Summary of Ph.D. Thesis

Expression of insulin and GLP-1 receptors in interneurons of the cerebral cortex

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II. Csajbók ÉA, Tamás G: Cerebral cortex: a target and source of insulin? *Diabetologia* 2016 59(8):1609-15. doi: 10.1007/s00125-016-3996-2. IF_{2016}: 6.08

III. Molnár G, Faragó N, Kocsis ÁK, Rózsa M, Lovas S, Boldog E, Báldi R, Csajbók É, Gardi J, Puskás LG, Tamás G: GABAergic Neurogliaform Cells Represent Local Sources of Insulin in the Cerebral Cortex *Journal of Neuroscience* 2014, 34(4): 1133-1137. doi:10.1523/JNEUROSCI.4082-13. IF_{2014}: 6.344

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INTRODUCTION

Insulin is present in the central nervous system in concentrations of 10 to 100 times higher than plasma levels depending on the area of the brain. Insulin regulates the metabolism, molecular composition and cognitive performance of microcircuits specific alterations in diabetes, aging, obesity and Alzheimer's disease. Since this first report suggesting the presence of both pancreatic and locally synthesized insulin in the brain a multitude of studies argued in favor of peripheral and central sources. Insulin can cross the blood-brain barrier as shown by increased insulin levels in the cerebrospinal fluid following infusion of insulin in the periphery and studies finding correlation between steady-state endogenous insulin levels in the plasma and cerebrospinal fluid suggesting that insulin enters CNS through the blood-brain barrier by a saturable transport system. However, local insulin synthesis in the central nervous system was suggested by variable brain versus blood insulin ratios in experimental paradigms and in pathological states and by in situ hybridization and immunocytochemical studies detecting insulin mRNA in developing and adult neurons and neuronal progenitor cells but the identity of neurons expressing insulin in terms of functional cell classes is not clear.

Glucagon-like peptide 1 (GLP-1) produced by L-cells of the intestine is important in blood glucose homeostasis acting through several classic mechanisms including the inhibition of gastric emptying, suppressing pancreatic glucagon secretion and enhancing insulin release in the pancreas. Direct action of circulating GLP-1 on G-protein coupled GLP-1 receptors located on pancreatic beta cells leads to glucose-dependent closure of ATP-sensitive K^+ channels with subsequent depolarization and Ca^{2+} influx and Ca^{2+} dependent release of Ca^{2+} from intra-cellular Ca^{2+} stores resulting in Ca^{2+}-dependent insulin secretion. It is of high clinical importance that GLP-1 reduces the concentrations of blood glucose only postprandially, when blood glucose levels exceed fasting concentrations. Such glucose-dependent action renders intravenously administered GLP-1 ineffective in producing hypoglycemia and, consequently, current treatment of type 2 diabetes mellitus include GLP-1 receptor agonists as therapeutic agents.

Circulating GLP-1 finds additional targets linked to insulin synthesis outside the pancreas. Native GLP-1 crosses the blood brain barrier and thus incretins arriving from the periphery, including GLP-1 produced by intestinal L-cells and GLP-1analogues prescribed in type 2 diabetes mellitus, have the possibility to act on neurons of the hippocampus and the neocortex known to express GLP-1 receptors. In addition, neurons in the brain might also receive GLP-1 from central sources according to results showing GLP-1 expression in neurons located in the nucleus of the solitary tract in the brainstem. On the other hand, accumulating evidence based on experiments performed mostly in the rat shows that insulin is synthesized by neurons of the cerebral cortex.

Insulin delivered intranasally to the brain is therapeutically promising against mild cognitive impairment and Alzheimer's disease and the GLP-1 analogues used in diabetes

treatment have preventive effects at the early stage of AD development Parkinson's disease and traumatic brain injury. We hypothesize that novel therapeutic strategies might include modulation of neural insulin production in the brain by GLP-1 agonists for counteracting diabetes, obesity and neurodegenerative diseases. Therefore, it is of potential importance whether GLP-1 of intestinal or neural origin or therapeutically applied GLP-1 receptor agonists find targets on neurons capable of insulin production

AIMS

We have focussed our studies on the following questions:

1. What is the number of insulin mRNAs in various cell classes in the cerebral cortex?
2. Can insulin be released in the local microcircuit of the cerebral cortex?
3. Do insulin-containing neurons express GLP-1 receptors?
4. Do GLP-1 receptors function on insulin expressing neurons?

MATERIALS AND METHODS

Electrophysiology and imaging. Brain slices (350 μm) were prepared from the somatosensory cortex of male Wistar rats (postnatal day 28-35). Recordings were obtained at 36 °C from cells visualised in layers 2-3 by infrared differential interference contrast videomicroscopy. Micropipettes were filled with an intracellular solution containing 126 mmol/l K-gluconate, 4 mmol/l KCl, 10 mmol/l HEPES, 10 mmol/l creatine phosphate and 8 mmol/l biocytin (pH 7.25, 300 mOsm), supplemented with RNase Inhibitor (1 U/μl) to prevent any RNA degradation. Slices were preincubated in 0.5 mmol/l glucose for 4 h prior to recording sessions under hypoglycaemic conditions. Pre-treatment with exendin-3(9-39) (1 μmol/l) was applied for 4 h prior to recording sessions under hyperglycaemic (10 mmol/l glucose) conditions. Detection of spontaneous EPSCs were performed with NeuroMatic functions for Igor Pro, separation of GABAergic currents was based on polarity. For imaging, neurogliaform cells were filled with 10 μM Alexa594 and 120 μM OGB-1 added to the ATP free intracellular solution with the application of the hypoglycaemic extracellular solution and detection of signals was performed with an Andor Revolution XD system.

Histology and reconstruction of neurons. Following electrophysiological recordings, slices were immersed in fixative containing 4% paraformaldehyde, 15% saturated picric acid and 1.25% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4). Brain slices were further sectioned into 60 μm slices for a standard ABC reaction for the visualization of biocytin inside the recorded cells and were stained in 1% uranyl acetate and dehydrated using an

ascending series of ethanol before being embedded into Durcupan on glass slides. Three-dimensional light microscopic reconstructions were carried out using a Neurolucida system with a ×100 objective.

Single-cell reverse transcription, QRT-PCR and digital PCR. At the end of electrophysiological recordings, the intracellular content was aspirated into recording pipettes while maintaining a tight seal and the content of the pipettes (~1.5 µl) was expelled into a test tube containing 0.5 µl SingleCellProtect solution in order to prevent nucleic acid degradation and to be compatible with direct reverse transcription reactions. Samples were snap-frozen in liquid nitrogen and used for reverse transcription. The first step of reverse transcription was performed for 5 min at 65 °C in a total reaction volume of 5 µl, containing 2 µl intracellular solution and SingleCellProtect mixed with the cytoplasmic contents of the neuron, 0.3 µl TaqMan reagent, 0.3 µl 10 mmol/l deoxynucleotide triphosphates (dNTPs), 1 µl 5X first-strand buffer, 0.3 µl 0.1 mol/l dithiothreitol, 0.3 µl RNase Inhibitor and 100 U reverse transcriptase (SuperScript III). The second step of the reaction was carried out at 55 °C for 1 h, following which the reaction was stopped by heating at 75 °C for 15 min. For single cell QRT-PCR, reactions were carried out after preamplification of cDNA in a total volume of 20µl (5 µl RT product, 1 µl of Taqman primer (*Rps18*: Rn01428913_gH; *Ins2*: Rn01774648_g1), 10µl TaqMan PreAmp Master Mix and 4.5µl nuclease-free water) in MyGenie 32 Thermal Block using standard protocols. For digital PCR analysis, half of the reverse transcription reaction mixture (2.5 µl), 2 µl TaqMan reagent, 10 µl OpenArray Digital PCR Master Mix and nuclease-free water (5.5 µl) were mixed, for a total volume of 20 µl. Processing of the OpenArray slide, cycling in the OpenArray NT cycler and data analysis were performed according to standard protocols.

Radioimmunoassay. Insulin extraction of cells was performed in the cold by the acid-ethanol technique. Radioimmunoassay (Sensitive Rat Insulin RIA kit, Millipore) was used to determine insulin contents with a sensitivity of 2 pg/tube. BCA protein assay kit (Pierce) was used for detecting total protein content.

Statistical analysis. Data are given as means ± SD. Datasets were statistically compared using one-way ANOVA, Kruskal–Wallis, Wilcoxon or Mann–Whitney U test with SPSS software. Differences were accepted as significant if $p < 0.05$.

RESULTS

Cell type dependent insulin mRNA expression in the cerebral cortex

We tested whether different neocortical neuron types, all of them identified by whole cell recordings and subsequent light microscopic assessment, express the mRNA of the *Ins2* gene encoding preproinsulin in the rat. After electrophysiological and anatomical identification of cell types based on characterization of membrane and firing properties, we harvested the cytoplasm of the recorded cells and applied conventional single cell QRT-PCR with pre-amplification protocol and detected *Ins2* mRNA in 15 out of 19 neurogliaform cells. In order to determine the number of *Ins2* mRNA molecules present in the harvested perisomatic cytoplasm of these cell types, we adapted the digital PCR method to single neurons without preamplification steps which would have decreased reliability. In high extracellular glucose concentration (10 mmol/l) which is standard for brain slice electrophysiology experiments, individual neurogliaform cells (n = 10) contained higher numbers of *Ins2* mRNAs (30 ± 13) compared to pyramidal (7 ± 2, n = 6) and fast spiking cells (5 ± 3, n = 5, p < 0.002). As a functional control, we lowered the glucose concentration to levels close to what was found in the brain during normoglycemia (2.4 mmol/l) and hypoglycemia (0.5 mmol/l) and this decreased the number of *Ins2* mRNA molecules in single neurogliaform cells to 14 ± 3 (n = 5, p < 0.008) and further to 7 ± 4 per cell (n = 5, p < 0.04). In contrast, copy numbers of *Rps18* mRNAs coding the homeostatic ribosomal protein S18 were similar in neurogliaform (n = 16, 65 ± 18), pyramidal (n = 14, 63 ± 26) and fast spiking cells (n = 15, 61 ± 25) regardless of external glucose concentrations. In further control experiments, we determined the number of *Rps18* (26 ± 6) and *Ins2* (1 ± 0.8) mRNAs in glial cells (n = 5 and 4, respectively) showing that our data on mRNA copy numbers exclude DNA contamination which might arise in small cells. The copy number of *Rps18* (p < 0.01) and *Ins2* (p < 0.04) mRNAs in glial cells was less than in any of the three neuron types we tested. In addition, we repeated conventional and digital PCRs amplifying both *Rps18* and *Ins2* without reverse transcriptase reaction and found no amplification and no PCR products meaning that genomic DNA amplification was negligible.

Insulin release from identified neurons

An increase in extracellular glucose level might act as a physiological trigger in releasing insulin from neurogliaform cells containing *Ins2* mRNAs. In order to test this hypothesis, we first searched for electrophysiologically measurable effects of external insulin in brain slices and administered insulin in the bath in concentrations (100 nmol/l) taking into account extra- and intracellular space ratios (0.18) and the ~140 μm diffusion into the slice pushing local concentrations down to a few nanomolar at our recording sites. Insulin reversibly decreased the frequency (from 13.0 ± 9.4 Hz to 7.3 ± 5.5 Hz, n = 16, p < 0.001) and amplitude (from 12.1 ± 8.13 pA to 10.1 ± 6.28 pA, n = 15, p < 0.005) of spontaneous EPSCs arriving to neocortical neurons in hypoglycemia (0.5 mmol/l) and application of the specific insulin

receptor antagonist S961 (20 nmol/l) prevented the effect (12.2 ± 8.6 Hz and 12.5 ± 9.47 pA). To test whether neurogliaform cells could mimic the reversible effect of externally added insulin, we performed simultaneous paired recordings in hypoglycaemic (0.5 mmol/l) conditions and puffed hyperglycaemic extracellular solution (10 mmol/l) locally to the soma of neurogliaform cells while measuring the frequency of spontaneous EPSCs arriving to neighbouring neurons (pyramidal cells (n = 5), fast spiking basket (n = 4) and axo-axonic (n = 1) cells, data are pooled as no differences were observed between cell types). Relative to control, the frequency (9.0 ± 8.3 Hz) of spontaneous EPSCs decreased following hyperglycaemic puffs to neurogliaform cells to 2.4 ± 1.6 Hz (n = 10, p < 0.004). When applying S961 before local hyperglycemia on neurogliaform cells, the frequency of spontaneous EPSCs remained unchanged (8.7 ± 2.9 Hz versus 8.6 ± 2.2 Hz, n = 7, p = 0.47). The effect of glucose puffs to neurogliaform cells was dependent on Y kinase signalling as shown by experiments in which lavendustin (5 μM) intracellularly applied in neighboring pyramidal cells prevented the glucose-induced decrease in sEPSC frequency and amplitude (6.67 ± 5.84 Hz vs. 7.12 ± 5.76 Hz, n = 5, p = 0.78 and 12.50 ± 4.45 pA vs. 12.92 ± 3.16 pA, p = 0.44). Paired recordings of layer 2/3 pyramidal cells and postsynaptic pyramidal cells (n = 5) and fast spiking basket cells (n = 4) showed that insulin decreased the amplitude of unitary EPSCs from 7.18 ± 5.02 to 4.61 ± 3.72 pA (n = 9, p < 0.004) but the paired pulse ratio remained stationary (0.82 ± 0.34 and 0.84 ± 0.36, respectively, p = 0.97) suggesting a postsynaptic site of action. Thus, local hyperglycemia on neurogliaform cells triggered insulin receptor mediated responses in the microcircuit mimicking the effect of external insulin.

Mechanisms leading to insulin-like effects of neurogliaform cells
Following previous studies showing that the ATP-sensitive potassium (K_{ATP}) channel blocker glibenclamide promotes both insulin expression and release, we confirmed the presence of K_{ATP} channels in neurogliaform cells using protocols established for cortical interneurons. Relative to control conditions having a partially suppressed activity of K_{ATP} channels due hypoglycaema (0.5 mmol/l) in the external solution, glibenclamide (20 μM) in the bath produced a current with current-voltage characteristics of K_{ATP} channels in neurogliaform cells (n = 8) with a reversal potential (-96.6 ± 2.9 mV) close to the potassium equilibrium potential. In addition, bath-applied glibenclamide (20 μM) increased intracellular Ca^{2+} concentration detected by changes in OGB-1 fluorescence averaged in 50 s time windows right before and 100-150 s after application (n = 5, 1.6 ± 0.4% $\Delta F/F_0$, p < 0.01). Glibenclamide (20 μM) puffs to the soma of neurogliaform cells in hypoglycemia (0.5 mmol/l) decreased the frequency of spontaneous EPSCs arriving to simultaneously recorded neighbouring pyramidal cells (n = 5) and fast spiking basket cells (n = 5) from 11.3 ± 7.3 Hz to 6.1 ± 5.3 Hz and S961 (20 nmol/l) reversed the effect to 9.2 ± 6.2 Hz (n = 11, p < 0.001). When applying S961 before glibenclamide, the frequency of spontaneous EPSCs remained unchanged (8.5 ± 7.8 Hz versus 9.7 ± 10.0 Hz, n = 9, p = 0.47). Moreover, intracellular application of BAPTA (4 mmol/l) in the neurogliaform cells targeted by glibenclamide also

prevented changes in the frequency of spontaneous EPSCs (7.2 ± 2.6 Hz versus 6.8 ± 2.7 Hz, n = 9, p > 0.30) confirming that the effect of glibenclamide was Ca^{2+} dependent. Neurogliaform cells potentially target $GABA_B$ receptors but $GABA_B$ blockade with CGP35348 (40 μM) did not prevent the suppressing effect of glibenclamide on spontaneous EPSC frequencies (10.4 ± 2.8 Hz versus 8.5 ± 3.4 Hz, n = 5, p < 0.01). In line with our single cell digital PCR data showing moderate *Ins2* RNA expression, we detected no effect on spontaneous EPSC frequencies recorded in nearby pyramidal cells (n = 14) or fast spiking basket cells (n = 6) when locally puffing glibenclamide to pyramidal cells (n = 11, 9.5 ± 4.5 Hz versus 9.1 ± 3.8 Hz , p = 0.76) or fast spiking interneurons (n = 9, 7.4 ± 2.8 Hz versus 7.1 ± 2.7 Hz , p = 0.65) in hypoglycemia (0.5 mmol/l). Finally, we added glibenclamide (20 μM) to hypoglycaemic (0.5 mmol/l) external solution of neocortical brain slices for 30 minutes and detected increased insulin levels with radioimmunoassay (80.8 ± 17.5 pg/mg protein, n = 10) in slices at the end of treatment compared to controls without glibenclamide (60.4 ± 21.7 pg/mg protein, n = 10, p < 0.04). Since glibenclamide could not trigger insulin receptor mediated effects around pyramidal and fast spiking cells, a fraction of this insulin, locally synthesized in acute brain slices in response to glibenclamide, could be produced by neurogliaform interneurons. Moreover, slices incubated in ACSF containing 2.4 or 10 mmol/l glucose showed increased insulin content relative to hypoglycemia (75.4 ± 14.1 and 104.2 ± 26.9 pg/mg protein, n = 10, p < 0.05 and p < 0.01, respectively) confirming local insulin synthesis.

Functional expression of GLP-1 receptors and related molecular characteristics of identified neurogliaform cells

The experiments presented above suggest modulations of the *Ins2* gene in neurogliaform cells in response to changes in the extracellular glucose concentration, indicating that these neurons of the cerebral cortex might have partially similar molecular and functional predispositions to those of pancreatic beta cells. Thus, we used the highly sensitive and quantitative method of single-cell digital PCR to test whether genes important in beta cell function and development are expressed in neurogliaform cells of the neocortex. In particular, GLP-1 receptors promote insulin secretion on pancreatic beta cells and we tested the expression of these receptors due to potential therapeutic implications. We detected the expression of GLP-1 receptors in electrophysiologically and anatomically identified neurogliaform cells using single-cell digital PCR (n = 11) with the homeostatic gene *S18* (also known as *Rps18*) as a reference. Moreover, we compared copy numbers of *Glp1r* mRNA in neurogliaform cells (n = 5) in hypoglycemia, and found that copy numbers in hyperglycemia exceeded those in hypogycaemia by 9.6 times when normalised to copy numbers of the homeostatic *S18* gene (0.0457 ± 0.0427 and 0.0048 ± 0.0066; p < 0.008).

We next asked whether GLP-1 receptors and insulin can be co-detected in individual neurogliaform cells. Our single-cell digital PCR method allows the exact measurement of mRNA copy numbers of no more than two genes, thus we replaced the homeostatic gene *S18*

with the *Ins2* gene in our protocol so as to test GLP-1 receptor and insulin co-expression. Similar to pancreatic beta cells, neurogliaform cells co-expressed mRNA of the *Ins2* and *Glp1r* genes. Neurogliaform cells tested for co-expression in hyperglycemia (n = 5) contained higher numbers of mRNA of both *Ins2* (8.60 ± 3.97) and *Glp1r* (8.40 ± 4.47) genes, compared with neurogliaform cells in hypoglycemia (n = 5; 2.60 ± 1.34 and 0.80 ± 1.30, respectively; p < 0.037 and p < 0.016, respectively). In showing that the external glucose concentration modulates the co-expression of insulin and GLP-1 receptors in neurogliaform cells, these results confirm our earlier report on insulin expression and its glucose modulation in neurogliaform cells and corroborate the results shown above for *Glp1r* referenced to a homeostatic gene.

In order to confirm the functional expression of GLP-1 receptors, we tested the effect of GLP-1 on electrophysiologically and anatomically identified neurogliaform cells using the hyperglycaemic extracellular glucose concentration, similar to previous experiments. Measuring the current required for –90 mV holding potential in whole-cell recordings before (–228 ± 39 pA, n = 11), during (–194 ± 49 pA, n = 11) and after (–214 ± 55 pA, n = 7) bath application of GLP-1 (100 nmol/l), we detected a decrease in the holding current (p < 0.003, Wilcoxon test), which was reversible upon washout (p < 0.022). Moreover, pre-treatment with the GLP-1 receptor-specific antagonist exendin-3(9-39) (1 µmol/l) was effective in blocking the response in identified neurogliaform cells (n = 7) to GLP-1 application (–171 ± 39 pA vs – 166 ± 32 pA; p = 0.205). Furthermore, changes in the holding current in neurogliaform cells (n = 6) did not occur during the application of GLP-1 in hypoglycaemic conditions (–201 ± 59 pA vs –204 ± 58 pA; p = 0.401). Accordingly, the moderate copy numbers relative to a homeostatic gene detected by single-cell digital PCR in neurogliaform cells appear sufficient for a functional GLP-1 response in neurogliaform interneurons.

The co-expression of GLP-1 receptors and insulin in neurogliaform cells gives rise to a potentially broader molecular similarity between pancreatic beta cells and neurogliaform neurons of the cerebral cortex. Indeed, the developmental lineage for pancreatic endocrine cells and neurons has been suggested to be related. Following these ideas, our final series of experiments using single-cell digital PCR on identified neurogliaform cells (n = 5) revealed the expression of transcription factors important in beta cell development (*Pdx1*, *Isl1* and *Mafb* copy numbers relative to *S18*: 0.0755 ± 0.0395, 0.0218 ± 0.0057 and 0.0279 ± 0.0254, respectively). In addition, we detected a significantly lower normalised mRNA copy numbers of *Pdx1* and *Isl1* in hypoglycemia (n = 5; 0.0073 ± 0.0163, 0.0051 ± 0.0072; p < 0.037 and p < 0.016 vs hyperglycemia, respectively).

DISCUSSION

Insulin expression and release by interneurons of the cerebral cortex

According to a textbook method for identifying a neurotransmitter, neurogliaform cells mimicked the reversible effect of externally added insulin by releasing a substance we identified as insulin based on the same specific receptor antagonist. It remains to be established how and when peptides in general are being released from interneurons. Neuropeptide release was shown to depend on dendritic Ca^{2+} entry, but does not necessarily require somatic action potentials. Failing to drive insulin release with somatic action potentials suggests that local dendritic electrogenesis, possibly in response to focal excitatory inputs to neurogliaform dendrites might be required. Action potentials in neurogliaform cells did not decrease sEPSCs during GABA receptor and NPY receptor blockade on the neighbouring and synaptically coupled cells (data not shown). However, local variations in glucose levels in physiologically relevant concentrations or targeted glibenclamide application were capable of triggering insulin receptor mediated action of neurogliaform cells without spikes as glibenclamide (4.2 ± 1.4 mV, n = 5, p < 0.02) or glucose (4.4 ± 0.6mV, n = 8, p < 0.04) depolarized the soma of neurogliaform cells, and these functions required Ca^{2+} entry. This suggests that GABAergic cells can contribute to local insulin release in conditions when pancreatic insulin supply temporarily or permanently does not match demand, e.g. the actual extracellular glucose availability.

GLP-1 receptor expression in insulin expressing neurons

Our results provide evidence for GLP-1 receptor expression in neurons also shown to release insulin in the cerebral cortex. Hyperglycemia increases the expression of GLP-1 receptors in neurogliaform cells, suggesting that endogenous incretins and therapeutic GLP-1 receptor agonists might have effects on these neurons, similar to those on pancreatic beta cells. In addition, we detected transcription factors (*Pdx1*, *Isl1*, *Mafb*) in neurogliaform cells known to be important in beta cell development.

The crucial gene in the GLP-1–insulin interaction, *Ins2*, shows significant variations under similar experimental conditions from cell type to cell type in the cerebral cortex; therefore, identifying the interneuron type(s) involved in an analysis is essential for appropriately interpreting the results. Our combined electrophysiological, neuroanatomical and molecular techniques allowed us to monitor transcriptional changes associated with experimentally controlled alterations in extracellular glucose concentrations, and to determine the identity of each neuron included in our dataset. Previous analyses of transcriptional changes in identified neurons in response to variable glucose concentrations are scarce, but are consistent regarding the functional effectiveness of approximately ten copies of the *Ins2* and *Glp1r* genes per neuron. The expression threshold for functional GLP-1 receptor response seems to be more than two copies of *Glp1r* mRNA in low external glucose concentrations. This suggests that genes with moderate expression levels detected by microarray or next-

generation techniques and potentially interesting in insulin/incretin action are worth testing in functional experiments.

Conjoint modulation of the expression of the *Ins2* and *Glp1r* genes reported here in identified neurogliaform interneurons suggests that mechanisms classically described in the pancreas for GLP-1-induced enhancement of insulin release might also operate in the brain. Application of glibenclamide, which is known to promote insulin release from pancreatic beta cells, has been successful in triggering insulin release from neurogliaform cells. Although the direct action of endogenous incretins or other GLP-1 receptor agonists in neuronal insulin release requires further experiments, the mode of GLP-1 action and the polarity of responses might be cell-type-specific. We speculate that the outward current in response to GLP-1 in neurogliaform cells at the holding potential applied in this study supports that activation of GLP-1 receptors leads to the opening of somatic K-channels possibly linked to gamma-aminobutyric acid (GABA) type B (GABAB) receptors and, as suggested in response to GLP-1 in hypothalamic neurons, to increased presynaptic GABA release. Moreover, considering the effect of GLP-1 in enhancing synaptic and tonic inhibitory currents arriving at hippocampal pyramidal cells, and taking into account the high expression of extrasynaptic GABA type A (GABAA) receptor delta subunits found on intermediate and distal dendrites of neurogliaform cells, we cannot exclude the possibility that our results also reflect the activation of GABAA channels located on distal dendrites and detected with suboptimal space-clamp due to the relatively low input resistance of neurogliaform cells and somatically placed electrodes. Possible tonic GABAA currents induced by GLP-1 on neurogliaform cells are in line with the involvement of neurogliaform cells shown to provide synaptic and extrasynaptic inhibition, and are further supported by insulin-triggered tonic inhibition through GABAA receptors. It is not yet clear whether neurogliaform cells receive innervation from GLP-1-releasing neurons of the brainstem; however, it is possible that intestinal-derived GLP-1 or therapeutic GLP-1 receptor agonists reach the cerebral cortex through the blood–brain barrier, similar to native GLP-1, and could modulate insulin release from neurogliaform cells. The inhibition of gastric emptying is considered a potential factor leading to weight loss caused by GLP-1 receptor agonist therapy. However, an alternative mechanism might emerge when considering imaging studies suggesting that the prefrontal cortex is important in the inhibitory control of food intake in humans and human brain-slice experiments showing that neurogliaform cells provide widespread inhibition in prefrontal microcircuits. Selective involvement of GABAergic interneuron subpopulations is likely in neurodegenerative diseases. Given that GLP-1 receptor agonists promise therapeutic effectiveness against neurodegeneration in models of traumatic brain injury and Alzheimer's and Parkinson's disease, the scenario of GLP-1 receptor-mediated insulin synthesis in the brain could be extended to the therapy of these diseases.

Pancreatic beta cells and neurogliaform cells of the cerebral cortex

A related developmental lineage for pancreatic endocrine cells and neurons has been implicated, and our results on the limited number of transcription factors tested in neurogliaform cells here support this idea. *Pdx1* is central in the regulation of pancreatic development and in the differentiation of beta cells from progenitor cells. The effects of GLP-1 on beta cell proliferation and secretory function depend on crosstalk with proteins in the insulin-signalling pathway and modulation of transcription factors including pancreatic and duodenal homeobox 1 (PDX1); thus, the co-expression of *Glp1r* and *Pdx1* found in neurogliaform cells suggests potential functional homology of neurogliaform and beta cells beyond development. Along the same vein, expression of the LIM homeodomain protein ISL1 (insulin gene enhancer binding protein, islet factor 1) is known from the developing pancreas and the central nervous system. Synergistically with the basic helix-loop-helix transcription factor BETA2, ISL1 activates the insulin promoter in beta cells and promotes pancreatic islet cell proliferation, and is required for the differentiation of interneurons in the spinal cord. The role of *Mafa* and *Mafb* genes is crucial in beta cells during development (*Mafb*) and adulthood (*Mafa* in mice and *MAFA* and *MAFB* in humans) and our results in neurogliaform cells confirm the widespread expression of MAFB reported earlier in developing and differentiated neocortical interneurons. Our results suggest that insulin and GLP-1 receptor-expressing neurogliaform interneurons of the cerebral cortex partially possess the transcription toolkit known to be instrumental in the development of insulin-synthesising pancreatic beta cells.

Neuron-derived insulin-based therapy

The effect of glibenclamide in triggering neuronal release of insulin also suggests that the delivery of substances known to enhance insulin release from pancreatic beta cells to the brain might have therapeutic implications. An as-yet untested strategy for increasing insulin concentrations in key areas affected by Alzheimer's disease, such as the hippocampus and neocortex, would be to boost insulin release from neurons or neuronal progenitors expressing insulin locally. Apart from sulfonylureas, incretins might represent a promising group of molecules to be tested for several reasons. GLP-1 receptors are expressed in neurons of the hippocampus and the neocortex, and our results show the expression of GLP-1 receptors on insulin-expressing neurons. Interestingly, GLP-1 agonists have effects on tonic inhibitory GABAergic currents similar to those reported for insulin, arguing for a hypothetical contribution of GLP-1 receptor-mediated insulin release. GLP-1 is produced in the brainstem, suggesting that centrally synthesised GLP-1 could be effective within the brain via the mechanisms outlined above. However, GLP-1 produced by L-cells of the intestine crosses the blood–brain barrier and thus incretins arriving from the periphery can possibly enhance insulin release from neurons in the brain. Importantly, these peripheral incretins include GLP-1 analogues prescribed in type 2 diabetes mellitus. We suggest that the weight loss caused by GLP-1 receptor analogue-based therapy (attributed primarily to the inhibition of gastric

emptying) might have an additional synergistic component through GLP-1 receptor-mediated insulin release from neurons of the brain. Human imaging studies suggest that the prefrontal cortex is crucial in the inhibitory control of food intake and human brain-slice experiments showing that neurogliaform cells provide widespread inhibition in prefrontal microcircuits. The expression of GLP-1 receptors on insulin-releasing neurogliaform neurons of the prefrontal cortex provides mechanistic support for this process. Selective involvement of GABAergic interneuron subpopulations is likely in neurodegenerative diseases. Given that GLP-1 receptor agonists promise therapeutic effectiveness against neurodegeneration in models of Alzheimer's, Huntington's and Parkinson's diseases and a scenario of GLP-1 receptor-mediated insulin synthesis in the brain could be extended to therapy for these diseases.

The evidence for insulin synthesis in the brain raises the question of whether brain-derived insulin could be used to replace peripheral insulin in type 1 diabetes. Insulin synthesised in the brain is unlikely to cross the blood–brain barrier in the brain-to-blood direction in the quantity required for euglycaemic control of plasma glucose concentrations; intranasal insulin delivery fails to increase plasma insulin levels significantly. An alternative approach might use autologous grafts of insulin-expressing neurons or neural progenitor cells as a potential replacement for lost pancreatic beta cells. Such neuron- or neural stem cell-based therapy of diabetes is suggested by experiments with neural stem cells isolated from the adult brain functionally replacing beta cells in diabetic patients. The suggested workflow for autologous neural stem cell-based therapy for diabetes is, critically, based on the suggestion that insulin-expressing neural stem cells might find similar molecular niches for their survival and insulin-expressing ability in the brain as well as in the pancreas. Neural stem cells can be transplanted directly into the pancreas of diabetic rats, where the pancreatic niche reprograms neuronal stem cells via Wnt signalling to express insulin. Isolating neural stem cells from models of type 1 diabetes (induced by streptozotocin in rats) or type 2 diabetes (in Goto–Kakizaki rats) followed by transplantation to the pancreas of animals of the corresponding model confirmed that grafted cells survive and produce insulin for long periods (> 10 weeks) and dramatically reduce blood glucose levels. The therapeutic potential for human diabetic patients is immense because no genetic manipulation is necessary and the procedure bypasses tumorigenic pluripotent stem cells and concerns inherent to chronic immunosuppression.

SUMMARY AND CONCLUSION

The action of insulin is not restricted to peripheral organs. Insulin receptors and signal transduction pathways described in the periphery are involved in a wide array of functions in the central nervous system. It is generally accepted that insulin produced by pancreatic beta cells in physiological conditions or applied intranasally with a therapeutic purpose for mild to moderate Alzheimer's disease finds its way to neurons of the cerebral cortex. The timescale of

external insulin transport to the vicinity of neurons is relatively slow, consistent with long-term homeostatic regulation of neural networks. Recent work has overwhelmingly shown that insulin is also synthesised locally in the cerebral cortex. Neuron-derived insulin is capable of rapid modulation of synaptic and microcircuit mechanisms and is suggested to regulate on-demand energy homeostasis of neural networks. Our results provide evidence for functional expression of GLP-1 receptors in neurons known to release insulin in the cerebral cortex. Hyperglycemia increases the expression of GLP-1 receptors in neurogliaform cells suggesting that endogenous incretins and therapeutic GLP-1 receptor agonists might have effects on these neurons similar to that of pancreatic beta cells. We suggest that novel therapeutic strategies might include modulation of neural insulin production in the brain by GLP-1 agonists for counteracting diabetes, obesity and neurodegenerative diseases. Recent experiments in which lost pancreatic beta cells were replaced by autologous transplants of insulin-producing neural progenitor cells signal the immense therapeutic potential of this approach for diabetes.

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