



Article

# Glucagon-like Peptide-1 Receptor in the Human Hypothalamus Is Associated with Body Mass Index and Colocalizes with the Anorexigenic Neuropeptide Nucleobindin-2/Nesfatin-1

Aristea Psilopanagiotti <sup>1,\*</sup>, Sofia Nikou <sup>1</sup>, Souzana Logotheti <sup>2</sup>, Marina Arbi <sup>3</sup>, Dionysios V. Chartoumpakis <sup>4</sup> and Helen Papadaki <sup>1</sup>

<sup>1</sup> Department of Anatomy-Histology-Embryology, School of Medicine, University of Patras, 26504 Patras, Greece

<sup>2</sup> Department of Pathology, School of Medicine, University of Patras, 26504 Patras, Greece

<sup>3</sup> Department of Biology, School of Medicine, University of Patras, 26504 Patras, Greece

<sup>4</sup> Service of Endocrinology, Diabetology and Metabolism, Lausanne University Hospital, 1011 Lausanne, Switzerland

\* Correspondence: aristep@upatras.gr



**Citation:** Psilopanagiotti, A.; Nikou, S.; Logotheti, S.; Arbi, M.; Chartoumpakis, D.V.; Papadaki, H. Glucagon-like Peptide-1 Receptor in the Human Hypothalamus Is Associated with Body Mass Index and Colocalizes with the Anorexigenic Neuropeptide Nucleobindin-2/Nesfatin-1. *Int. J. Mol. Sci.* **2022**, *23*, 14899. <https://doi.org/10.3390/ijms232314899>

Academic Editor: Jean-François Tanti

Received: 31 October 2022

Accepted: 25 November 2022

Published: 28 November 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Abstract:** Data on animals emphasize the importance of the neuronal glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) for feeding suppression, although it is unclear whether astrocytes participate in the transduction of anorectic GLP-1R-dependent signals. In humans, the brain circuitry underlying these effects remains insufficiently investigated. The present study aimed to explore GLP-1R protein expression in the human hypothalamus and its correlation with body mass index (BMI). Sections of hypothalamus from 28 autopsy cases, 11 with normal weight (BMI < 25 kg/m<sup>2</sup>) and 17 with non-normal weight (BMI ≥ 25 kg/m<sup>2</sup>), were examined using immunohistochemistry and double immunofluorescence labeling. Prominent GLP-1R immunoreactivity was detected in neurons of several hypothalamic nuclei, including paraventricular, supraoptic, and infundibular nuclei; the lateral hypothalamic area (LH); and basal forebrain nuclei. Interestingly, in the LH, GLP-1R was significantly decreased in individuals with BMI ≥ 25 kg/m<sup>2</sup> compared with their normal weight counterparts ( $p = 0.03$ ). Furthermore, GLP-1R was negatively correlated ( $r = -0.347$ ,  $p = 0.024$ ) with BMI levels only in the LH. GLP-1R extensively colocalized with the anorexigenic and antiobesogenic neuropeptide nucleobindin-2/nesfatin-1 but not with the astrocytic marker glial fibrillary acidic protein. These data suggest a potential role for GLP-1R in the regulation of energy balance in the human hypothalamus. In the LH, an appetite- and reward-related brain region, reduced GLP-1R immunoreactivity may contribute to the dysregulation of homeostatic and/or hedonic feeding behavior. Possible effects of NUCB2/nesfatin-1 on central GLP-1R signaling require further investigation.

**Keywords:** hypothalamus; GLP-1 receptor; nucleobindin-2; nesfatin-1; obesity; astrocytes; GFAP

## 1. Introduction

The incretin hormone glucagon-like peptide-1 (GLP-1) is secreted from intestinal enteroendocrine L cells in response to nutrient ingestion and potentiates insulin release, suppresses glucagon secretion, delays gastric emptying, increases satiety, and reduces appetite and body weight [1]. The GLP-1 anorexigenic effect is transduced via GLP-1 receptor (GLP-1R) signaling in several brain areas implicated in the control of energy homeostasis, including the hypothalamus [2]. Although a considerable number of studies have investigated the peripheral actions of GLP-1 in humans [2], information about the effects of GLP-1 and the levels of GLP-1R in the human central nervous system is limited. GLP-1R has been localized in the human hypothalamus using *in situ* hybridization techniques [3,4] and immunohistochemistry [5]. In the aforementioned studies, GLP-1R showed a particularly

heterogenous mRNA and protein expression with strong interindividual variations. Interestingly, a decreased expression of GLP-1R in the paraventricular (PVN) and infundibular (IFN) (arcuate) hypothalamic nuclei was observed in patients with type 2 diabetes mellitus [4]. Furthermore, according to Ten Kulve et al. [4], GLP-1R was sporadically colocalized with energy balance-related neuropeptides, such as neuropeptide Y (NPY), agouti-related peptide (AgRP), and proopiomelanocortin (POMC), in the human IFN.

The astrocytes that represent an important central nervous system cell population have been proposed to contribute to the transduction of anorectic GLP-1R-dependent signals in rats [6]. GLP-1R expression in astrocytes has been described within rat brains [6–8] and in a human astrocyte cell line [9]. On the other hand, no colocalization of GLP-1R with the astrocytic marker glial fibrillary acidic protein (GFAP) was observed in any area of the mouse brain examined, including the hypothalamus [10].

In humans, GLP-1R activation reduces neuronal responses to food cues in appetite- and reward-related brain areas, resulting in feeding suppression [11]. Furthermore, it has been suggested that vagal afferents are involved in mediating the effects of GLP-1 on food intake [12]. However, it is unknown whether central GLP-1R acts directly and/or recruits other neuropeptide mediators to exert its anorexigenic and antiobesogenic effects in humans [2].

Nesfatin-1, an 82-amino acid peptide derived from the precursor nucleobindin-2 (NUCB2), is highly expressed both in human and rodent hypothalami [13–16]. Animal model studies have provided evidence for the important physiological role of NUCB2/nesfatin-1 in food intake, glucose and lipid metabolism, energy expenditure, and stress response as well as cardiovascular, reproductive, and gastrointestinal functions [13–15]. NUCB2/nesfatin-1 is widely expressed in the mammalian gastrointestinal tract, and it has been shown to reduce inflammation and oxidative stress during ulceritis, necrotizing enterocolitis, and acute mesenteric ischemia [17]. NUCB2/nesfatin-1 has also been proposed to play a role in cancer progression by enhancing proliferation, migration, invasion, and evasion of apoptosis in most cancer types although few studies report that NUCB2/nesfatin-1 inhibits cell proliferation and increases apoptosis [18].

Nesfatin-1 neurons have been shown to contribute to the anorexigenic effects of GLP-1 in an animal model of GLP-1R-mediated feeding suppression [19,20]. In rats, intraperitoneal administration of GLP-1 resulted in a significant increase in the number of activated nesfatin-1 neurons in hypothalamic nuclei, and GLP-1-induced food intake reduction was significantly attenuated by pretreatment with intracerebroventricular administration of antisense nesfatin-1 [21].

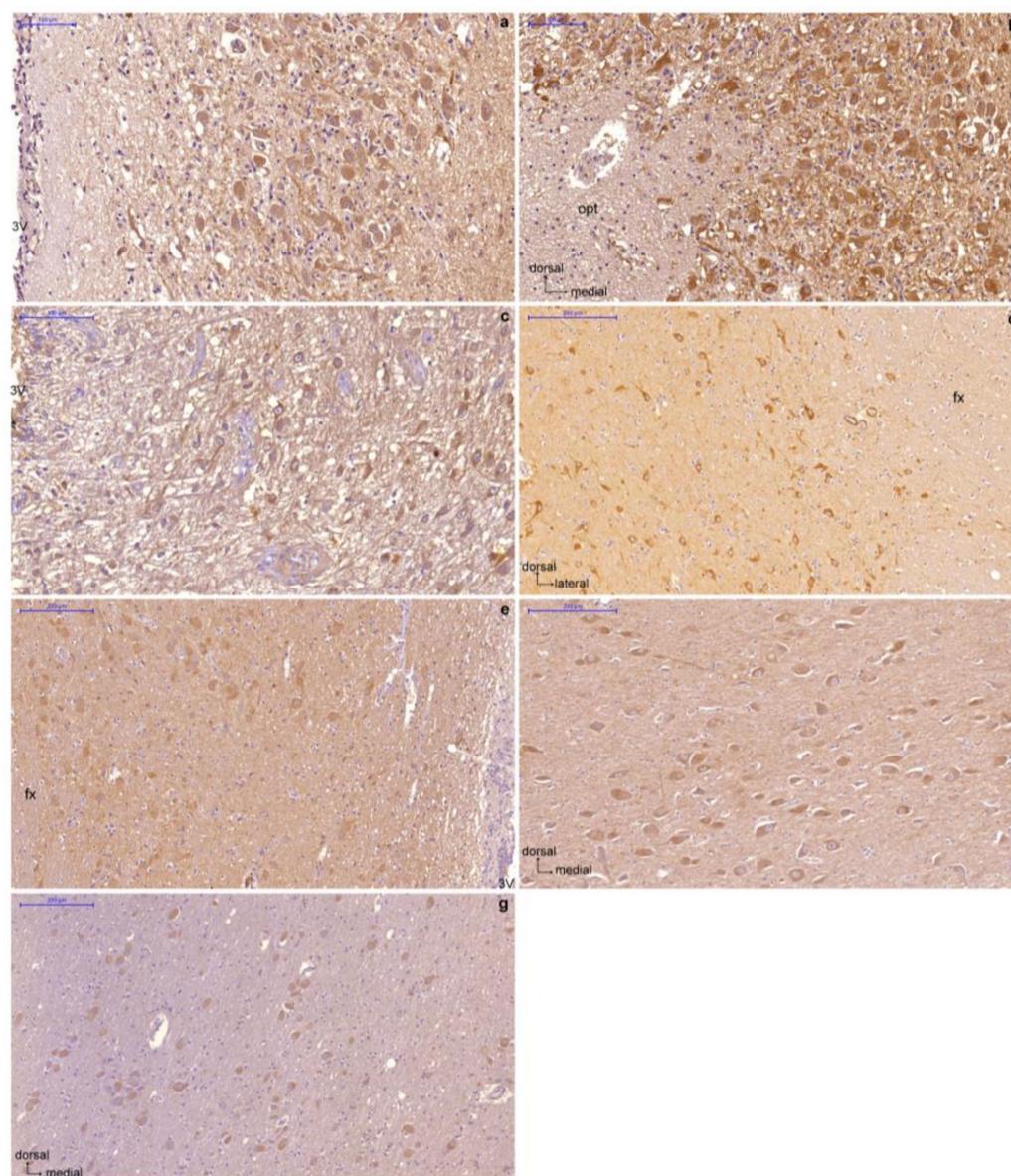
Given the increasing evidence that brain circuits regulate energy homeostasis and body weight, the widespread use of GLP-1-based therapies for obesity and type 2 diabetes treatment [2] and the variable response of patients to these therapies [22], further studying of GLP-1R levels variations in key cell subpopulations of the human hypothalamus is of clinical interest. We hypothesized that patients would show differential protein levels and patterns of GLP-1R localization with reference to their body weights. Herein, we investigated the association between GLP-1R immunorexpression and clinicopathological data, including body weight. In addition, we examined the colocalization of GLP-1R with the potent anorexigenic neuropeptide NUCB2/nesfatin-1 as well as with the astrocytic marker GFAP.

## 2. Results

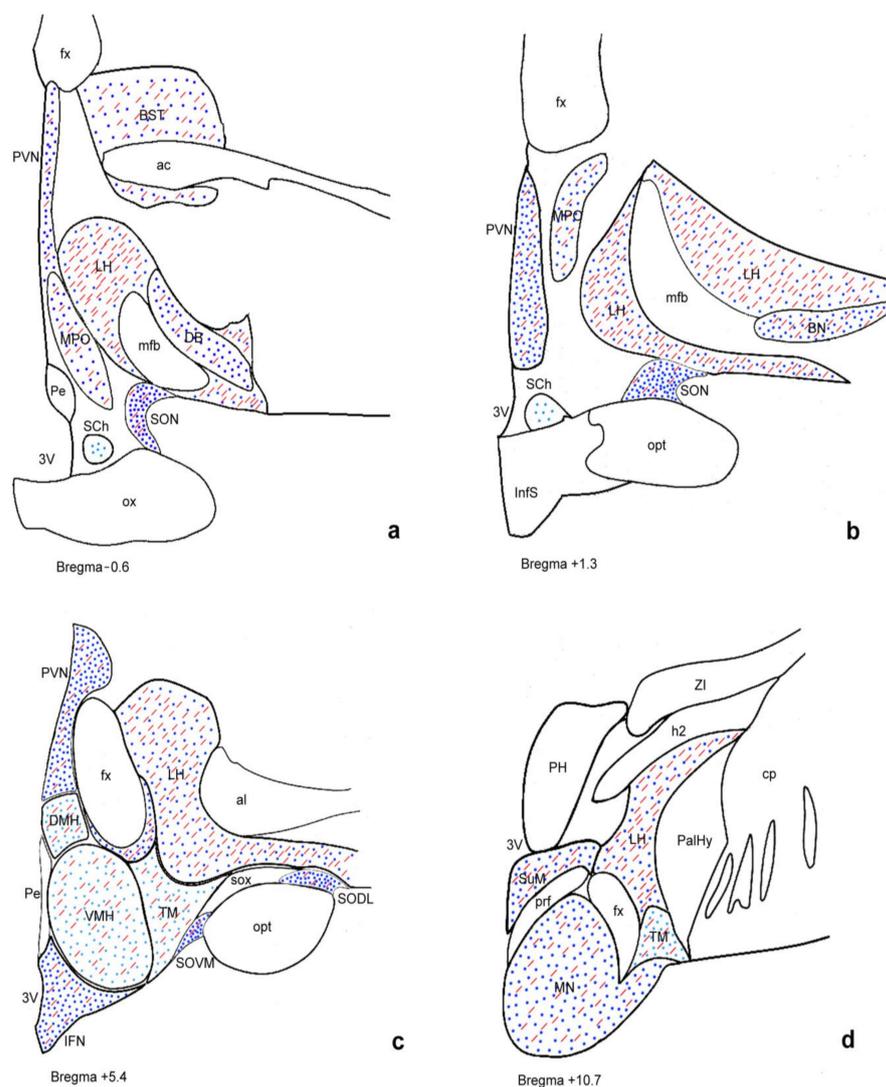
### 2.1. Distribution of GLP-1R Immunoreactivity in the Human Hypothalamus Is Different between Normal Weight and Overweight or Obese Subjects

GLP-1R immunoreactive (GLP-1R-ir) neurons exhibited diffuse cytoplasmic immunoperoxidase labeling and widespread distribution in several hypothalamic nuclei and areas (Figure 1). The PVN (magnocellular and parvicellular neuronal populations), supraoptic (SON) (dorsolateral, dorsomedial, and ventromedial parts), and IFN nuclei; the lateral hypothalamic area (LH); and the cholinergic basal forebrain nuclei, including the basal

nucleus of Meynert and the diagonal band of Broca, showed the most prominent GLP-1R immunoreactivity. In addition, GLP-1R-ir neurons showing moderate, weak, and, in a few cases, negative immunostaining were detected in the dorsomedial (DMH), ventromedial (VMH), perifornical, tuberomammillary (TM), suprachiasmatic, and mammillary hypothalamic nuclei as well as in the bed nucleus of the stria terminalis. No immunopositivity for GLP-1R was observed in human glial cells. GLP-1R-ir fibers were widely distributed in the hypothalamus. A schematic representation of GLP-1R-ir cell bodies and fibers is depicted in Figure 2.



**Figure 1.** Representative sections of human hypothalamus showing GLP-1R immunoreexpression in neurons of the paraventricular nucleus (a), supraoptic nucleus (b), infundibular nucleus (c), lateral hypothalamic area (d), dorsomedial nucleus (e), basal nucleus (f), diagonal band (g). The 3V, third ventricle; fx, fornix; opt, optic tract. Scale bars indicate 100 μm (a–c) and 200 μm (d–g).



**Figure 2.** Schematic illustration of GLP-1R distribution in the human hypothalamus. Depiction of GLP-1R-ir cell bodies (blue dots) and GLP-1R-ir fibers (red lines) in four representative coronal sections of human hypothalamus (a–d) arranged from rostral (a) to caudal (d). Dark blue coloring indicates areas showing high staining intensity for GLP-1R and light blue indicates less intense staining. The 3V, third ventricle; ac, anterior commissure; al, ansa lenticularis; BN, basal nucleus; BST, bed nucleus of the stria terminalis; cp, cerebral peduncle; DB, diagonal band; DMH, dorsomedial hypothalamic nucleus; fx, fornix; h2, lenticular fascicle; ic, internal capsule; IFN, infundibular nucleus; InfS, infundibular stalk; LH, lateral hypothalamic area; mfb, medial forebrain bundle; mfb, medial forebrain bundle; MN, mammillary nucleus; MPO, medial preoptic nucleus; opt, optic tract; ox, optic chiasm; PalHy, pallidohypothalamic nucleus; Pe, periventricular nucleus; PeF, perifornical nucleus; PH, posterior hypothalamic area; prf, principal fasciculus; PVN, paraventricular nucleus; SCh, supraoptic nucleus; SON, supraoptic nucleus; SODL, supraoptic nucleus (dorsolateral part); SOVM, supraoptic nucleus (ventromedial part); sox, supraoptic commissure; SuM, supramammillary nucleus; TM, tuberomammillary nucleus; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta.

Clinicopathological parameters of all examined cases are summarized in Table 1. The GLP-1R distribution pattern was highly similar among all subjects, although an interindividual variation in staining intensity was noticed in a few hypothalamic nuclei. Interestingly, in the LH, immunohistochemical expression of GLP-1R displayed a significantly different distribution between the two BMI groups. Normal weight subjects showed significantly higher immunoreactivity scores compared to their overweight or obese counterparts with  $\text{BMI} \geq 25 \text{ kg/m}^2$  ( $p = 0.03$ ). The results of the comparison of immunoreactivity scores

between BMI categories are presented in Table 2. Figure 3 illustrates the difference in GLP-1R immunoreactivity score in the LH between a normal weight (case 4) and an excess adiposity BMI subject (case 28), both males with similar age, postmortem intervals, and cause of death. GLP-1R protein expression in all other hypothalamic nuclei was not significantly correlated with BMI weight status category ( $p > 0.05$ ). No statistically significant association between GLP-1R immunoreactivity and sex, age, postmortem interval, or cause of death was noticed ( $p > 0.05$ ). Regional differences in GLP-1R immunostaining scores for all examined cases are summarized in Supplemental Tables S1 and S2.

**Table 1.** Clinicopathological data.

Case	Sex	Age (Years)	BMI (kg/m <sup>2</sup> )	PMI (Hours)	Cause of Death, Medical History
1	M	30	21.5	10	Homicide
2	F	65	21.6	24	Automobile accident
3	M	66	23.0	16	Cardiac tamponade, myocardial infarction, hypertension
4	M	53	23.0	10	Myocardial infarction
5	M	40	23.3	24	Myocardial infarction
6	M	57	23.9	12	Myocardial infarction
7	F	27	24.2	11	Automobile accident
8	M	31	24.8	24	Dilated cardiomyopathy
9	F	55	24.9	24	Myocardial infarction
10	F	56	24.9	11	Myocardial infarction
11	M	24	24.9	5	Automobile accident
12	M	64	27.7	17	Myocardial infarction
13	M	37	27.8	3	Automobile accident, hypertension
14	F	58	29.0	24	Automobile accident
15	F	77	29.1	12	Automobile accident, hypertension
16	M	86	29.1	15	Gastric cancer/hemorrhage
17	F	22	29.3	8	Automobile accident
18	M	60	29.4	24	Automobile accident
19	F	64	29.4	3	Cor pulmonale
20	M	58	30.5	24	Myocardial infarction
21	M	63	30.7	12	Gastric hemorrhage, alcoholism
22	M	56	31.6	12	Aspiration pneumonia, hypertension
23	M	39	31.7	2	Myocardial infarction
24	M	59	32.4	12	Myocardial infarction
25	M	27	32.4	24	Myocardial infarction, familial hypercholesterolemia
26	M	42	33.7	6	Myocardial infarction
27	M	58	35.5	10	Electrocution
28	M	54	46.3	12	Myocardial infarction

Abbreviations: BMI, Body Mass Index; PMI, Postmortem Interval.

Furthermore, GLP-1R immunoreactivity did not negatively correlate with BMI in the LH (Kendall's Tau-b =  $-0.347$ ,  $p = 0.024$ ). BMI was not correlated with GLP-1R in any other hypothalamic nuclei/areas analyzed in the present study ( $p > 0.05$ ) (Table 3). In addition, no statistically significant correlation between GLP-1R and clinicopathologic parameters, such as sex, age, cause of death, and postmortem interval, was observed ( $p > 0.05$ ).

Moreover, we verified our results regarding GLP-1R protein levels by using another antibody against GLP-1R (Merck-Millipore) that targets a nonoverlapping epitope instead of the one originally used (Origene). Similar results regarding the GLP-1R topography and staining intensity in the hypothalamus were obtained using both antibodies, as shown in Supplemental Table S2.

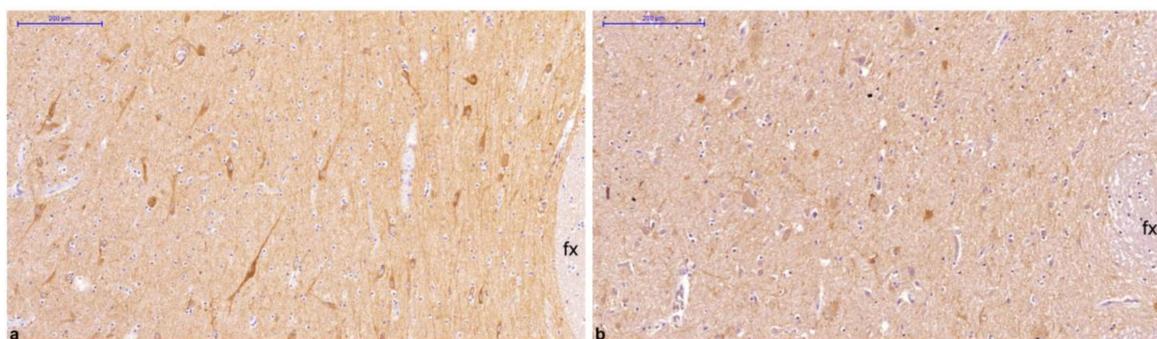
For control purposes, the GLP-1R protein levels were examined in the pancreas. As expected, intense GLP-1R immunoreactivity was observed in the human pancreas.

Omitting the primary antibody or substituting GLP-1R antiserum with normal rabbit serum resulted in no immunoreactivity in the human pancreas and hypothalamus.

**Table 2.** Comparison between the semiquantitatively expressed GLP-1R immunoreactivity scores of normal body mass index (BMI) (<25 kg/m<sup>2</sup>) and non-normal BMI (≥25 kg/m<sup>2</sup>) cases in the lateral hypothalamic area. Immunoreactivity score is expressed as follows: +++, >50% of cells exhibit strong immunoreactivity; ++, >50% of cells exhibit moderate immunoreactivity; +, >50% of cells exhibit weak immunoreactivity.

	Cases (n)	GLP-1R Immunoreactivity		
		+++	++	+
Normal BMI	11	5 (46%)	4 (36%)	2 (18%)
Non-normal BMI *	17	1 (6%)	8 (47%)	8 (47%)

\* p < 0.05, compared with the normal BMI group, using the Mann-Whitney U test.



**Figure 3.** Comparison of GLP-1R immunoreactivity in lateral hypothalamic area from a normal weight and a non-normal weight individual, both with similar clinicopathological characteristics. Images taken in similar rostrocaudal levels (medial zone of the tuberal region) are derived from a male with BMI 23.0 kg/m<sup>2</sup>, displaying high GLP-1R immunoreactivity, (a) and a male with BMI 46.3 kg/m<sup>2</sup>, showing low GLP-1R immunoreactivity (b). Bar denotes 200 µm.

**Table 3.** Correlations between BMI and immunoreactivity of GLP-1R in human hypothalamic nuclei.

	Kendall's Tau-b Correlation Coefficient														
	BMI	PVN	SON	IFN	LH	PeF	VMH	DMH	TM	MPO	SCh	MN	BN	DB	BST
BMI	1.00	-0.134	-0.178	-0.034	-0.347 *	-0.246	0.092	-0.041	0.087	-0.055	-0.242	0.048	-0.252	-0.110	-0.058
τb		0.395	0.268	0.831	0.024	0.109	0.557	0.796	0.647	0.720	0.531	0.765	0.119	0.506	0.743
p															

Abbreviations: BMI, Body Mass Index; BN, basal nucleus; BST, bed nucleus of the stria terminalis; DB, diagonal band; DMH, dorsomedial hypothalamic nucleus; IFN, infundibular nucleus; LH, lateral hypothalamic area; MN, mammillary nucleus; MPO, medial preoptic nucleus; PeF, perifornical nucleus; PVN, paraventricular nucleus; SCh, supraoptic nucleus; SON, supraoptic nucleus; TM, tuberomammillary nucleus; VMH, ventromedial hypothalamic nucleus; τb, Kendall's coefficient of rank correlation. \* Correlation is significant at the 0.05 level (2-tailed).

### 2.2. GLP-1R Colocalizes with the Anorexigenic and Antiobesogenic Neuropeptide NUCB2/Nesfatin-1 but Not with the Astrocytic Marker GFAP

GLP-1R colocalized extensively with NUCB2/nesfatin-1 in several hypothalamic nuclei, namely PVN, SON, IFN, LH, and in basal forebrain nuclei (Figure 4). In the PVN, the vast majority of GLP-1R-ir neurons overlapped with NUCB2/nesfatin-1 (91.2 ± 2.4%). The numbers represent mean value ± Standard Error of the Mean (SEM). On average, in the SON, of all GLP-1R-ir perikarya, 96.7 ± 0.9% exhibited NUCB2/nesfatin-1 immunoreactivity. Similarly, in the IFN, GLP-1R-ir neurons colocalized with NUCB2/nesfatin-1 at a percentage of 89.7 ± 3.9%. In the LH, of all GLP-1R-ir neurons, 97.0 ± 1.9% contained NUCB2/nesfatin-1. Furthermore, in the basal nucleus, 95.9 ± 1.4% of GLP-1R-ir cells over-

lapped with NUCB2/nesfatin-1. Conversely, the percentages of NUCB2/nesfatin-1-ir neurons coexpressing GLP-1R in the PVN, SON, IFN, LH, and basal nucleus, were  $55.1 \pm 2.8\%$ ,  $60.3 \pm 4.3\%$ ,  $62.2 \pm 4.0\%$ ,  $57.7 \pm 6.5\%$ , and  $75.3 \pm 7.7\%$ , respectively. Percentages of GLP-1R-ir neurons colocalizing with NUCB2/nesfatin-1 as well as of NUCB2/nesfatin-1-ir neurons colocalizing with GLP-1R are shown in Tables 4 and 5, respectively. In all hypothalamic nuclei/areas examined, comparing medians of percentages of GLP-1R-ir neurons colocalized with NUCB2/nesfatin-1 as well as of NUCB2/nesfatin-1-immunopositive neurons coexpressing GLP-1R revealed no statistically significant difference between normal weight and non-normal weight BMI groups ( $p > 0.05$ ). Furthermore, there was no statistically significant association between percentages of colocalized proteins (GLP-1R and NUCB2/nesfatin-1) and clinicopathological parameters, including BMI. In addition, GLP-1R did not colocalize with GFAP in any of the examined cases (Figure 5). Omission of the primary antibodies resulted in signal absence. Similar results regarding GLP-1R colocalization with (a) NUCB2/nesfatin-1 and (b) GFAP in human hypothalamic nuclei were obtained using both anti-GLP-1R antibodies.

**Table 4.** Double immunofluorescence analysis of GLP-1-receptor-immunoreactive neurons colocalizing with NUCB2/nesfatin-1 in human hypothalamus. Numbers denote the percentage of GLP-1-receptor-immunoreactive neurons colocalized with NUCB2/nesfatin-1 (%).

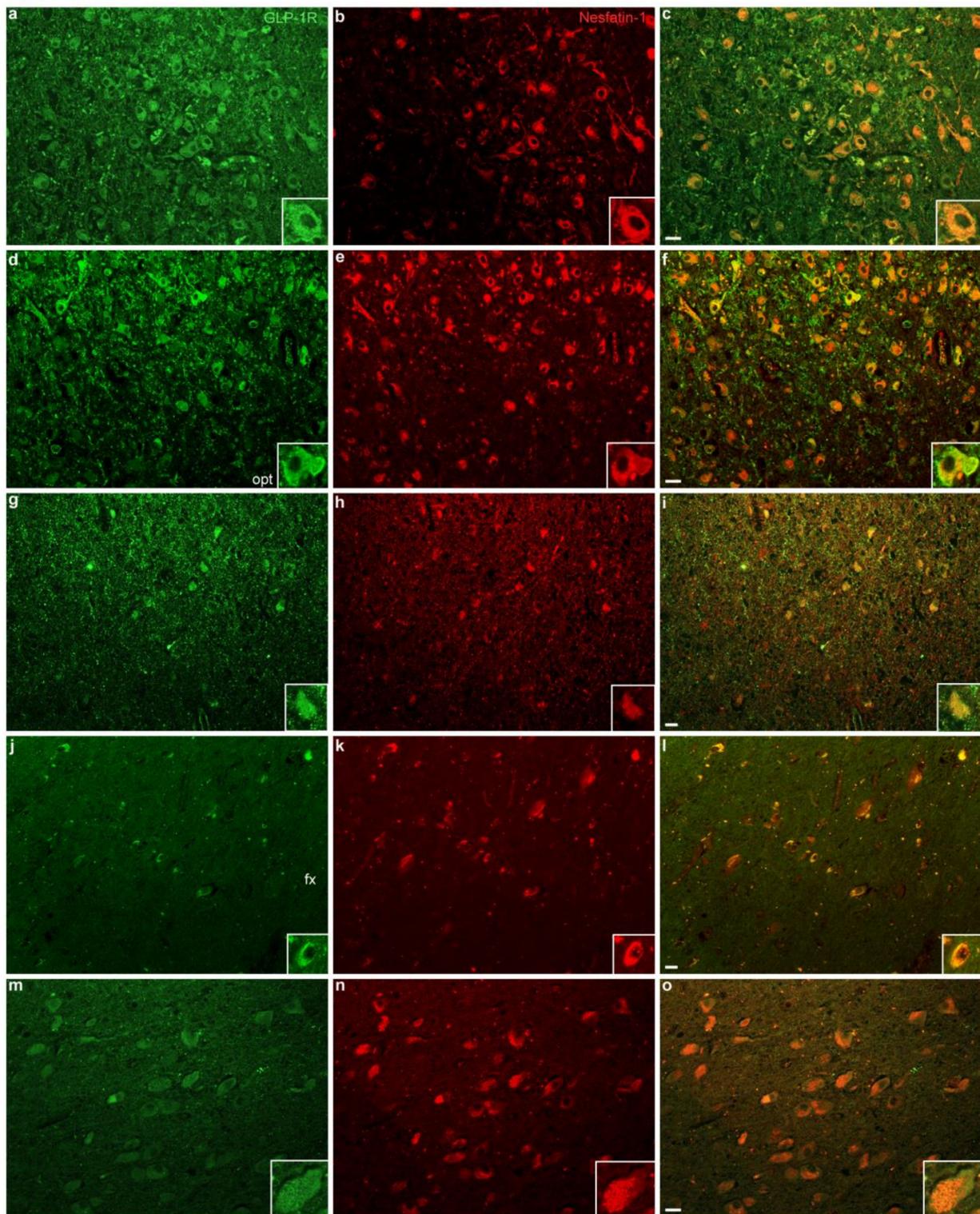
	Case	BMI (kg/m <sup>2</sup> )	PVN	SON	IFN	LH	BN
Normal weight group	2	21.6	81.2	94.7	95.2	85.6	95.1
	4	23.0	99.0	99.0	n/a	98.7	97.0
	6	23.9	94.7	95.0	99.1	n/a	n/a
	9	24.9	88.9	93.8	81.9	98.5	99.0
Mean value $\pm$ SEM		23.4 $\pm$ 0.7	91.0 $\pm$ 3.9	95.6 $\pm$ 1.2	92.1 $\pm$ 5.2	94.3 $\pm$ 4.3	97.0 $\pm$ 1.1
Non-normal weight group	14	29.0	94.5	93.8	85.5	99.0	90.4
	18	29.4	99.9	99.4	99.0	99.5	99.0
	23	31.7	85.2	98.9	95.5	98.7	91.7
	25	32.4	86.0	99.0	71.4	99.0	99.0
Mean value $\pm$ SEM		30.6 $\pm$ 0.8	91.4 $\pm$ 3.5	97.8 $\pm$ 1.3	87.9 $\pm$ 6.2	99.0 $\pm$ 0.2	95.0 $\pm$ 2.3
Total Mean value $\pm$ SEM		27.0 $\pm$ 1.5	91.2 $\pm$ 2.4	96.7 $\pm$ 0.9	89.7 $\pm$ 3.9	97.0 $\pm$ 1.9	95.9 $\pm$ 1.4

Abbreviations: BMI, Body Mass Index; BN, basal nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; IFN, infundibular nucleus; LH, lateral hypothalamic area; SEM, Standard Error of the Mean; n/a, not assessed.

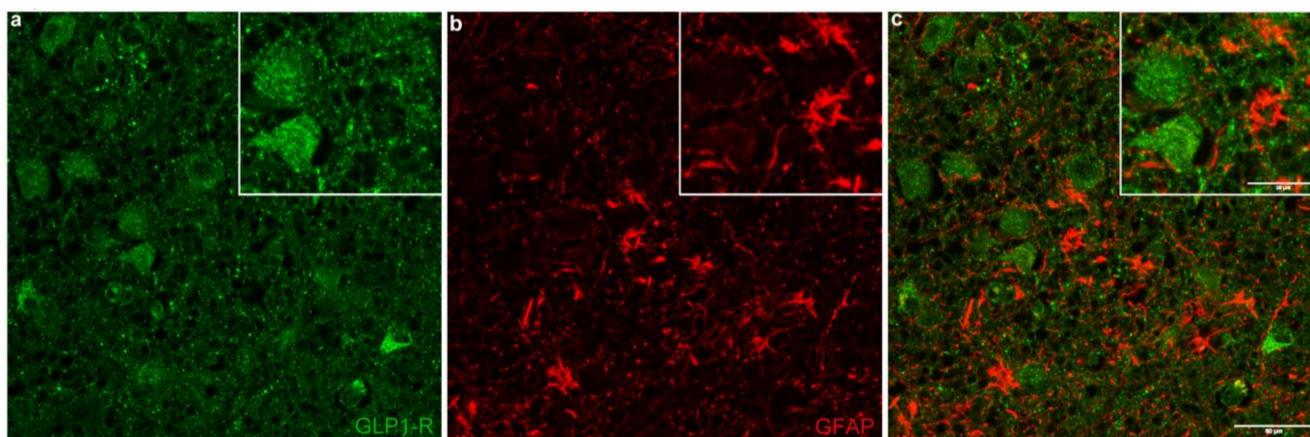
**Table 5.** Double immunofluorescence analysis of NUCB2/nesfatin-1-immunoreactive neurons colocalizing with GLP-1 receptor in human hypothalamus. Numbers present the percentage of NUCB2/nesfatin-1-immunoreactive neurons colocalized with GLP-1 receptor (%).

	Case	BMI (kg/m <sup>2</sup> )	PVN	SON	IFN	LH	BN
Normal weight group	2	21.6	60.6	72.2	61.0	63.6	72.9
	4	23.0	62.9	46.6	n/a	70.3	77.5
	6	23.9	55.2	68.4	82.0	n/a	n/a
	9	24.9	58.1	42.3	60.1	64.7	71.8
Mean value $\pm$ SEM		23.4 $\pm$ 0.7	59.2 $\pm$ 1.7	57.4 $\pm$ 7.6	67.7 $\pm$ 7.2	66.2 $\pm$ 2.1	74.1 $\pm$ 1.7
Non-normal weight group	14	29.0	62.2	75.0	69.4	29.5	n/a
	18	29.4	39.2	53.5	52.5	62.1	89.1
	23	31.7	50.0	67.7	51.3	75.5	98.0
	25	32.4	52.2	56.5	58.8	38.3	42.7
Mean value $\pm$ SEM		30.6 $\pm$ 0.8	50.9 $\pm$ 4.7	63.2 $\pm$ 5.0	58.0 $\pm$ 4.1	51.4 $\pm$ 10.6	76.6 $\pm$ 17.0
Total Mean value $\pm$ SEM		27.0 $\pm$ 1.5	55.1 $\pm$ 2.8	60.3 $\pm$ 4.3	62.2 $\pm$ 4.0	57.7 $\pm$ 6.5	75.3 $\pm$ 7.7

Abbreviations: BMI, Body Mass Index; BN, basal nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; IFN, infundibular nucleus; LH, lateral hypothalamic area; SEM, Standard Error of the Mean; n/a, not assessed.



**Figure 4.** Representative light microscopic fluorescent images depicting GLP-1R and NUCB2/nesfatin-1 colocalization in the human hypothalamus. Representative sections of paraventricular nucleus (a–c), supraoptic nucleus (d–f), infundibular nucleus (g–i), lateral hypothalamic area (g–l), and basal nucleus (m–o) double stained for GLP-1R (green) and NUCB2/nesfatin-1 (red). Merged images depict double stained neurons (c,f,i,l,o) (200×). Insets represent neurons at higher magnification (400×). fx, fornix; opt, optic tract. Scale bars indicate 50 μm.



**Figure 5.** Confocal image showing GLP-1R and GFAP double immunofluorescence labeling in human hypothalamus. Panel (a) shows GLP-1R-immunoreactivity (green) and panel (b) displays GFAP-immunoreactivity (red). The merged image (c) shows the lack of colocalization of GLP-1R and GFAP. Scale bar equals 50  $\mu\text{m}$  (25  $\mu\text{m}$  in insets).

### 3. Discussion

In the present study, we showed that GLP-1R protein is localized in several human hypothalamic nuclei implicated in the orchestration of energy metabolism and neuroendocrine and autonomic functions [23]. The most pronounced detection of GLP-1R was observed in the PVN, SON, IFN, LH, and in cholinergic basal forebrain nuclei. GLP-1R-ir cells were also detected, although to a lesser extent, in other nuclei such as the VMH, DMH, TM, perifornical, medial preoptic, and mammillary as well as in the bed nucleus of the stria terminalis. Importantly, in the LH, GLP-1R protein expression was significantly lower in overweight or obese subjects compared with normal weight individuals and did not negatively correlate with BMI. Interestingly, GLP-1R-immunopositive neurons extensively overlapped with the anorexigenic and antiobesogenic neuropeptide NUCB2/nesfatin-1 in hypothalamic nuclei, namely PVN, SON, IFN, and LH, and in basal nucleus. In all the aforementioned nuclei, the percentages of colocalization between GLP-1R and NUCB2/nesfatin-1 were not found to be altered in relation to BMI. Additionally, GLP-1R was not localized in hypothalamic GFAP-positive astrocytes.

GLP-1R localization, as described herein, is similar with previous studies in humans and animals [3–5,10,24–31]. According to our data, GLP-1R-ir cells, although in general agreement between studied cases, showed an interindividual variation in staining intensity in a few hypothalamic nuclei. These findings are consistent with previous reports in humans showing variations in hypothalamic GLP-1R mRNA and protein expression [4,5]. The prominent immunoreactivity of GLP-1R in the PVN, SON, IFN, and LH is in agreement with neuroanatomical evidence and functional analyses from human and animal studies, revealing that GLP-1R eating-inhibitory and antiobesogenic effects are possibly mediated by various brain areas, including hypothalamic nuclei [2–5,10,24–31]. In the present study, GLP-1R was also localized, although to a lesser extent, in the perifornical nucleus, VMH, DMH, and TM, which have discrete roles in feeding behavior. GLP-1R immunoreactivity was also detected in the medial preoptic area, suprachiasmatic nucleus, mammillary nucleus, and bed nucleus of the stria terminalis, which participate in food intake modulation in response to temperature change, biological rhythms, memory, and emotional and behavioral responses to stress, respectively [23,32–35]. The significance of this finding is unclear at present, and further investigation is warranted. It can be hypothesized that GLP-1R might play a role in the behavioral act of feeding or in so-far undefined physiological actions.

Furthermore, herein we showed that BMI had a significant moderate negative correlation with GLP-1R in the LH. Interestingly, GLP-1R immunoreactivity was lower in

overweight or obese subjects compared with normal weight ones. As one of the most extensively interconnected brain areas, the LH is involved in different aspects of food consumption, including hedonic feeding and body weight regulation [36,37]. It should be noted that direct administration of GLP-1 into the LH of rats acutely suppresses feeding [38]. Additionally, the decrease in GLP-1R immunoreactivity in the LH of overweight or obese individuals, shown herein, is comparable to the decreased expression of GLP-1R mRNA reported in the human PVN and IFN of type 2 diabetic patients [4]. It may be hypothesized that reduced GLP-1R in the LH contributes to dysregulation of homeostatic and/or hedonic feeding behavior and, consequently, to body weight gain. Although it has been suggested that the effects of central GLP-1R activation on food intake and energy expenditure are mediated by at least partially separate hypothalamic areas and circuitries [2], it is unclear whether regional variations in LH GLP-1R expression might influence GLP-1R signaling in the hypothalamus. The factors contributing to these variations in hypothalamic nuclei and the concomitant functional implications in humans need to be further elucidated.

Interestingly, GLP-1R was also found to widely colocalize with the anorexigenic and antiobesogenic neuropeptide NUCB2/nesfatin-1 in human hypothalamic nuclei involved in the regulation of energy metabolism, namely the PVN, SON, IFN, and LH. In a previous study by Ten Kulve et al. on the human hypothalamus [4], GLP-1R was only sporadically colocalized with energy balance-related neuropeptides, such as NPY, AgRP, and POMC. It is intriguing to hypothesize that the extensive immunolocalization of GLP-1Rs on neurons expressing NUCB2/nesfatin-1, as observed herein, might indicate that GLP-1 affects energy homeostasis by acting on these hypothalamic neurons. Furthermore, it has been reported that nesfatin-1 hypothalamic neurons mediate GLP-1 anorexigenic effects in rodents [19,20]. In addition, intraperitoneal administration of GLP-1 has been described to significantly increase activated nesfatin-1 neurons in hypothalamic nuclei, while GLP-1-induced food intake reduction has been significantly attenuated by pretreatment with intracerebroventricular administration of antisense nesfatin-1 [19]. GLP-1 and NUCB2/nesfatin-1 share similar physiologic properties in energy homeostasis and body weight [2,11,15,31]. The possibility of a protein-to-protein physical interaction cannot be claimed based on colocalization [39]; thus, possible effects of NUCB2/nesfatin-1 on central GLP-1R signaling require further investigation.

Furthermore, we found no immunoreexpression of GLP-1R in human hypothalamic astrocytes. GLP-1R localization in the human hypothalamus by means of immunohistochemistry and in situ hybridization has been previously reported only in neurons [4,5]. On the other hand, GLP-1R expression has been described in a human astrocytic cell line [9]. In this latter study, astrocytes displayed GLP-1R after being subjected to mechanical or metabolic stress. However, the complexity of neuronal and non-neuronal compartments of brain circuits may more accurately be described in vivo and/or ex vivo compared to in vitro.

Interestingly, in our study, GLP-1R immunoreexpression was observed in the cholinergic basal forebrain nuclei, a brain region known to participate in cognitive processes, memory, and feeding [23]. Although research has primarily focused on GLP-1R's metabolic role, it is becoming increasingly obvious that GLP-1R activation has additional functions [2]. Experimental models and preclinical studies show beneficial effects of GLP-1R agonism on Alzheimer's disease's neuropathological features, including cholinergic basal forebrain nuclei degeneration and cognition [40–42]. Recent reports suggest that brain–gut peptides, including GLP-1 and nesfatin-1, share neuroprotective properties and might have therapeutic potential for neurodegenerative disorders [43–45]. The presence of GLP-1R in the human basal nucleus and its colocalization with nesfatin-1, as described in our study, is in line with this notion. However, the functional importance of these findings in the human cholinergic basal forebrain nuclei remains to be determined.

Animal models have strongly improved our understanding of hypothalamic responses as well as of factors contributing to body weight gain. It remains, however, difficult to assess whether these data can be extrapolated to neuroendocrine mechanisms underlying

obesity in the human hypothalamus. Elucidating these neuronal pathways in humans entails technical difficulties and has limitations. Postmortem studies allow for a detailed anatomical and neurochemical examination of the energy-balance-related circuitry albeit causation cannot be determined. Conditions before and during death vary and cannot be controlled as in animal experiments. Furthermore, the availability of well-documented material is limited, resulting in a relatively low number of observations derived from a heterogeneous patient population. However, despite these limitations, we found GLP-1R immunoexpression to be negatively correlated with BMI in the human lateral hypothalamus and to extensively colocalize with the anorexigenic and anti-obesogenic neuropeptide NUCB2/nesfatin-1.

The essential molecular mechanisms underlying body weight gain remain largely unknown [46]. In our research, important biological information into the chemical neuroanatomy of the normal weight human hypothalamus and the hypothalamus affected by excess adiposity may be derived, providing an opportunity to gain insight into the mechanism underlying the considerable variation in achieving significant weight reduction observed between patients receiving GLP-1R agonists [47] as well as to decipher potential central molecular targets to therapeutically modify obesity-associated maladaptive physiologic alterations in neuronal networks and thereby adjust the control of appetite and metabolism in the brain.

Further studies are warranted to clarify the functional significance of reduced GLP-1R protein expression in overweight or obese individuals in certain hypothalamic regions as well as any possible role of non-neuronal cells and the potential effects of NUCB2/nesfatin-1 in GLP-1R signaling in human hypothalamic circuits controlling energy homeostasis.

## 4. Materials and Methods

### 4.1. Tissue Collection

Hypothalami from the level of optic chiasm to mammillary bodies were dissected at autopsy, fixed in neutrally buffered 10% formalin, dehydrated, and paraffin-embedded. Twenty-eight subjects (20 males and 8 females, aged 22–86 years) with no history or post-mortem evidence of neuropsychiatric disease were analyzed. Exclusion criteria included age less than 18 years old, history of neurological and psychiatric disorders, diabetes mellitus, postmortem delay of more than 24 h, trauma to the brain, use of neuroleptic and antiobesity medication prior to death, and exposure to toxins. Cases were classified into two groups according to body mass index (BMI) as follows: “normal weight”, including subjects with BMI < 25 kg/m<sup>2</sup>, and “non-normal weight”, with subjects having a BMI ≥ 25 kg/m<sup>2</sup> (being overweight or obese). BMI of the examined cases varied between 21.5 and 46.3 kg/m<sup>2</sup>. Postmortem interval ranged from 3 to 24 h. Clinicopathological data of examined cases are presented in Table 1. The study protocol was reviewed and approved by the Ethics Committee of the University Hospital of Patras (approval number 94/10.5.07). The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

### 4.2. Histochemistry

Standard H and E and luxol fast blue-cresyl violet stains were performed on representative sections for morphological and topographic assessment of brain areas.

### 4.3. Immunohistochemistry

Consecutive 4 µm coronal sections of hypothalamus (preoptic area, anterior hypothalamic area, tuberal region, and mammillary region) were analyzed. Three to six hypothalamic sections per nucleus per case taken at regular intervals throughout the rostrocaudal axis of each nucleus were deparaffinized in a series of xylene and ethanol. Immunohistochemistry was performed as previously described [16,48]. In brief, after blocking endogenous peroxidase activity by incubating the sections in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min and performing antigen retrieval in citrate buffer (pH 6), sections were incubated overnight at

4 °C using a rabbit polyclonal anti-GLP-1R antibody (1:50; Origene, Rockville, MD, USA, TA336864). Specificity of GLP-1R antiserum has been previously described in detail [49–51]. Color was developed using the EnVision Flex Kit (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, after incubation with the primary antibody, sections were washed in TBS and incubated with the secondary antibody coupled with activation reagent EnVision Flex horseradish peroxidase (HRP) for 20 min. Visualization was achieved by incubating slides 10 min with 3,3'-diaminobenzidine (DAB). Slides were counterstained with Harris' hematoxylin, dehydrated in ascending alcohol row, and permanently mounted. All sections were simultaneously processed in the presence of appropriate positive and negative controls.

Hypothalamic structures were identified with reference to *Atlas of the Human Brain* by Mai J. et al. [52]. Qualitative analysis of protein expression was performed independently and graded blindly by two researchers (H.P. and A.P.). Images were captured on 3D HISTECH Ltd., Hungary Panoramic DESK Scanner 1.16 and analyzed using Panoramic Viewer Software Version 1.15.4 C3D HISTECH Ltd., Budapest, Hungary. Immunostaining score was calculated in a semiquantitative fashion, using a four-point scale system as follows: +++ (more than 50% of cells are positive and staining intensity is high); ++ (more than 50% of cells are positive and staining intensity is moderate); + (more than 50% of cells are positive and staining intensity is low); – (no staining).

In order to further validate the results and confirm specificity, an additional rabbit polyclonal anti-GLP-1R antibody was used (1:50; Merck Millipore, Darmstadt, Germany, AB9433-I).

Pancreas was used as positive control for GLP-1R. Negative controls were prepared by omitting the primary antibody or by substituting GLP-1R antiserum with normal rabbit serum.

#### 4.4. Double Immunofluorescence Labeling

Double immunofluorescence analysis of human hypothalamus was performed to further characterize GLP-1R-expressing cells and to define whether (a) GLP-1R and nesfatin-1 and (b) GLP-1R and GFAP reside at the same physical location within the context of cellular compartments of different hypothalamic nuclei-implicated in energy homeostasis.

Hypothalamic sections from 8 individuals, 4 with normal and 4 with non-normal BMI (cases 2, 4, 6, 9, 14, 19, 23, 25 in Table 1), were double immunostained using the following antisera couples: (a) rabbit polyclonal anti-GLP-1R (1:50; Origene, Rockville, MD, USA) and mouse monoclonal anti-NUCB2/nesfatin-1 antibody (1:50; Merck Millipore, Darmstadt, Germany, MABS1164) and (b) rabbit polyclonal anti-GLP-1R (1:50; Origene, Rockville, MD, USA) and mouse monoclonal anti-GFAP antibody (1:500; Merck Millipore, Darmstadt, Germany, MAB360). Specificity of antibodies used for colocalization studies was previously published [16,49–51,53].

Hypothalamic sections were deparaffinized, blocked in phosphate-buffered saline containing 10% fetal bovine serum, 3% bovine serum albumin, and 0.1% Tween-20, for 2 h, and incubated overnight with couples of primary antibodies at 4 °C. After washing, sections were incubated at room temperature for 1 h with secondary antibodies Alexa Fluor 568 goat antimouse IgG and Alexa Fluor 488 goat antirabbit IgG (1:500; Invitrogen Life Technologies, Rockford, IL, USA), counterstained with Hoechst 33,258 (1:1500; Sigma-Aldrich Chemie GmbH, Germany), and cover slipped with fluorescent mounting medium (Merck Millipore, Darmstadt, Germany). Staining specificity was determined by omission of primary antibodies. Slides were analyzed in the Advanced Light Microscopy Facility of Patras Medical School with the use of a Nikon Eclipse TE 2000-U inverted microscope. Images were captured using the INFINITY software package (Lumenera Corporation, Ottawa, Canada). In hypothalamic and basal forebrain nuclei, the percentage of double-labeled neurons with GLP-1R and NUCB2/nesfatin-1 was counted by examining ten high-power fields per nucleus per section at a magnification of 400× and averaged to obtain a single value. Figures from double immunofluorescence labeling of GLP-1R and

GFAP proteins were obtained on a Confocal Leica SP5 microscope (Leica Microsystems, Paris, France).

To further validate the results regarding colocalization of GLP-1R and NUCB2/nesfatin-1 and of GLP-1R and GFAP, double immunofluorescence labeling was performed using another antibody against GLP-1R (Merck Millipore, Darmstadt, Germany) that targets a nonoverlapping epitope instead of the one originally used (Origene, Rockville, MD, USA).

#### 4.5. Statistical Analysis

All data were analyzed with IBM SPSS Statistics for Windows version 25.0 (IBM Corp., Armonk, NY, USA). Ordinal variables, such as protein expression-immunoreactivity scores, were presented as frequency distributions with percentages. Quantitative variables were defined as mean value  $\pm$  Standard Error of the Mean (SEM). Normality was tested both graphically and using the Shapiro–Wilk test. Normal distribution was not met, and therefore non-parametric statistical methods were employed. Group differences in the distribution of immunoreactivity scores were evaluated with Mann–Whitney U test. Correlations between GLP-1R immunoreactivity and clinicopathological parameters were examined using the Kendall's Tau-b correlation coefficient. Correlations between percentages of colocalized protein and clinicopathological parameters were analyzed using Pearson correlation coefficient ( $r$ ).  $p < 0.05$  was considered statistically significant.

## 5. Conclusions

GLP-1R protein localization and expression varied regarding the body mass index of the subjects. Specifically, GLP-1R was negatively correlated with BMI levels only in the later hypothalamic area. These data may be useful for gaining useful insights into the considerable variation in achieving significant weight reduction observed between patients receiving GLP-1R agonists and/or for developing new safe and effective antiobesity therapies. Further experimental work regarding the physiological significance of reduced GLP-1R immune expression in the LH, an appetite- and reward-related brain region, as well as any possible effects of NUCB2/nesfatin-1 in GLP-1R signaling are warranted to translate these findings into clinical applications.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms232314899/s1>, Table S1: Distribution of GLP1R protein expression in the human hypothalamus of all examined cases, presented as immunostaining score, using rabbit polyclonal anti-GLP-1R antibody (Origene, Rockville, MD, USA, TA336864); Table S2: Distribution of GLP1R protein expression in the human hypothalamus of all examined cases, presented as immunostaining score, using rabbit polyclonal anti-GLP-1R antibody (Merck Millipore, Darmstadt, Germany, AB9433-I).

**Author Contributions:** Conceptualization, A.P.; methodology, A.P. and H.P.; validation, A.P., D.V.C. and H.P.; formal analysis, A.P.; investigation, S.N., S.L. and M.A.; data curation, A.P.; writing—original draft preparation, A.P.; writing—review and editing, A.P., D.V.C.; visualization, A.P., S.L. and M.A.; supervision, H.P. and A.P.; internal funding acquisition, H.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital of Patras (approval number 94/10.5.07).

**Informed Consent Statement:** Patient consent was waived because specific informed consent was not required at the time of tissue sample collection for immunocytochemical analysis of archival tissue specimens since all cases were anonymized and genetic analysis was not carried out, and the study was approved by the Scientific Council of the University Hospital of Patras (approval number 249/25.5.07).

**Data Availability Statement:** The original contributions presented in the study are included in the article’s materials. Further inquiries can be directed to the corresponding author.

**Acknowledgments:** The present study is dedicated to the memory of Professor John Varakis, who passed away on 14 September 2020, aged 79 years. Professor Varakis was an excellent mentor, colleague, and friend to students and scientific staff of the University of Patras Medical School. We are sincerely grateful for his academic integrity and mental acuity which guided, inspired, and motivated us towards the search for scientific truth. The authors would also like to gratefully acknowledge the Advanced Light Microscopy Facility of Patras Medical School for sharing equipment.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Andersen, A.; Lund, A.; Knop, F.K.; Vilsbøll, T. Glucagon-like peptide 1 in health and disease. *Nat. Rev. Endocrinol.* **2018**, *14*, 390–403. [[CrossRef](#)] [[PubMed](#)]
2. Müller, T.D.; Finan, B.; Bloom, S.R.; D’Alessio, D.; Drucker, D.J.; Flatt, P.R.; Fritsche, A.; Gribble, F.; Grill, H.J.; Habener, J.F.; et al. Glucagon-like peptide 1 (GLP-1). *Mol. Metab.* **2019**, *30*, 72–130. [[CrossRef](#)] [[PubMed](#)]
3. Alvarez, E.; Martinez, M.D.; Roncero, I.; Chowen, J.A.; Garcia-Cuartero, B.; Gispert, J.D.; Sanz, C.; Vazquez, P.; Maldonado, A.; De Caceres, J.; et al. The expression of GLP-1 receptor mRNA and protein allows the effect of GLP-1 on glucose metabolism in the human hypothalamus and brainstem. *J. Neurochem.* **2005**, *92*, 798–806. [[CrossRef](#)]
4. Ten Kulve, J.S.; van Bloemendaal, L.; Balesar, R.; IJzerman, R.G.; Swaab, D.F.; Diamant, M.; la Fleur, S.E.; Alkemade, A. Decreased Hypothalamic Glucagon-Like Peptide-1 Receptor Expression in Type 2 Diabetes Patients. *J. Clin. Endocrinol. Metab.* **2016**, *101*, 2122–2129. [[CrossRef](#)] [[PubMed](#)]
5. Farr, O.M.; Sofopoulos, M.; Tsoukas, M.A.; Dincer, F.; Thakkar, B.; Sahin-Efe, A.; Filippaios, A.; Bowers, J.; Srnka, A.; Gavrieli, A.; et al. GLP-1 receptors exist in the parietal cortex, hypothalamus and medulla of human brains and the GLP-1 analogue liraglutide alters brain activity related to highly desirable food cues in individuals with diabetes: A crossover, randomised, placebo-controlled trial. *Diabetologia* **2016**, *59*, 954–965.
6. Reiner, D.J.; Miettlicki-Baase, E.G.; McGrath, L.E.; Zimmer, D.J.; Bence, K.K.; Sousa, G.L.; Konanur, V.R.; Krawczyk, J.; Burk, D.H.; Kanoski, S.E.; et al. Astrocytes Regulate GLP-1 Receptor-Mediated Effects on Energy Balance. *J. Neurosci.* **2016**, *36*, 3531–3540. [[CrossRef](#)] [[PubMed](#)]
7. Chowen, J.A.; de Fonseca, F.R.; Alvarez, E.; Navarro, M.; García-Segura, L.M.; Blázquez, E. Increased glucagon-like peptide-1 receptor expression in glia after mechanical lesion of the rat brain. *Neuropeptides* **1999**, *33*, 212–215. [[CrossRef](#)]
8. Iwai, T.; Ito, S.; Tanimitsu, K.; Udagawa, S.; Oka, J. Glucagon-like peptide-1 inhibits LPS-induced IL-1beta production in cultured rat astrocytes. *Neurosci. Res.* **2006**, *55*, 352–360. [[CrossRef](#)]
9. Bułdak, Ł.; Machnik, G.; Skudrzyk, E.; Bołdys, A.; Okopień, B. The impact of exenatide (a GLP-1 agonist) on markers of inflammation and oxidative stress in normal human astrocytes subjected to various glycemic conditions. *Exp. Ther. Med.* **2019**, *17*, 2861–2869. [[CrossRef](#)]
10. Graham, D.L.; Durai, H.H.; Trammell, T.S.; Noble, B.L.; Mortlock, D.P.; Galli, A.; Stanwood, G.D. A novel mouse model of glucagon-like peptide-1 receptor expression: A look at the brain. *J. Comp. Neurol.* **2020**, *528*, 2445–2470. [[CrossRef](#)]
11. Van Bloemendaal, L.; IJzerman, R.G.; Ten Kulve, J.S.; Barkhof, F.; Konrad, R.J.; Drent, M.L.; Veltman, D.J.; Diamant, M. GLP-1 receptor activation modulates appetite- and reward-related brain areas in humans. *Diabetes* **2014**, *63*, 4186–4196. [[CrossRef](#)] [[PubMed](#)]
12. Plamboeck, A.; Veedefald, S.; Deacon, C.; Hartmann, B.; Wettergren, A.; Svendsen, L.B.; Meisner, S.; Hovendal, C.; Vilsbøll, T.; Knop, F.K.; et al. The effect of exogenous GLP-1 on food intake is lost in male truncally vagotomized subjects with pyloroplasty. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **2013**, *304*, G1117–G1127. [[CrossRef](#)] [[PubMed](#)]
13. Oh-I, S.; Shimizu, H.; Satoh, T.; Okada, S.; Adachi, S.; Inoue, K.; Eguchi, H.; Yamamoto, M.; Imaki, T.; Hashimoto, K.; et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* **2006**, *443*, 709–712. [[CrossRef](#)] [[PubMed](#)]
14. Stengel, A.; Goebel, M.; Taché, Y. Nesfatin-1: A novel inhibitory regulator of food intake and body weight. *Obes. Rev.* **2011**, *12*, 261–271. [[CrossRef](#)] [[PubMed](#)]
15. Dore, R.; Levata, L.; Lehnert, H.; Schulz, C. Nesfatin-1: Functions and physiology of a novel regulatory peptide. *J. Endocrinol.* **2017**, *232*, R45–R65. [[CrossRef](#)]
16. Psilopanagioti, A.; Nikou, S.; Papadaki, H. Nucleobindin-2/nesfatin-1 in the human hypothalamus is reduced in obese subjects and colocalizes with oxytocin, vasopressin, melanin-concentrating hormone, and cocaine- and amphetamine-regulated transcript. *Neuroendocrinology* **2019**, *108*, 190–200. [[CrossRef](#)]
17. Kras, K.; Muszyński, S.; Tomaszewska, E.; Arciszewski, M.B. Minireview: Peripheral Nesfatin-1 in Regulation of the Gut Activity-15 Years since the Discovery. *Animals* **2022**, *12*, 101. [[CrossRef](#)]
18. Kmiecik, A.M.; Dzięgiel, P.; Podhorska-Okołów, M. Nucleobindin-2/Nesfatin-1-A New Cancer Related Molecule? *Int. J. Mol. Sci.* **2021**, *22*, 8313. [[CrossRef](#)]

19. Katsurada, K.; Maejima, Y.; Nakata, M.; Kodaira, M.; Suyama, S.; Iwasaki, Y.; Kario, K.; Yada, T. Endogenous GLP-1 acts on paraventricular nucleus to suppress feeding: Projection from nucleus tractus solitarius and activation of corticotropin-releasing hormone, nesfatin-1 and oxytocin neurons. *Biochem. Biophys. Res. Commun.* **2014**, *451*, 276–281. [[CrossRef](#)]
20. Katsurada, K.; Yada, T. Neural effects of gut- and brain-derived glucagon-like peptide-1 and its receptor agonist. *J. Diabetes Investig.* **2016**, *7* (Suppl. S1), 64–69. [[CrossRef](#)]
21. Saito, R.; So, M.; Motojima, Y.; Matsuura, T.; Yoshimura, M.; Hashimoto, H.; Yamamoto, Y.; Kusuhara, K.; Ueta, Y. Activation of Nesfatin-1-Containing Neurons in the Hypothalamus and Brainstem by Peripheral Administration of Anorectic Hormones and Suppression of Feeding via Central Nesfatin-1 in Rats. *J. Neuroendocrinol.* **2016**, *28*. [[CrossRef](#)]
22. Brown, E.; Wilding, J.P.H.; Barber, T.M.; Alam, U.; Cuthbertson, D.J. Weight loss variability with SGLT2 inhibitors and GLP-1 receptor agonists in type 2 diabetes mellitus and obesity: Mechanistic possibilities. *Obes. Rev.* **2019**, *20*, 816–828. [[CrossRef](#)]
23. Swaab, D.F. Part I: Nuclei of the human hypothalamus: Cytoarchitecture, chemoarchitecture, functional neuroanatomy, topographic neuropathology. In *Handbook of Clinical Neurology. The Human Hypothalamus: Basic and Clinical Aspects*; Aminoff, M.J., Boller, F., Swaab, D.F., Eds.; Elsevier: Amsterdam, The Netherlands, 2003; Volume 79, pp. 3–59.
24. Göke, R.; Larsen, P.J.; Mikkelsen, J.D.; Sheikh, S.P. Distribution of GLP-1 binding sites in the rat brain: Evidence that exendin-4 is a ligand of brain GLP-1 binding sites. *Eur. J. Neurosci.* **1995**, *7*, 2294–2300. [[CrossRef](#)]
25. Merchanthaler, I.; Lane, M.; Shughrue, P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. *J. Comp. Neurol.* **1999**, *403*, 261–280. [[CrossRef](#)]
26. Richards, P.; Parker, H.E.; Adriaenssens, A.E.; Hodgson, J.M.; Cork, S.C.; Trapp, S.; Gribble, F.M.; Reimann, F. Identification and characterization of GLP-1 receptor-expressing cells using a new transgenic mouse model. *Diabetes* **2014**, *63*, 1224–1233. [[CrossRef](#)]
27. Cork, S.C.; Richards, J.E.; Holt, M.K.; Gribble, F.M.; Reimann, F.; Trapp, S. Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain. *Mol. Metab.* **2015**, *4*, 718–731. [[CrossRef](#)]
28. Shugrue, P.J.; Lane, M.; Merchanthaler, I. Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. *Endocrinology* **1996**, *137*, 5159–5162. [[CrossRef](#)]
29. Jensen, C.B.; Pyke, C.; Rasch, M.G.; Dahl, A.B.; Knudsen, L.B.; Secher, A. Characterization of the Glucagonlike Peptide-1 Receptor in Male Mouse Brain Using a Novel Antibody and In Situ Hybridization. *Endocrinology* **2018**, *159*, 665–675. [[CrossRef](#)]
30. Heppner, K.M.; Kirigiti, M.; Secher, A.; Paulsen, S.J.; Buckingham, R.; Pyke, C.; Knudsen, L.B.; Vrang, N.; Grove, K.L. Expression and distribution of glucagon-like peptide-1 receptor mRNA, protein and binding in the male nonhuman primate (*Macaca mulatta*) brain. *Endocrinology* **2015**, *156*, 255–267. [[CrossRef](#)]
31. Van Bloemendaal, L.; Ten Kulve, J.S.; la Fleur, S.E.; Ijzerman, R.G.; Diamant, M. Effects of glucagon-like peptide 1 on appetite and body weight: Focus on the CNS. *J. Endocrinol.* **2014**, *221*, T1–T16. [[CrossRef](#)]
32. Yu, S.; François, M.; Huesing, C.; Münzberg, H. The Hypothalamic Preoptic Area and Body Weight Control. *Neuroendocrinology* **2018**, *106*, 187–194. [[CrossRef](#)] [[PubMed](#)]
33. Lebow, M.A.; Chen, A. Overshadowed by the amygdala: The bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatry* **2016**, *21*, 450–463. [[CrossRef](#)] [[PubMed](#)]
34. Vann, S.D.; Aggleton, J.P. The mammillary bodies: Two memory systems in one? *Nat. Rev. Neurosci.* **2004**, *5*, 35–44. [[CrossRef](#)] [[PubMed](#)]
35. Tsujino, N.; Sakurai, T. Role of orexin in modulating arousal, feeding, and motivation. *Front. Behav. Neurosci.* **2013**, *7*, 28. [[CrossRef](#)] [[PubMed](#)]
36. Bernardis, L.L.; Bellinger, L.L. The lateral hypothalamic area revisited: Ingestive behavior. *Neurosci. Biobehav. Rev.* **1996**, *20*, 189–287. [[CrossRef](#)]
37. Stuber, G.D.; Wise, R.A. Lateral Hypothalamic Circuits for Feeding and Reward. *Nat. Neurosci.* **2016**, *19*, 198–205. [[CrossRef](#)]
38. Schick, R.R.; Zimmermann, J.P.; vom Walde, T.; Schusdziarra, V. Peptides that regulate food intake: Glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2003**, *284*, R1427–R1435. [[CrossRef](#)]
39. Wu, Y.; Eghbali, M.; Ou, J.; Lu, R.; Toro, L.; Stefani, E. Quantitative determination of spatial protein-protein correlations in fluorescence confocal microscopy. *Biophys. J.* **2010**, *98*, 493–504. [[CrossRef](#)]
40. Vargas-Soria, M.; Carranza-Naval, M.J.; Del Marco, A.; Garcia-Alloza, M. Role of liraglutide in Alzheimer’s disease pathology. *Alzheimers Res. Ther.* **2021**, *13*, 112. [[CrossRef](#)]
41. Arendt, T.; Bigl, V.; Tennstedt, A.; Arendt, A. Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in Alzheimer’s disease. *Neuroscience* **1985**, *14*, 1–14. [[CrossRef](#)]
42. Salcedo, I.; Tweedie, D.; Li, Y.; Greig, N.H. Neuroprotective and neurotrophic actions of glucagon-like peptide-1: An emerging opportunity to treat neurodegenerative and cerebrovascular disorders. *Br. J. Pharmacol.* **2012**, *166*, 1586–1599. [[CrossRef](#)]
43. Dong, D.; Xie, J.; Wang, J. Neuroprotective Effects of Brain-Gut Peptides: A Potential Therapy for Parkinson’s Disease. *Neurosci. Bull.* **2019**, *35*, 1085–1096. [[CrossRef](#)]
44. Arabacı Tamer, S.; Koyuncuoğlu, T.; Karagöz Köroğlu, A.; Akakın, D.; Yüksel, M.; Yeğen, B.Ç. Nesfatin-1 ameliorates oxidative brain damage and memory impairment in rats induced with a single acute epileptic seizure. *Life Sci.* **2022**, *294*, 120376. [[CrossRef](#)]
45. Kim, D.S.; Choi, H.I.; Wang, Y.; Luo, Y.; Hoffer, B.J.; Greig, N.H. A New Treatment Strategy for Parkinson’s Disease through the Gut-Brain Axis: The Glucagon-Like Peptide-1 Receptor Pathway. *Cell Transplant.* **2017**, *26*, 1560–1571. [[CrossRef](#)]

46. Tschöp, M.; DiMarchi, R. Single-Molecule Combinatorial Therapeutics for Treating Obesity and Diabetes. *Diabetes* **2017**, *66*, 1766–1769. [[CrossRef](#)]
47. Lee, Y.H.; Lee, H.W.; Choi, H.J. GLP-1 Based Combination Therapy for Obesity and Diabetes. *J. Obes. Metab. Syndr.* **2017**, *26*, 155–160. [[CrossRef](#)]
48. Psilopanagioti, A.; Makrygianni, M.; Nikou, S.; Logotheti, S.; Papadaki, H. Nucleobindin 2/nesfatin-1 expression and colocalisation with neuropeptide Y and cocaine- and amphetamine-regulated transcript in the human brainstem. *J. Neuroendocrinol.* **2020**, *32*, e12899. [[CrossRef](#)]
49. Zhou, D.; Chen, Y.-W.; Zhao, Z.-H.; Yang, R.-X.; Xin, F.-Z.; Liu, X.-L.; Pan, Q.; Zhou, H.; Fan, J.-G. Sodium butyrate reduces high-fat diet-induced non-alcoholic steatohepatitis through upregulation of hepatic GLP-1R expression. *Exp. Mol. Med.* **2018**, *50*, 1–12. [[CrossRef](#)]
50. Timper, K.; del Río-Martín, A.; Cremer, A.L.; Bremser, S.; Alber, J.; Giavalisco, P.; Varela, L.; Heilinger, C.; Nolte, H.; Trifunovic, A.; et al. GLP-1 Receptor Signaling in Astrocytes Regulates Fatty Acid Oxidation, Mitochondrial Integrity, and Function. *Cell Metab.* **2020**, *31*, 1189–1205.e13. [[CrossRef](#)]
51. Rocha-Ferreira, E.; Poupon, L.; Zelco, A.; Leverin, A.L.; Nair, S.; Jonsdotter, A.; Carlsson, Y.; Thornton, C.; Hagberg, H.; Rahim, A.A. Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy. *Brain* **2018**, *141*, 2925–2942. [[CrossRef](#)]
52. Mai, J.; Majtanik, M.; Paxinos, G. Part 2: Myelo- and cytoarchitectonic atlas of the human brain in stereotaxic (MNI) space. In *Atlas of the Human Brain*, 4th ed.; Mai, J., Majtanik, M., Paxinos, G., Eds.; Academic Press: Cambridge, MA, USA; Elsevier: Amsterdam, The Netherlands, 2015; pp. 181–233, 427–431.
53. Xing, L.; McDonald, H.; Da Fonte, D.F.; Gutierrez-Villagomez, J.M.; Trudeau, V.L. Dopamine D1 receptor activation regulates the expression of the estrogen synthesis gene aromatase B in radial glial cells. *Front. Neurosci.* **2015**, *9*, 310. [[CrossRef](#)] [[PubMed](#)]