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Title: Glucagon Like Peptide-1 and Its Receptor in Human Brain: Distribution of Expression, Functional Implications, Age Related Changes & Species Specific Characteristics

Running title: Glucagon like peptide-1 and its receptor in human brain

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Abstract

Emerging evidence has shown that the Glucagon like peptide-1 (GLP-1) agonist can be used for treating Alzheimer's disease, but knowledge of its neural targets is limited. To understand the neural substrates of GLP-1, we have done whole brain mapping for GLP-1 and its receptor (GLP-1R), in 30 human brains. GLP-1 expression was studied by immuno-histochemistry and confirmed by western blot method. GLP-1R gene expression was studied by RT-PCR. GLP-1 expression was seen in most of the cortical areas (maximum in frontal, prefrontal & parietal cortex), diencephalon and brainstem, but not in cerebellum. Protein expression studies validated these results. Highest expression of GLP-1R was found in the frontal cortex. The orbito-frontal cortex and cerebellum had negligible expression. Hippocampus demonstrated significant presence of GLP-1R but patchy immunoreactivity to GLP-1. GLP-1R presence in most of the human cortical regions and absence in cerebellum is the major deviation from the animal brain. Sites which might be of interest in Alzheimer's have been identified. GLP-1 demonstrated age related decline in most of the areas after 5th decade. At 60yrs GLP-1 was not found in any of the cortical areas except in the prefrontal cortex but it was present in the sub-cortical areas. Age related profiling of GLP-1 in various brain areas has been analysed, which can have important bearing on understanding the Alzheimer's. This study provides detailed description of GLP-1 and GLP-1R locations by complete human brain mapping for the first time and may lead to novel treatment options targeting the GLP-1 receptors.

Keywords: Glucagon like peptide-1, Glucagon like peptide -1 receptor, Alzheimer's disease, Human brain

Introduction

Glucagon like peptide (GLP-1) is secreted by L cells of the intestine. It is classified as an incretin, as it increases the glucose dependent release of insulin in response to food. GLP-1 increases the synthesis and secretion of insulin from the pancreatic β cells, inhibits the release of glucagon; have anabolic effect on liver; exert a cardio protective effect; It is said to produce satiety through its action on nucleus of tractus solitarius and hypothalamus (Andersen & Knop, 2018; Drucker, 2018). GLP-1 acts via a trans-membrane receptor (GLP-1R). GLP-1R agonists are being used as anti-diabetic drugs for many years' now (Gupta, 2013). Recent studies have shown that GLP-1 agonists have neuro protective properties (Gejl et al., 2016; Holubová et al., 2019; Nakajima, Numakawa, Adachi, & Shin, 2016; Salcedo, Tweedie, Li, & Greig, 2012). Many studies have proven the neuro trophic effects of GLP-1 or GLP-1 receptor agonist on cellular and animal models of neurodegenerative diseases like Alzheimer's and Parkinson's disease; acute cerebrovascular disorders; and traumatic brain injury (Athauda & Foltynie, 2016; Batista, Bodart, Felice, & Ferreira, 2018; Gao, Liu, Jiang, Ding, & Li, 2014). This strongly suggests that GLP-1 & GLP-1R have widespread distribution in anatomically and functionally distinct areas of CNS. In a very recent study by Farkas et al, 2020, localization of GLP-1R protein was determined in rat's brain. Immunoreactivity was seen in the circumventricular organs and areas around the arcuate nucleus and nucleus tractus solitarii (Farkas et al., 2020). Moreover, neuronal profiles of GLP-1R were distinguished in telencephalon, diencephalon, brainstem and cerebellum. Graham et al, 2020, have reported GLP-1R-expressing cells in the lateral septum, hypothalamus, amygdala, bed nucleus of the stria terminalis, hippocampus, ventral midbrain, periaqueductal gray, and cerebral cortex of transgenic mice (Graham et al., 2020).

Detailed information about the GLP-1 location and its potential sites of action is an important first step in evaluating the function of GLP-1 in the brain. To the best of our knowledge complete mapping of human brain for GLP-1 and its receptor has not been done. There have been few studies on GLP-1 receptors (GLP-1R) in specific areas of brain, which regulate the feeding, like hypothalamus and brain stem. Complete mapping of brain GLP-1 receptors was done first time in rodent brain by Gu et.al, 2013 which was followed by mapping of nonhuman primate brain by Heppner et al, 2015 (Gu et al., 2013; Heppner

et al., 2015). The majority of studies examining central GLP-1 effects have been performed on rodents (Trapp & Richards, 2013).

The aim of the present study is to provide detailed information about the location of GLP-1 and its receptors in the human brain. In this study the samples were taken from 14 different sites, encompassing the whole human brain. The gene expression pattern of GLP-1R was studied by qPCR, while GLP-1 was detected by immuno-histochemistry using specific monoclonal antibody as well as by studying the protein expression pattern by western blot. These sites can be pharmacologically manipulated for treating neurological disorders.

Materials & Methods:

The study was approved by the Institute ethical committee vide INT/IEC/2015/441, dated 11.09.2015.

Samples: The study was conducted on adult human brains (n=30), obtained from autopsy (within 4-6 hrs of death) after fully informed consent. Samples were collected from the following sites:

1. Cortex: Orbito frontal cortex, Medial frontal cortex, Pre-frontal cortex, Frontal cortex, Parietal cortex, Occipital cortex, Temporal cortex and Hippocampus
2. Diencephalon: Thalamus (area containing - medial dorsal nucleus and midline nucleus) & Hypothalamus (area containing - paraventricular nucleus)
3. Brain stem: Midbrain (area containing - superior colliculus), Pons (area containing - ventral tegmentum), Medulla (area containing - Inferior olivary nucleus, raphe nuclei & pyramidal fibres)
4. Cerebellum

Patients with history of any neurological disease, diabetes, head injury or brain surgery were excluded from the study. The brain samples were collected in saline solution. Samples were divided and preserved in 10% buffered formalin for Immuno-histochemistry (IHC) (n=30), RNA later for m-RNA study at 4°C (n=10) and frozen at -80°C for protein expression study (n=10).

Tissue location confirmation: Each tissue was subjected to detailed histological examination to confirm the exact location of tissue being studied.

Procedures:

Messenger RNA (mRNA) expression of GLP-1R for different regions was studied by RT-PCR (Real Time-polymerase chain reaction). Protein expression was studied by western blotting for validation. GLP-1 localization was done by IHC.

RNA isolation and cDNA synthesis:

Total RNA was isolated from the sample of fresh brain tissue stored in the RNA later with a commercially available kit (*RiboPure™, Ambion, Life Technologies, USA*) according to the manufacturer's instructions. The yield and purity of RNA was assessed by spectrophotometer by measuring absorbance at 260 nm and 280 nm. Integrity of RNA was verified by denaturing agarose gel electrophoresis. RNA bands on the gel were visualized under UV light; presence of 18S as well as 28S rRNA bands confirmed good quality of the yield. A total RNA measuring 500 ng was used to generate first strand cDNA as initial step of a two-step Reverse Transcriptase - PCR. Contamination with genomic DNA was eliminated by pre-treatment of cellular RNA with DNaseI (*Invitrogen, San Diego, CA*). Synthesis of cDNA was performed using a commercially available kit according to the manufacturer's instructions. cDNA of each sample was amplified using primers of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to check the efficiency of reverse transcription.

Relative gene expression assay by RT-PCR for expression of GLP-1R:

Selection of the house keeping gene: Both GAPDH and β -actin were properly validated with brain samples to determine that their gene expression is unaffected by the experimental treatment. Both were run against GLP-1R. The Ct value and melt curve of GAPDH was least variable among all the samples as compared to β -actin, therefore it was selected as the housekeeping gene.

Quantitative PCR was performed using sequence specific predesigned primers & housekeeping gene using SYBR green chemistry -

GLP-1R (XM_017010751.1): Forward-5' ATCCAAACTGAAGGCCAATC3'

Reverse-5' AGCTGGACCTCATTGTTGA3'

GAPDH (NC_000012.12): Forward-5' ACACCGCCCTGGATCTCATA3'

Reverse-5' ACGAACGTGTGCGGAATCTT 3'

Relative quantification of genes was carried out on *LightCycler® 480* Real-time PCR system (*Roche Diagnostics, Germany*). The amount of GLP-1R m-RNA was normalized to

GAPDH. Following cycling parameters were used - for 7 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. Reaction volume used was 10 μ l. The analysis of data was done using the Δ Ct method. In order to nullify the due variation in samples, the data has been normalized to the housekeeping gene, GAPDH, which served as an internal control. The samples were loaded in triplicates. To calculate Δ Ct, the average of triplicate Ct values was taken and the mean Ct value of housekeeping gene (GAPDH) was subtracted from mean Ct value of gene of interest (GLP-1R).

We have chosen to use Δ Ct for analysis instead of $\Delta\Delta$ Ct which is more commonly used. $\Delta\Delta$ Ct can be calculated by comparing the change in the gene expression relative to the diseased case in the same organ (different areas of brain in our case). We did not have diseased brain samples. Another method to calculate $\Delta\Delta$ Ct is to use expression of the same mRNA in another organ as calibrator. It provides tissue comparison, but the results are difficult to analyse from biological point of view. The single relative quantity reported actually reflects, variation in both target and reference transcripts across a variety of cell types that might be present in any particular tissue (Schmittgen & Livak, 2008). As our aim is to record the expression of the GLP-1R mRNA in different regions of brain, which can be further used, we have chosen to present our results as Δ Ct.

Western blot to check the protein expression of GLP-1:

The tissue was incubated on ice for 15 minutes and lysed with chilled 1X RIPA lysis buffer and protease inhibitor (*Merck-Millipore*). The suspension was centrifuged at 14000 rpm for 25 minutes at 4°C and the supernatant was collected. The protein concentration was determined by BCA assay using bicinchoninic acid assay kit (*ThermoFisher Scientific*). Thirty μ g of total protein sample was mixed with the 2X Laemmli's sample buffer and heated at 100°C for 5 minutes. The protein samples were immediately put onto the ice. Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For immune detection, total proteins on the gel were electro-transferred to polyvinylidene difluoride membrane (PVDF) membrane (*ImmobilonTM-P, Millipore*) using a trans-blot transfer cell (Bio-Rad). The polyacrylamide gel was stained with Commassie Brilliant Blue G-250 (CBB) for 1 hour and destained with double distilled water overnight. The membrane was blocked with 5% BSA (bovine serum albumin) in TBST buffer (solution of tris-buffered saline & 0.1% Tween 20) for 1 hour at room temperature. The membrane was washed for 3 times, 5 minutes each with TBST buffer and was incubated with primary antibody (*anti-GLP-1, 1:1500 dilution, mouse monoclonal, ab36598*) in 5%

BSA buffer overnight at 4°C with β actin (*anti- β actin, 1:5000 dilution, mouse monoclonal, MA5-15739*) as control. The membrane was washed for 3 times, 5 minutes each with TBST buffer followed by incubation with horseradish peroxidase conjugated secondary antibody (*anti-mouse, 1:40,000 dilution, ab97046*). The membrane was again washed for 3 times, 5 minutes each with TBST buffer. Visualization of blot was done with enhanced chemiluminescence substrate (ECL) (*Bio-Rad laboratories, USA*) by Protein Simple Imaging System.

Densitometric analysis was done using Image J software by selecting band width and measurement of intensity. The quantification of the bands was then performed using β -actin as control. The ratio of area of interest/ β -actin of the band intensities was taken.

Localization of GLP-1 expression was done by immunohistochemistry (IHC):

For IHC tissue sections were fixed in 10% buffered formalin, processed and embedded in paraffin following a standard protocol. The sections were deparaffinised and hydrated gradually through graded alcohols (100%, 70% and 50%) & washed in de-ionised water. Endogenous blocking was done by hydrogen peroxide to quench endogenous peroxidase activity. Antigen was retrieved in citrate buffer at pH 6.1 for 12 minutes to increase immunogenicity. The slides were then incubated with GLP-1 monoclonal (*anti-GLP-1, 5 μ g/ml dilution, mouse monoclonal, ab36598*) primary antibody at 4°C for overnight and followed by secondary antibody (*anti-mouse, 1:2000 dilution, Abcam, ab97046*) for 1 hour at room temperature. Peroxidase activity was developed in 0.5% 3, 3'-diaminobenzidine (DAB). Counter staining was done with haematoxylin. Pancreatic tissue was used for positive control and negative control was obtained by excluding the primary antibody. Sections were cleared in xylene and mounted with di-n-butyl phthalate in xylene (DPX).

Results:

GLP-1 Immuno-histochemistry (Fig. 1 & 2): Thirty tissue samples were divided in three groups; cases with, age less than 30 years in group 1 (group 1a upto 20 yrs & group 1b > 20 yrs), 30 to 50 years in group 2 (group 2a upto 40 yrs & group 2b >40 yrs) and more than 50 years in group 3 (group 3a <60 yrs & group 3b - 60 yrs) (Table I).

Group 1 (Age < 30 years):

Cortex: In 5 cases of below 20 years age group (14-16years), immunoreactivity (IR) to GLP-1 monoclonal antibody was not observed in any region of the cerebral cortex. In cases from 20 to 30 years, IR of mild intensity was detected in cortical regions in patchy fashion. In the prefrontal cortex patchy IR was found in the large pyramidal cells and neuropil (data not shown). In frontal, parietal and temporal cortical areas scattered immuno-staining of punctate cytoplasmic pattern was seen only in large pyramidal cells along with neuropil staining. Temporal cortex showed scanty IR. No IR was seen in the occipital cortex. Hippocampus staining was in marginally extensive area, including few pyramidal cells in stratum pyramidalis and scattered neuropil.

Diencephalon: The thalamic sample was taken from magnocellular part of the medial dorsal nucleus and the midline nuclei. In group 1a neurons were not stained but scattered mild IR was seen in the white matter and choroid plexus. In group 1b IHC picture was same, but at few places groups of 2-3 neurons were also stained. The hypothalamic sample was taken from the paraventricular nucleus (medial part), which showed scattered magnocellular neurons in the background of small neurons. Scanty mild IR was seen in few magnocellular neurons, in all group 1 cases.

Brain stem: In Pons small patches of mild IR involving 1-2 pontine neurons was found. In medulla the tissue sample has been taken from the open part of medulla as shown in figure 2. This medullary tissue included, medial part of the inferior olivary nucleus, pyramidal fibres and raphe nuclei of medullary reticular formation. In the lateral part, corresponding to the central reticular nucleus of medulla, mid IR patches encompassing 3-5 neurons and the surrounding neuropil were seen. Inferior olivary nucleus depicted mild IR of neurons and hilar fibres. Pyramidal fibres were unstained.

Cerebellum: No IR was seen in the cerebellum

Group 2 (Age 30 - 50 years):

Cortex: Immunoreactivity (IR) to GLP-1 monoclonal antibody was observed in all areas of cortex, though quantity, intensity and location pattern was variable. Scattered IR involving cortical layers 2 to 6 was seen in all of the cortical areas. The staining intensity was more in layer 3 and 5 where, moderate staining was seen in the scattered areas involving 2-5 granule cells and their neuropil, and mild punctate cytoplasmic staining was seen in some

large pyramidal cells and their processes. The orbital frontal cortex was exception to this as it only showed scattered mild IR in the cortical layer 3. The cortical layer 1 was not stained in most of the areas except the medial frontal and parietal cortices. Least IR was seen in the occipital cortex. Hippocampus showed moderate intensity staining of patches of neuropil near surface. The large pyramidal cells in stratum pyramidalis showed moderate staining of cytoplasm and associated neurites at places.

Diencephalon: In the thalamus scattered IR of mild to moderate intensity was seen, involving the fibres mainly but at places neurons were also stained. Some large neurons showed cytoplasmic granular staining in part of the cytoplasm while in few neurons the IR was seen in whole of the cytoplasm and their arborisation. The hypothalamic sample showed scattered magnocellular neurons in the background of small neurons. The small neurons did not take the stain. The magnocellular IR was in the form of cytoplasmic stippling, forming a crescent, which variably covered $1/4^{\text{th}}$, $1/3^{\text{rd}}$ or almost the whole cell.

Brain stem: In medulla the lateral part, corresponding to the central reticular nucleus of medulla, had moderate IR patches encompassing 3-5 neurons and the surrounding neuropil. This was the area of maximum immunoreactivity in the medullary tissue. Inferior olivary nucleus with typical crumpled bag appearance was seen. Its neurons showed cytoplasmic punctate staining of moderate intensity, covering $1/2$ to total cellular area. Scattered patches of IR, of moderate intensity, were also seen in the fibres seen at the hilum of the Inferior olivary nucleus. Pyramidal fibres are unstained. In the median part of the tissue intense punctate staining was seen in the median raphe reticular nuclei. In Pons moderate intensity patchy staining of 4-8 pontine nuclei and nerve fibres around them was seen.

Cerebellum: No IR was seen in the cerebellum

Group 3 (Age > 50 years):

The pattern of the IHC results in group 3a were similar to that found in the group 2 except for the variations in the intensity of IR; the prefrontal, temporal and hippocampal areas showed lesser intensity while higher intensity IR was seen in the brain stem.

In 3b group there was a major difference in the cortical immunoreactivity as none of the cortical areas showed the IR except the prefrontal cortex. The prefrontal cortex had mild staining, scattered in all the cortical layers and in few large pyramidal neurons and their

processes. The diencephalon also depicted much lesser IR in this group. Thalamus IR was very mild and only fibres were stained. In Hypothalamus mild IR was seen in occasional magnocellular neuron covering half to three fourths of its cytoplasmic area. The IR pattern was similar to the group 2 but with decreased intensity.

GLP-1 Protein expression (Fig. 3):

To analyze the protein expression of GLP-1, separate regions from the human brain were classified into 4 groups. Quantification of Western Blot images with the aid of Image J software revealed that the ratios of the net band to net loading control (β actin) for the regions of the first group i.e. Orbito-frontal cortex, medial frontal cortex and temporal cortex were 0.267, 0.76, and 0.619 respectively. The second group comprising of proteins from midbrain, medulla and pons displayed the ratios of 7.0, 3.77 and 6.22 respectively. For the third group (frontal cortex, prefrontal cortex and parietal cortex) values of the ratios were found out to be 17.0, 11.2, and 5.2 respectively. The ratio of the intensities of the bands observed in the fourth group comprising of hippocampus, thalamus and hypothalamus was calculated to be 0.418, 0.905 and 0.108 respectively. On comparing all the regions the data revealed that the highest expression of GLP-1 was found in frontal cortex and the lowest in hypothalamus. No results were found in the occipital cortex and cerebellum. This data is diagrammatically represented in the Figures 3.

GLP-1 receptor (GLP-1R) (Fig. 4):

The gene expression of GLP-1R in 14 different brain regions was assessed in 10 cases (age 27- 60 years). The brain regions included were orbito-frontal cortex, medial-frontal cortex, pre-frontal cortex, frontal cortex, parietal cortex, occipital cortex, temporal cortex, hippocampus, thalamus, hypothalamus, midbrain, medulla, pons and cerebellum. The mean of delta Ct values for these regions were 0, 3, 12.92, 17, 13.35, 1.4, 1.58, 8.92, 7.8, 8.768, 12.25, 3.7, 8.408 and 0 respectively. The data revealed that the highest expression of GLP-1R was found in the frontal cortex. The pre-frontal cortex, midbrain, parietal cortex, has lower expression than the frontal cortex. Diencephalon and the hippocampus along with pons have moderate expression whereas medial frontal cortex, occipital cortex, temporal cortex and medulla expressed at low levels. Orbito-frontal cortex and cerebellum did not have any expression.

Discussion:

In our study the GLP-1 expression has been observed by dual methods and the results of the proteomics study are in concordance with immuno-histochemical expressions. Moderate intensity IHC staining, seen in the pre-frontal cortex, frontal cortex, parietal cortex, and the brain stem is reflected in the quantified protein expression in these sites. Similarly in the hypothalamus amount of protein expressed is less, which is in agreement with the GLP-1 immunoreactivity (IR) seen only in the scattered magnocellular neurons.

GLP-1 receptor (GLP-1R) is a G protein-coupled receptor. Messenger RNA coding for this receptor is widely expressed in rodents (Le et al., 2018; Merchenthaler, Lane, & Shughrue, 1999; Ohshima, Hotsumi, Holscher, & Seki, 2015; Reiner et al., 2016) and non-human primates (Heppner et al., 2015). We also found widespread gene expression of GLP-1R in the human brain. Most of the cerebral cortical regions and diencephalon expressed GLP-1 mRNA, while the expression in the brainstem decreased inferiorly, with high expression in the mid brain, moderate in the pons, and low in the medulla.

On comparing the gene expression profile of GLP-1R with the GLP-1 sites mapped by immunoblotting and IHC, we observed that the GLP-1 location profile matches its receptor location in most of the cortical areas and in the brain stem. In the hippocampus and the diencephalon m-RNA expression far exceeded the protein expression. This might indicate that the hippocampus and diencephalon are responsible for production of GLP-1 which is then being transported to other sites. The IHC finding of moderate intensity staining of neuropil in the hippocampus seems to support this conclusion.

Functional considerations of GLP-1 and GLP-1R:

The distribution profile of GLP-1 and its receptor found in the present study is consistent with possible areas of interest in Alzheimer's disease. The pre-frontal and parietal cortices were found to have maximum GLP-1 and GLP-1R concentration. Prefrontal cortex is involved in planning, social insight & judgement while the parietal cortex integrates, analyse & contextualize sensory input (Datta, 2000). Hippocampus is responsible for new memory formation & retrieval of old memories in association with the prefrontal cortex (Preston & Eichenbaum, 2013). Both the thalamic nuclei which have shown moderate amounts of GLP-1 and its receptor, in the present study, have behavioural functions.

Thalamic medial dorsal nucleus lends emotional tone to the behaviour while mid line nuclei are believed to play a role in memory (Datta, 2000). In the present study the hypothalamic paraventricular nuclei (PVN), which is considered to be a major area for energy homeostasis in brain showed moderate presence of GLP-1 and GLP-1R. Reduced glucose utilization and disturbed energy metabolism occurs early in the course of Alzheimer's disease and correlates with impaired cognition (Monte, 2012; Simsir, Soyaltin, & Cetinkalp, 2018). Thus the distribution pattern of GLP-1 and its receptor described in this study provide anatomical basis for the clinical observations made by many authors that GLP1-R agonist might prove to be therapeutic in sporadic Alzheimer's disease (Gao et al., 2014; Gejl et al., 2016).

Age related changes in GLP-1 (Table II):

Age related decline in GLP-1 in human brain was also observed in the present study. GLP-1 was absent in cortical areas including the hippocampus below 20 years. From 20 to 50 years it increased with age in all cortical areas studied; but started decreasing after the age of 50 years. At 60 yrs of age immunoreactivity to the GLP-1 was absent in all cortical area except in the prefrontal cortex. In the sub cortical regions positive immunoreactivity for GLP-1 was seen as early as 13 -18 years of age; then it increased with age till 55 years. At 55 years about 30% decline in the GLP-1 immunoreactivity was noticed in the all subcortical regions studied; these levels persisted till 60 years. At 60 yrs GLP-1 was seen in reduced amount in prefrontal cortex & in subcortical areas only (We did not have any sample older than 60 years). Age related reduction of GLP-1 in mouse prefrontal cortex has been demonstrated, which was found to be associated with impairment of spatial cognitive memory, but unlike the present study the hippocampus did not show the age related decline in GLP-1 (Ohshima et al., 2015). Age related decline in GLP-1 might have some bearing on development of Alzheimer's disease in elderly population.

Species specific differences in GLP-1R (Table III):

The GLP-1 receptor (GLP-1R) has been mapped in the rodent brain (Gu et al., 2013) and recently in the nonhuman primate central nervous system (Heppner et al., 2015). GLP-1R distribution in the primate brain has been found to be similar to that of the rodent with few exceptions. GLP-1 receptors were absent in the hippocampus and were much more in the

amygdala, in the primate brain, as compared with the rodent (Heppner et al., 2015; Ohshima et al., 2015). In animals most abundant expression was seen in the hypothalamus.

The GLP-1R m-RNA mapping of the human brain in the present study highlights many differences, when the data is compared to the animal studies. Most important being presence of GLP-1R mRNA in the different areas of cortex. Previous studies on monkey and rat (Heppner et al., 2015; Le et al., 2018; Merchenthaler et al., 1999) did not find GLP-1R mRNA in these cortical areas, except in the mouse prefrontal cortex (Ohshima et al., 2015). On the other hand we did not find the GLP-1R in the orbitofrontal cortex though it has been found in the basal portion of the frontal cortex in the rat brain (Merchenthaler et al., 1999). The expression of GLP-1R in the hippocampus and hypothalamic PVN is highly conserved between rodents, monkeys and humans. GLP-1R expression in subcortical areas studied in the present study is similar to that reported by animal studies (Heppner et al., 2015; Le et al., 2018; Merchenthaler et al., 1999; Ohshima et al., 2015). Absence of GLP-1R mRNA in human cerebellum is similar to that found in rat (Rinaman, 2020), but is a major deviation from non human primate brain (Heppner et al., 2015). Most abundant expression of GLP-1R was seen in the frontal cortex in humans but in the hypothalamus in animals, this probably denotes higher cerebral control present in humans.

Conclusion

Mapping of human brain for GLP-1 and its receptor has been done for the first time as per our knowledge. The quantitative analysis of protein expression has provided data by which relative significance of different brain regions can be assessed in context of the GLP-1 (Fig 3). This information can be utilised for therapeutic application by insight into cellular localisation of the GLP-1 provided by the IHC (Fig 1&2). This study has provided detailed and novel information about the age related changes in GLP-1 profile (Table-II). This will aid in understanding the role of GLP-1 in diseases like Alzheimer's and Parkinsonism, which are associated with senescence. To understand the species specific differences human brain mapping data has been compared to the data of primate and non primate animals. This study is an important step in evaluating the function of GLP-1 in the brain.

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Figure 1: Microscopic anatomy of various cerebral cortical areas after immuno staining using GLP-1 monoclonal antibody. The macroscopic view of the cerebrum in mid sagittal section depicting the Prefrontal (PF), the Frontal (F) and the Parietal (P) cortical areas and below that, an inferior view of the cerebrum dissected to demonstrate the Hippocampus is shown in left lower corner. The Parietal cortex at 40x magnification depicts moderate immune staining in neuropil and scattered mild staining in neurons. The Frontal cortex at 40x magnification depicts cytoplasmic staining in large pyramidal cells of cortical layer 5. The Prefrontal cortex shows moderate immunoreactivity (IR) in the granule cells and surrounding neuropil including a vessel wall. The Hippocampal trilaminar cortex with scattered IR is seen at 4x magnification. At 40x magnification moderate patchy IR is seen in large pyramidal cells & few fibres in the stratum pyramidalis of the Hippocampus.

Figure 2: The upper panel depicts macroscopic mid sagittal view of the thalamus and the hypothalamus. Diagrammatic representation of the thalamic medial dorsal nucleus (MDN) and the hypothalamic paraventricular nucleus (PVN) is shown in the section. The thalamic MDN at 20x magnification depicts moderated immunoreactivity (IR) in the fibres and few large neurons. The hypothalamic PVN shows magnocellular neurons in the background of small neurons at 40x. IR is seen in the magnocellular neurons as cytoplasmic stippling forming a crescent. Small neurons did not take the stain. The lower panel depicts macroscopic view of the olive in the medulla, followed by the microscopic view of the inferior olivary nucleus. A typical crumpled bag appearance of the inferior olivary nucleus can be seen at 10x. In the inset the pattern of staining in the neuron can be appreciated in the form of cytoplasmic punctate moderate intensity immunoreactivity, at 40x magnification. Pons at 40 x magnification shows group of pontine nuclei and surrounding neuropil with moderate IR. Immuno staining has been done using GLP-1 monoclonal antibody.

Figure 3: Total protein separated by SDS PAGE was resolved on 12% resolving gel, stained with commassie blue. 10 wells gel was loaded with 9 samples & protein marker (M) of known molecular weight, to check the presence of protein and their resolution. Representative images of the protein blot of different areas of brain and β -actin, for GLP-1 expression. At the bottom, a graphical representation of the GLP-1 protein expression is seen. Densitometric analysis was done using Image J software by selecting band width and measurement of intensity. The quantification of the bands was then performed using β -actin as a control. Each of the brain areas studied is shown on the X axis and the ratio of band intensities of sample (from each brain area)/ β -actin is shown on Y axis.

Figure 4: In the upper panela melt curve analysis is seen - performed at the end of the PCR cycle, to confirm specificity of primer annealing. In the lower panel a graphical representation of GLP-1 receptor (GLP-1R) distribution pattern in different areas of the brain is shown. The Y axis represents mean of delta Ct values for these areas.

Table I: Demographic data of cases

S. No	Group 1	Group 2	Group 3
	Age < 30 yrs	Age 30 - 50 yrs	Age >50 yrs
1	14/F	30/M	51/M
2	15/M	31/M	52/F
3	15/M	32/M	52/M
4	16/M	38/M	53/M
5	16/M	40/M	60/M
6	20/M	40/F	60/M
7	22/M	42/M	60/M
8	24/F	45/M	60/M
9	27/M	45/M	60/M
10	29/M	47/M	
11		50/M	

M: Male, F: Female

Brain Area	Group 1 Age < 30 yrs		Group 2 Age 30-50 yrs		Group 3 Age >50 yrs	
	upto 20 yrs	> 20 yrs	upto 40yrs	>40yrs	<60 yrs	60 yrs
Cerebral cortex						
Prefrontal cortex	-	+	++	++	+	+
Frontal cortex	-	+	++	++	++	-
Parietal cortex	-	+	++	++	++	-
Temporal cortex	-	+	++	++	+	-
Hippocampal	-	+	++	++	+	-
Diencephalon						
Thalamus	+	+	+++	++	++	+
Hypothalamus	+	+	++	+++	+++	+
Brain stem						
Mid brain	+	+	++	++	+++	++
Pons	+	+	++	+	+++	++
Medulla	+	+	++	++	+++	++

Table II: Age related decline in GLP-1 immunoreactivity in human brain

No immunoreactivity -, mild immunoreactivity +, moderate immunoreactivity ++ and Intense immunoreactivity +++.

Brain area	Human	Non-human primate	Rodents
Cerebral cortex	Present in all areas except orbitofrontal cortex	Absent	Absent except in mouse prefrontal cortex
Hippocampus	Present	Present	Present
Amygdala	Present	Present (Much higher than rodents)	Present
Diencephalon			
Thalamus	Present	Present	Present
Hypothalamus	Present	Most abundant expression	Most abundant expression
Brain stem	Present	Present	Present
Cerebellum	Absent	Present	Present

Table III: Species specific differences in GLP-1 receptor