was obtained from The Netherlands Brain Bank at The Netherlands Institute for Neuroscience in accordance with the formal permissions for brain autopsy and for the use of human brain material and clinical information for research purposes.

Histology
Brains were dissected at autopsy and the (hemi-) hypothalamus was fixed in 4% phosphate-buffered formalin at room temperature for 4–15 weeks (Table 1). After dehydration in graded ethanol series, tissues were cleared in toluene, and embedded in paraffin. Serial coronal sections (6 µm) were cut from the level of the lamina terminalis to the caudal border of the mamillary bodies. For anatomical orientation, every 100th section was collected and mounted on Superfrost Plus slides (Menzel Glaser, Braunschweig, Germany) dried for at least 2 days at 37 °C and stained with thionine. In experiment 1 (distribution study) GLP-1R ISH was performed using systematic sampling (600mm intervals) over the entire rostro-caudal axis of the hypothalamus. In experiment 2 (quantification), we used thionine staining for anatomical orientation, and GLP-1R ISH was performed on a central cross section of the IFN and PVN.

Locked Nucleic Acid probe
A Locked Nucleic Acid (LNA) probe (LNA-2’-O-methyl-RNA) specific for the human GLP-1R mRNA sequence was designed (15). This technique has been applied successfully to post-mortem human brain tissue (16). We applied an antisense probe 3’-GTCCTTGAGGTTGTACTTG-5’ complementary to bases 953-972 of the human GLP-1R (GenBankNM_002062.3). A scrambled sequence was used as a negative control. Probes were FAM tagged at the 5’ end and custom ordered (Ribotask, Langeskov, Denmark). Specificity of the probe was further assessed by testing of a concentration gradient ranging from 1pM to 25nM.

In situ hybridization
Sections were deparaffinised in xylene and rehydrated through a graded series of ethanol followed by rinsing in phosphate-buffered saline (PBS). Sections were equilibrated for 2 min in 0.01 M sodium citrate buffer (pH 6.0) and microwaved at 800 W for 2x 5 min. After cooling and rinsing in PBS, sections were deproteinated with 0.2N HCL for 20 min followed by 15 min proteinase K digestion at 2µg/ml at 37 °C. Reaction was stopped in a glycine buffer. Delipidation was performed using 0.1% Triton X-100 for 10 min, which was followed by a 30-60 min prehybridization in a humidified
Table 1 | Clinicopathological data

<table>
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<th>Fix (d)</th>
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<th>Cause of death, clinical diagnoses</th>
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<td>93-061</td>
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<th>Fix (d)</th>
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<td>41</td>
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<td>21:30</td>
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<td>T2DM</td>
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<td>38.5</td>
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†, excluded for IFN analysis due to tissue damage; *, excluded for all analyses due to tissue damage; F, Female; M, male; Fix, fixation duration; PMD, post-mortem delay; NA, not applicable; CO, controls; T2DM, type 2 diabetes mellitus; COPD, chronic obstructive pulmonary disease; CVA, cardiovascular accident.
chamber at 55 °C in hybridization buffer (HBF, not containing probe) with final concentrations of 50% (v/v) deionised formamide, 600 mM NaCl, 10 mM HEPES (pH 7.5), 5x Denhardt’s, 1 mM EDTA, and 200 µg/ml denatured herring sperm DNA (Invitrogen Life Technologies, cat. no. 15634-017). For hybridization, antisense GLP-1R probe was diluted in HBF to a final concentration of 3 nM, denatured at 95 °C for 5-10 min, and cooled on ice. Sections were covered and hybridised overnight at 55 °C and subsequently washed for 5 min each in 2x standard sodium citrate buffer (2x SSC; 0.3 M NaCl, 0.03 M trisodium citrate dihydrate), 0.5x SSC, and 0.2x SSC at 55 °C and for 5 min in Tris-buffered saline (TBS; 0.05 M Tris-HCl pH 7.6, 0.15 M NaCl) at room temperature. Next, sections were incubated with Anti-Fluorescin-Alkaline Phosphatase Fab fragments (Roche Life Science, cat.no. 11426338910) at 1:3000 in Supermix (0.25% (w/v) gelatin and 0.5% (v/v) Triton X-100 in TBS), for 3 h at room temperature. Slides were washed 3x 5 min in TBS, rinsed in buffer 2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂), followed by colour development using NBT-BCIP solution (buffer 2 containing 0.34 mg/ml NitroBlueTetrazolium Chloride (NBT; Roche Life Science, cat.no. 1585029001), 0.18 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Life Science cat. no. 11585002001) and 0.24 mg/ml levamisole (Sigma Aldrich, cat. no. L9756) for 20-30 min under dark conditions.

**Figure 1** | Photomicrographs showing staining with sense and scrambled probe

Photomicrographs showing staining with sense probe (left) and scrambled probe (right) in IFN and PVN. Scalebar 100 µm.

IFN, infundibular nucleus; PVN, paraventricular nucleus.
conditions. Sections were then washed in distilled water, rinsed in methanol (Sigma Aldrich, cat. no. 34860) for 5 min, and rinsed again in distilled water. Finally, slides were coverslipped using Kaiser’s glycerine (Merck Millipore, cat.no. 109242) and stored at 4 °C for until further processing.

**Distribution**

In experiment 1 staining intensity was scored by visual inspection under a microscope in a semi-quantitative fashion: - (no staining), +/- (few positive cells showing light staining intensity), + (the majority of cells are positive and cells show a moderate to strong signal), ++ (the majority of cells are positive and staining intensity is high). Every 100 th 6µm section was scored, systematically over the entire rostro-caudal axis. Two researchers scored staining intensities (JStK and LvB).

**Quantitative analysis**

All sections were hybridised within the same experiment. Sections were digitally scanned and GLP-1R staining was quantified using a thresholding procedure in ImageJ (V1.49n, ImageJ.net). The threshold settings were determined experimentally and set to 116 for all further analyses. Multiplication of the mean optical density and the fraction of the outlined surface covered by the mask resulted in a quantitative value expressed in Arbitrary Units (a.u.).

**Double labelling**

To further characterize GLP1R expressing neurons in the IFN, colocalization studies were performed for pro-opiomelanocortin (POMC), agouti-related peptide (AgRP) and neuropeptide Y (NPY). Paraffin IFN sections were stained from four control subjects, one male and three females, ranging in age from 77 to 100 years. Antibody staining was performed before GLP1R mRNA detection. The following antibodies were applied: anti-AGRP at 1:750 (cat. no. H-003-053, Phoenix Pharmaceuticals), anti-NPY at 1:500 (Niepke, bleeding 091188, Netherlands Institute for Neuroscience) (17) and anti-a-melanocyte stimulating hormone (aMSH) as a marker for POMC neurons at 1:2000 (NHI4372, bleeding 230475, Netherlands Institute for Neuroscience) (18;19). Antibody specificity has been described previously for all antibodies including pre-adsorption studies with the homologous peptide, and cross-adsorption with independent peptides (17;18). Double labeling experiments and subsequent analyses using spectral analyses have been described previously, with minor adaptations (16;20). For AgRP and NPY, the peroxidase-anti-peroxidase (PAP) detection was employed and the ABC method was used for POMC. Staining was visualised with 0.5 mg/ml DAB. The ISH procedure was performed as describe above. The GLP1R LNA probe was applied at concentrations of 20-40 nM. ISH signal was developed using NBT/BCIP.

We used the Nuance multispectral imaging system (NuanceFX camera [PerkinElmer, USA] connected to a Zeiss Axioskop microscope with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Jena, Germany) for spectral analyses. For each chromogen, a spectral library was defined using single-stained sections. Colocalization was evaluated by unmixing spectral libraries on double stained sections. Pseudofluorescence was used for visualization purposes.
**Statistical analysis**

Analyses of quantitative data were performed with the Statistical Package for the Social Sciences (SPSS) version 20. We did not assume normal distribution of the data, and performed conservative non-parametric statistical analyses. Group differences were analysed using Mann-Whitney U-test. P< 0.05 was considered statistically significant.

**RESULTS**

**Specificity**

ISH specificity was supported by the absence of signal using the scrambled sequence (Figure 1). Specificity was additionally supported by the decrease in signal observed with increased dilution of the probe. Specificity of the antibodies used for co-localization studies was published previously (17,19).

**Distribution of GLP-1R mRNA**

GLP-1R mRNA positive cells were present in a number of hypothalamic nuclei (Figure 2). The distribution pattern of GLP-1R mRNA was in general agreement between the 6 subjects, although a strong interindividual variation was observed in staining intensity (Table 2). Intense ISH signal was present in the PVN, supraoptic nucleus (SON), diagonal band of Broca (DBB), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and nucleus basalis of Meynert (NBM). Staining was also present in the IFN, suprachiasmatic nucleus (SCN), lateral hypothalamic area (LHA), bed nucleus striaterminalis(BST), preoptic area (POA), tuberomammillary nucleus (TMN) and mamillary body (MB).

Co-localization studies revealed sporadic co-localization in the IFN between GLP 1R mRNA and NPY, AGRP, and POMC (Figure 3).

**Table 2 | Distribution of GLP-1R mRNA in the human hypothalamus**

<table>
<thead>
<tr>
<th>Subject</th>
<th>PVN</th>
<th>IFN/ME</th>
<th>VMN/DMN</th>
<th>SON</th>
<th>SCN</th>
<th>LHA/PFo</th>
<th>DBB</th>
<th>NBM</th>
<th>BST</th>
<th>POA</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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PVN, paraventricular nucleus; IFN, infundibular nucleus; VMN, ventromedial nucleus; DMN, dorsomedial nucleus; SON, supraoptic nucleus; SCN, suprachiasmatic nucleus; LHA, lateral hypothalamic area; Pfo, perifornical nucleus; DBB, diagonal band of Broca; NBM, nucleus basalis of Meynert; BST, bed nucleus striaterminalis; ME, median eminence; POA, preoptic area; TMN, tuberomammillary nucleus; MB, mammillary body; -, no staining; +/-, few positive cells showing light staining intensity; +, the majority of cells are positive and show a moderate to strong signal; ++, the majority of cells are positive and staining intensity is high.
Figure 2 | Distribution of the GLP-1R in coronal sections of human hypothalamus

A) Schematic illustration of distribution of GLP-1R in coronal sections of human hypothalamus. Dark grey colouring indicates areas showing intense staining for GLP-1R represents staining for GLP-1R, light grey indicate less intense staining.

B) Photomicrographs showing GLP-1R staining throughout the hypothalamus including detailed photomicrographs of PVN, IFN and SON.

AC, anterior commissure; BST, bed nucleus of the striae terminalis; DBB, diagonal band of Broca; DMN, dorsomedial nucleus; Fx, fornix; IFN, infundibular nucleus; LV, lateral ventricle; OT, optic tract; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus; SON, supraoptic nucleus; TMN, tuberomamillary nucleus; VMN, ventromedial nucleus; III, third ventricle.

Figure 3 | Co-localization of GLP-1R expression and NPY, AgRP and POMC in the infundibular nucleus (IFN)
Photomicrographs showing GLP-1R staining (in green) combined with respectively NPY, AGRP and POMC (in red). Scalebar 50 μm.

NPY, neuropeptide Y; AGRP, agouti related protein; POMC, pro-opiomelanocortin
Comparison of GLP-1R mRNA expression between T2DM and control subjects

Eight subjects with T2DM and eight control subjects were compared for GLP-1R expression in the PVN and IFN. Tissue damage was observed in the PVN in two T2DM patients, who were deemed unsuitable for further analyses (#02-087 and 98-055). We decided not to retest these two subjects in view of the significant interassay variation we observed. Therefore, subjects were not closely matched and a conservative Mann Whitney-U test was used for statistical analyses. GLP-1R expression in the PVN was lower in T2DM patients compared to control subjects (U = 4.0; p = 0.008) (Figure 4a). For the quantification of GLP-1R mRNA in the IFN, 2 subjects from the control group were excluded due to damaged specimens. GLP-1R expression in the IFN was also lower in T2DM patients compared to control subjects (U = 0.0; p = 0.002) (Figure 5a). Examples of the differences in GLP-1R staining in PVN and IFN between T2DM and control subjects are illustrated in Figures 4b and 5b. Age, sex, post-mortem delay and fixation duration were not significantly different between groups (p > 0.2). Data on BMI was available in 2 control subjects (24 and 32 kg/m²) and in 4 T2DM patients (range 20-35 kg/m²), and could therefore not be analysed in a reliable fashion.

DISCUSSION

We show that GLP-1R mRNA is expressed throughout the human hypothalamus, comparable to what has been reported in rodents and nonhuman primates (9;10) with most pronounced expression in the PVN, SON, DBB and NBM. Additional staining was observed in other nuclei involved in the regulation of energy metabolism such as VMN, DMN, IFN and SCN. Moreover, we found a decreased expression of the GLP-1R in both the PVN and IFN of T2DM patients. A minority of GLP-1R mRNA expressing neurons showed co-expression of NPY, AGRP and POMC. Future studies will be needed to further characterize these neurons.

Some differences with the localisation of GLP1R in rodents and non-human primates should be noted. Expression of GLP-1R in the VMN has not been reported in rodents or nonhuman primates (9;10). In the present study, we did observe a clear expression of GLP-1R mRNA in the human VMN. Our observation is in line with an earlier report on GLP-1R expression in the human brain (14). Whether this observation represents an interspecies difference remains unclear.

From rodent studies it is clear that GLP-1 affects energy metabolism by acting on the ARC (IFN) and the PVN. Chemical ablation of the ARC in rats prevents the inhibitory action of GLP-1 on feeding (21); GLP-1 infusion into the ARC lowers hepatic glucose production, thereby reducing blood glucose (12); and direct injection of GLP-1 in the PVN of rats leads to decreased food intake (13). It is possible that decreased GLP-1R expression in the IFN and PVN in T2DM patients...
GLP-1 receptor in the human hypothalamus contributes to dysregulation of glucose homeostasis and feeding behaviour as reported in T2DM. Alternatively, in rats a hyperglycaemic state decreases GLP1R expression in pancreatic islets (22), which could indicate that the decreased GLP-1R expression could be a consequence of a hyperglycaemic state.

We observed sporadic co-localization of GLP-1R expression and NPY/AGRP and POMC. Although speculative, the expression of GLP-1Rs on neurons expressing NPY/AGRP and POMC may indicate that GLP-1 affects energy metabolism via actions on these hypothalamic neurons. We have shown in our previous studies that alterations in NPY and AGRP expression were related to BMI, whereas expression of the POMC derived alpha melanocyte stimulating hormone (aMSH)
was related to T2DM (23). Interestingly, it has been shown in rats that GLP-1 directly stimulates POMC neurons and that the GLP-1R on POMC neurons in the ARC plays a role in mediating the anorectic effects of GLP-1RA (24-26). Furthermore, fasting GLP-1 levels were shown to be positively associated with body fat mass (27). In the present study, we were unable to relate our data to BMI, since these data were only available in a small number of the subjects included. Additionally co-localization was only sporadic, and therefore functional implications may be limited.

The use of human post-mortem tissues has a number of inherent limitations. The availability of well-documented material is limited, resulting in relatively small sample sizes. Despite a low number of observations, we found significant differences in GLP-1R expression. All T2DM patients in our study were treated with glucose lowering agents, i.e. metformin and/or a sulphonylurea derivative. We cannot exclude that these compounds may have influenced GLP-1R expression. However, it was shown that metformin increases GLP-1R mRNA expression in murine islet cells (28). Since we observed a decrease in GLP-1R expression in T2DM patients, potential effects of metformin may have led to an underestimation of the observed effect. To our knowledge, no effects of sulphonylurea derivatives on GLP-1R expression have been reported in literature.

To conclude, we describe the systematically determined distribution of the GLP-1R in the human hypothalamus. In addition, we show a decreased expression of the GLP-1R in T2DM patients in the IFN and PVN. This decreased expression of the GLP-1R may be related to the dysregulation of feeding behaviour and glucose homeostasis in T2DM patients.
**REFERENCE LIST**


