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

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TABLE OF CONTENTS

INTRODUCTION

Discovered almost a century ago [1], insulin is widely known for its essential role in reducing blood glucose levels through the stimulation of glucose uptake by muscle, liver and adipose tissues. Pancreatic beta cells secrete insulin, a process that is crucial for determining blood glucose levels. Beta cells detect extracellular glucose concentrations through the glucose transporter GLUT2. Importantly, unlike the glucose transporters in insulin-sensitive tissues (predominantly GLUT4), GLUT2 is not regulated by insulin, thus the drop in blood glucose level due to the action of GLUT4 triggers a negative feedback mechanism suppressing insulin secretion through GLUT2 positioned on beta cells. Type 1 diabetes mellitus is a consequence of the partial or complete loss of beta cells, which leads to impaired insulin secretion and uncontrolled blood glucose levels (hypoinsulinaemic hyperglycemia). In type 2 diabetes, insulin levels are indirectly disturbed. Insufficient insulin action on receptors in insulin-sensitive tissues allows blood glucose levels to rise, leading to enhanced insulin release by beta cells. This insulin release is initially capable of restoring normal blood glucose concentrations (hyperinsulinaemic euglycemia). When beta cells reach their maximal insulin-producing capacity and are no longer able to maintain normal blood glucose, the state of hyperinsulinaemic hyperglycemia known as type 2 diabetes arises.

The function of insulin, however, is not restricted to peripheral organs. In the brain, the insulin-independent GLUT1 and GLUT3 are predominantly responsible for glucose uptake in glial cells and neurons, respectively [2]. Accordingly, brain metabolism has been considered insulin independent for decades, but the discovery of insulin receptors in the brain [3] now indicates that the cerebral functions of insulin are more complex. Insulin receptors expressed in the brain are similar to those found in the periphery [4], with the highest expression detected in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus and cerebellum [3]. Insulin receptor levels are higher in neurons than in glial cells [5]. Importantly, there are similarities in the periphery and the brain in the major signal transduction pathways linked to the insulin receptor. The phosphoinositide-3 kinase (PI3)/Akt and Ras/mitogen-activated protein kinase (MAPK) routes crucial to peripheral glucose, lipid and protein metabolism [6] are also essential in the mechanism of insulin action in the brain [4, 7].

External insulin reaches the brain

As outlined above, a normal supply of insulin in the brain appears to be crucial for neural function, including metabolism, and, consequently, dynamic or persistent alterations in insulin-dependent mechanisms could contribute to pathological processes. The sources of insulin found in the brain are not completely clear. It is generally accepted that insulin synthesised by pancreatic beta cells is delivered to the brain [8–13], but an accurate picture of this process is missing (Fig. 1) [14]. Pancreatic insulin circulating in the plasma has two ways into the interstitial fluid immediately surrounding neurons and glial cells of the brain.

The first pathway delivers relatively small amounts of plasma insulin through the choroid plexus to the cerebrospinal fluid. Plasma concentrations of insulin are an order of magnitude higher compared with those measured in the cerebrospinal fluid [15, 16]. Interestingly, this difference is increased in obesity [17], despite the higher plasma insulin concentrations in obese individuals. This process is saturable [13, 18], but it is not clear whether saturation is caused by the potential involvement of insulin receptors of the choroid plexus or by the suspected contribution of megalin, a transporter known to mediate leptin transport across the choroid plexus and to be involved in insulin transport [19] in the epithelial cells of renal tubules [20].

The second pathway takes insulin from plasma into the endothelial cells of the brain microvasculature. Based on experiments showing that aortic endothelial cells outside the brain are able to concentrate insulin [21], the second pathway is hypothesised to transport the bulk of peripheral insulin to the brain. Mechanisms of transendothelial insulin transport in the brain have not been directly studied to date, but one can speculate that a vesicular trafficking process, beginning with insulin binding to its receptor followed by the involvement of caveolae and promoted by NO signalling [21–24], could be involved according to experiments with peripheral endothelia. The two pathways join at the Virchow–Robin space surrounded by endothelial cells, astrocytic endfeet and pericytes. Peripheral insulin then passes the line of astrocytic endfeet, an effective filter and limiter of movement speed for larger molecules [25], before reaching the interstitial space around neurons and glia. Absolute insulin concentrations are difficult to measure reliably in the interstitial space of the brain, but the relative changes detected in response to food intake were independent of plasma insulin concentration [26, 27], raising the possibility that pancreas-independent local insulin synthesis occurs in the brain [10].

The speed of pancreatic insulin transport into the cerebrospinal fluid and then to the interstitial space of the brain is orders of magnitude slower than the operating speed of neural

networks estimated according to the lowest frequency of brain oscillations. Insulin levels in the cerebrospinal fluid remain below fasting levels in response to several hours of peripheral hyperinsulinaemic euglycaemic clamping [16, 18] and, moreover, fasting insulin levels in the cerebrospinal fluid (~7 pmol/l) [28] are insufficient for signal transduction through insulin receptors. Even if insulin concentrations in the cerebrospinal fluid are elevated to effective levels, it is estimated that the slow circulation of cerebrospinal fluid limits insulin delivery to the interstitial space of the brain at a rate of ~1/600 of that to skeletal muscle and at < 1/30,000 of that to the liver [14]. Alternatively, insulin might move directly from the plasma through the blood–brain barrier to the Virchow–Robin space and the interstitial fluid. Studies examining the involvement of this route measured the tissue content of radiolabelled insulin in brain regions [29], which did not allow the insulin concentration in the interstitial fluid to be determined. To date, estimations of the speed by which insulin moves across the blood–brain barrier are limited by brain microvessels binding insulin with high affinity without significant insulin degradation [30]. Nevertheless, insulin finds its way from the plasma to the immediate vicinity of neurons, but equilibration with the interstitial space in the brain is achieved at timescales consistent with long-term homeostatic regulation outside the frequency range (~0.1–200 Hz) of changes in membrane potential in neural networks.

The limited speed by which external insulin is distributed is also a factor to consider when delivering insulin to the brain intranasally [28]. This process has gained particular relevance following encouraging reports [31] and clinical trials [32] that provide evidence for cognitive improvements with daily intranasal insulin administration in patients with mild cognitive impairment or mild to moderate Alzheimer's disease. Levels of insulin are reduced in Alzheimer's disease [33, 34], and intranasally applied insulin raises concentrations in the cerebrospinal fluid within 10 min of application, with maximal levels achieved after 30 min, while plasma insulin and glucose levels remain unaffected [28]. How intranasal insulin reaches the brain remains mechanistically unclear [35], but the process can be stimulated by inhibiting protein kinase C [36]. A different 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.

Local insulin synthesis in the brain

Whether insulin is produced locally in the central nervous system is not a trivial question to answer. Initial studies on the subject suggested that immunoreactive insulin is present in the rat brain in concentrations 10 to 100 times higher than in the plasma [37], but this was

challenged by subsequent findings [38], leading to the conclusion that 'little or no insulin is produced in the brain' [39]. The heart of the problem is that experiments must be able to differentiate between insulin of pancreatic origin and insulin synthesised locally. Anti-insulin antibodies recognise the same epitopes on pancreas- and brain-derived insulin, thus methods such as anti-insulin immunocytochemistry or radioimmunoassay capable of detecting insulin in small amounts are not adequate. Increasing the resolution to allow cellular or subcellular localisation of anti-insulin immunoreaction signals is of little help as receptor-bound and internalised insulin pools are degraded or recycled to the plasma membrane at intracellular locations that potentially overlap with those of locally synthesised peptide [40]. Immunoreactions detecting peptides involved in the different steps of insulin synthesis might overcome these limitations. Indeed, C-peptide, an integral part of proinsulin, was localised to the same neurons as insulin [41–43], and proinsulin-like immunoreactivity was documented in samples derived from the central nervous system [44], arguing for local synthesis in the brain.

Another strategy for detecting insulin production in the brain is to search for the mRNA of insulin-coding genes: *Ins1* and *Ins2* in mice; *Ins2* in rat; and *INS* in humans. A pioneering RT-PCR study detected widespread *Ins2* expression in the rat brain throughout development [45] and the same laboratory confirmed it in rabbit, showing *Ins2* expression in neurons of the hippocampus and olfactory bulb [46]. More recently, hippocampal granule cells from adult rats and neuronal progenitor cells derived from the hippocampus or olfactory bulb were also found to express insulin mRNAs [43]. Furthermore, expression of *Ins2*, but not *Ins1*, was found in cortical and subcortical areas of the mouse brain [47, 48] and *INS* mRNA expression characterised human samples of the hippocampus, amygdala and temporal lobe in addition to the olfactory bulb, cerebellar and pontine regions [48]. Recently developed methods to precisely quantify mRNA copy numbers in single neurons [49] have provided an effective tool for determining *Ins2* levels in several cell types and astrocytes in the cerebral cortex.

Glucagon-like peptide 1 and the brain

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, suppression of pancreatic glucagon secretion and enhancement of insulin release in the pancreas [50]. 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 depolarisation and Ca^{2+} influx, and Ca^{2+}-dependent release of Ca^{2+} from intracellular Ca^{2+} stores, resulting in Ca^{2+}-dependent insulin secretion [50]. It is of high clinical importance that GLP-1 reduces the concentrations of blood glucose only postprandially, when blood glucose levels exceed fasting concentrations [51]. Such glucose-dependent action means that intravenously administered GLP-1 does not result in hypoglycemia and, consequently, the current treatment of type 2 diabetes mellitus includes GLP-1 receptor agonists as therapeutic agents.

Outside of the pancreas, circulating GLP-1 has additional targets that are linked to insulin synthesis. Native GLP-1 crosses the blood–brain barrier [50, 52] and, thus, it is possible for incretins arriving from the periphery, including GLP-1 produced by intestinal L cells and GLP-1 analogues prescribed in type 2 diabetes mellitus [53], to act on neurons of the hippocampus and the neocortex, which are known to express GLP-1 receptors [54, 55]. 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 [56, 57]. On the other hand, accumulating evidence based on experiments performed mostly in rats shows that insulin is synthesised by neurons of the cerebral cortex [14, 43, 46, 58].

Insulin delivered intranasally to the brain is therapeutically promising against mild cognitive impairment and Alzheimer's disease [32], and the GLP-1 analogues used in diabetes treatment have preventive effects in the early stage of Alzheimer's disease development [59, 60], Parkinson's disease [59, 61] and traumatic brain injury [62, 63]. We suggest that novel therapeutic strategies might include modulation of neural insulin production in the brain by GLP-1 agonists in order to counteract diabetes, obesity and neurodegenerative diseases. Therefore, it is of potential importance to determine whether GLP-1 of intestinal or neural origin or therapeutically applied GLP-1 receptor agonists find targets on neurons capable of insulin production. In the current study, we tested whether interneurons of the rat neocortex have the molecular components to be involved in GLP-1 signalling.

MATERIALS AND METHODS

Animals

All procedures were performed with the approval of the University of Szeged (no. I-74-8/2016) and in accordance with the *Guide for the Care and Use of Laboratory Animals* (2011) (http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf).

Male Wistar rats (postnatal day 22-35, 190–220 g; Charles River, Isaszeg, Hungary) were kept in individually ventilated cages (0.25 m^2) with biological bedding and ad libitum dry food and water.

Electrophysiology and imaging

Animals were anaesthetized by intraperitoneal injection of ketamine (30 mg/kg) and xylazine (10 mg/kg) and, following checking of the cessation of pain reflexes and decapitation, coronal slices (350 μm) were prepared from the somatosensory cortex. Animals used for this study also provided brain slices for other projects performed in parallel in the laboratory. Slice preparation and recordings were performed as previously described [49]. Recordings were obtained at approximately 36 °C from cells visualised in layers I–III by infrared differential interference contrast videomicroscopy, at depths 60–130 μm from the surface of the slice. Micropipettes (5–7 MΩ) 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, Life Technologies, Budapest, Hungary) 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, Tocris, Bristol, UK) was applied for 4 h prior to recording sessions under hyperglycaemic (10 mmol/l glucose) conditions. Voltage clamp protocols were applied according to [64]. Detection of spontaneous EPSCs were performed with NeuroMatic functions for Igor Pro (Wavemetrics). In our low chloride recording conditions, reversal potential of unitary inhibitory postsynaptic potential was -73.3 ± 3mV thus, separation of GABAergic currents was based on polarity. After Gaussian filtering, EPSC events were detected with the threshold detection algorithm as described by Kudoh and Taguchi (2002) [65] and events were reviewed after automatic detection. Threshold was set to 3 pA, onset time limit was set to 2 ms, which defines the maximum interval from the baseline to the deflection reaches the threshold. Peak time limit was set to 3 ms. For imaging, neurogliaform cells were filled with 10 μM Alexa594 and 120 μM OGB-1 (Invitrogen) added

to the ATP free intracellular solution with the application of the hypoglycaemic extracellular solution and detection of signals was performed with a Revolution XD system and IQ Software (Andor). Data are presented as mean ± S.D. throughout, n values refer to the number of neurons, statistical test are defined for each paradigm. Access resistance was monitored with –10 mV voltage steps in between experimental epochs and neurons were excluded from data analysis if access resistance exceeded 35 MΩ. Signals were filtered at 8 kHz, digitised at 16 kHz and analysed with PULSE software (HEKA, Lambrecht/Pfalz, Germany). Traces shown are single sweeps for firing patterns.

Histology and reconstruction of neurons

Following electrophysiological recordings, slices were immersed in fixative containing 4% (wt/vol.) paraformaldehyde, 15% (vol./vol.) saturated picric acid and 1.25% (vol./vol.) glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4; Sigma-Aldrich, Saint Louis, MO, USA), at 4 °C for at least 12 h. After several washes with 0.1 mol/l phosphate buffer, slices were frozen in liquid nitrogen and then thawed in 0.1 mol/l phosphate buffer, embedded in 10% (wt/vol.) gelatine and further sectioned into 60 μm slices. Sections were incubated in a solution of conjugated avidin–biotin horseradish peroxidase (1:100; Vector Labs, Burlingame, CA, USA) in Tris-buffered saline (pH 7.4) at 4 °C overnight. The enzyme reaction was revealed by 3′3-diaminobenzidine tetrahydrochloride (0.05% wt/vol.) as the chromogen and 0.01% (vol./vol.) H_2O_2 as the oxidant. Sections were post-fixed with 1% OsO_4 (wt/vol.) in 0.1 mol/l phosphate buffer. After several washes in distilled water, sections were stained in 1% (wt/vol.) uranyl acetate and dehydrated using an ascending series of ethanol. Sections were infiltrated with epoxy resin (Durcupan; Sigma-Aldrich) overnight and embedded on glass slides. Three-dimensional light microscopic reconstructions were carried out using the Neurolucida system (MicroBrightField, Williston, VT, USA) 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 by applying a gentle negative pressure while maintaining a tight seal [49]. The pipettes were then delicately removed to allow outside-out patch formation and the content of the pipettes (~1.5 μl) was expelled into a low-adsorption test tube (Axygen, Corning, NY, USA) containing 0.5 μl SingleCellProtect (Avidin, Szeged, Hungary) 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 stored or

immediately used for reverse transcription. Reverse transcription of the harvested cytoplasm was carried out in two steps. The first step 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 (Life Technologies) and 100 U reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA, USA). 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. The reverse transcription reaction mix was stored at –20 °C until PCR amplification.

For single cell QRT-PCR, reactions were carried out after preamplification of cDNA in a total volume of 20µl (5µl RT product, 1µl of Taqman primer (*Rps18*: Rn01428913_gH; *Ins2*: Rn01774648_g1), 10µl TaqMan PreAmp Master Mix (Life Technologies) and 4.5µl nuclease-free water) in MyGenie 32 Thermal Block (Bioneer) using protocols as described [49]. We repeated QRT-PCRs (traditional and digital) amplifying both the control gene *Rps18* and *Ins2* without reverse transcriptase reaction and found no amplification and no PCR products meaning that possible genomic DNA amplification background under our conditions was negligible. To further eliminate the possibility of amplifying genomic DNA, we tried to amplify the read through of *Ins2-Igf2* mRNA transcript and also multiple introns of the *Ins2* as well as intergenic region at the *Ifngr1* gene locus on chromosome 1 and neither of these primer sets gave positive results during QRT-PCR.

For digital PCR analysis, half of the reverse transcription reaction mixture (2.5 µl), 2 µl TaqMan reagent (Life Technologies), 10 µl OpenArray Digital PCR Master Mix (Life Technologies) 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 as previously described [49]. For our digital PCR protocol for amplification, reactions with C_t confidence values < 100 were considered not different from background noise and were excluded from the dataset. In addition, reactions with C_t values < 23 were considered primer dimers and those > 33 were considered false reactions originated from non-template amplifications and were excluded from the dataset.

Sequencing

We sequenced 4 individual PCR products from 4 individual neurogliaform cells using capillary electrophoresis sequencing on 3500 Genetic Analyzer (Life Technologies). After purification of the products, we used 4 different sequencing primers using the following

primer sequences: FOR1: 5’- 5’-cccatgtcccgccgcg-3’ (16) FOR2: 5’-gtggaggacccacaagtg-3’ (18) REV1: 5’-tgccaaggtctgaaggtcac-3’ (20) REV2: 5’-ttctgccgggccacctcc-3’ (18).

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 or the Kruskal–Wallis or Wilcoxon tests. The Mann–Whitney U test was used for electrophysiological measurements with SPSS software (IBM, Armonk, NY, USA). Differences were accepted as significant if $p < 0.05$. Randomisation of samples and blinding was not carried out for outcome assessment.

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 (Fig. 2A), express the mRNA of the *Ins2* gene encoding preproinsulin in the rat [66]. After electrophysiological and anatomical identification of cell types based on characterization of membrane and firing properties (Fig. 2A), 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 (Fig. 2C). To exclude any possibilities in amplifying DNA fragments other than *Ins2*, we sequenced four individual PCR products from n = 4 neurogliaform cells and found 100% match (84/84; 47/47; 42/42, 31/31) to the ref|NM_019130.2| Rattus norvegicus insulin 2 (*Ins2*) mRNA sequence. 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 [49] (Fig. 2C). 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, $p < 0.002$, Kruskal-Wallis test). 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) [67] and this decreased the number of *Ins2* mRNA molecules in single neurogliaform cells to 14 ± 3 (n = 5, $p < 0.008$, Kruskal-Wallis test) 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 [66] 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 either of the three neuron types we tested (Fig. 2C). 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 (Fig. 2C).

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 [37, 68]. Insulin reversibly decreased the frequency (from 13.0 ± 9.4 Hz to 7.3 ± 5.5 Hz , n = 16, $p < 0.001$, Wilcoxon-test, Fig. 3A) 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) [69] 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) (Fig. 3B). 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, $p < 0.004$, Wilcoxon-test). 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 [70] 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; Fig. 4C). 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; Fig. 3D) 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 [71], we confirmed the presence of K_{ATP} channels in neurogliaform cells using protocols established for cortical interneurons [72]. Relative to control conditions having a partially suppressed activity of K_{ATP} channels due hypoglycaema (0.5 mmol/l) in the external solution [71], 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 (Fig. 4A). 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% $\Delta F/F_0$, $p < 0.01$, Wilcoxon-test, Fig. 4B). 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; Fig. 4C) 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, $p < 0.001$, Wilcoxon-test, Fig. 4C). 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, Fig. 4C). 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, $p > 0.30$, Fig. 4C) confirming that the effect of glibenclamide was Ca^{2+} dependent. Neurogliaform cells potentially target $GABA_B$ receptors [73, 74] 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, Fig. 4C) 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, $p < 0.04$, Mann–Whitney U test, Fig. 4D). 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.

Morphophysiological characteristics of identified neurogliaform cells in hyper- and hypoglycemia

In the next series of experiments, we searched for interneurons showing characteristics of neurogliaform cells in layers 1–3 using the whole-cell patch-clamp mode of brain slices prepared from the somatosensory cortex of male rats (P28-35). Acute brain slices were maintained in artificial cerebrospinal fluid containing glucose either in the concentration used as standard for in vitro brain slices (10 mmol/l, hyperglycemia) or at a concentration of 0.5 mmol/l, determined as the hypoglycaemic external glucose concentration in the rat brain [67]. Differential interference contrast microscopy was used to select putative interneurons based on perisomatic morphology, and the identity of neurogliaform cells was first confirmed according to their late-spiking firing characteristics in response to depolarising current pulses (Fig. 5A-D). The use of biocytin in the patch-clamp recording pipettes allowed us to recover the morphology of the recorded cells, and the identity of each neurogliaform cell included in this study (n = 87) was additionally confirmed by post hoc anatomical assessment of axonal morphology (Fig. 5A-D). Quantitative morphological analysis was beyond the scope of this study; however, an extremely dense axonal arborisation with small and frequently spaced boutons, the hallmark of neurogliaform cells [73], could be readily observed in samples recorded and biocytin filled in hyper- and hypoglycaemic conditions. In addition, no somatodendritic differences seemed to emerge between the two experimental groups and no morphological features considered pathological were observed.

We compared the basic electrophysiological properties of neurogliaform cells recorded in 10 mmol/l (n = 10) and 0.5 mmol/l (n = 10) external glucose concentrations (Fig. 5E-M). Hyper- vs hypoglycaemic conditions had no significant effect on the resting membrane potential (–67.08 ± 2.66 mV vs –69.52 ± 5.28 mV; p = 0.35, Mann–Whitney U test), input resistance (113.15 ± 34.03 mΩ vs 85.82 ± 17.91 mΩ; p = 0.079), amplitude of action potentials (75.76 ± 6.84 mV vs 72.30 ± 6.1 mV; p = 0.39), interspike interval (0.103 ± 0.030 ms vs 0.078 ± 0.033 ms; p = 0.094), half width of action potentials (0.82 ± 0.27 ms vs 0.56 ± 0.12 ms; p = 0.079), action potential threshold (–30.89 ± 1.77 mV vs –30.9 ± 4.8 Mv; p =

0.71) or action potential accommodation (131.86 ± 56.51% vs 155.25 ± 43.51%; p = 0.15). However, significant differences emerged between neurogliaform cells recorded in 10 mmol/l vs 0.5 mmol/l glucose when measuring accommodation in the amplitudes of successive action potentials (90.04 ± 6.18% vs 81.44 ± 6.47%; p = 0.010; Fig. 5L) and accommodation in the half widths of successive action potentials (129.73 ± 10.75% vs 145.53 ± 12.96%; p = 0.019; Fig. 5M). Thus, apart from minor differences possibly due to the relatively lower metabolic supply in hypoglycemia slightly affecting the amplitude and duration of action potentials during sustained activity, electrophysiological and morphological features of neurogliaform cells appeared stable in our experimental conditions.

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 [49] 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, Fig. 6A) 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, Mann–Whitney U test; Fig. 6A).

We next asked whether GLP-1 receptors and insulin can be co-detected in individual neurogliaform cells (Fig. 6B). 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, Mann–Whitney U test; Fig. 6B). In showing that the external glucose concentration modulates the co-expression of insulin and GLP-1 receptors in neurogliaform cells, these results confirm our experiments presented 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 (Fig. 6C-E), similar to previous experiments [54]. 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) [54], we detected a decrease in the holding current ($p < 0.003$, Wilcoxon test), which was reversible upon washout ($p < 0.022$; Fig. 6C-E). 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; $p = 0.205$, Wilcoxon test; Fig. 6F). 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; $p = 0.401$, Wilcoxon test; Fig. 6G). 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 [75]. 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; Fig. 7) [76, 77]. 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, Mann–Whitney U test; Fig. 7).

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 [78]. 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.

Insulin action in the brain

Given the parallel expression of insulin-activated PI3/Akt and Ras/MAPK pathways in the periphery and the brain, a logical question is whether insulin-mediated effects on neurons involve metabolic regulation. Insulin receptors and the insulin-sensitive glucose transporter GLUT4 have been shown to co-localise on neurons [79], and cellular mechanisms supporting neuronal metabolic functions of insulin involve translocation of GLUT4 to the cell surface [80], so providing an alternative to insulin-independent glucose uptake through GLUT3. The insulin dependence of brain metabolism at the neural network level has also been revisited by a number of human in vivo studies [81–83], which suggest that insulin can effectively stimulate glucose uptake in the medial temporal lobe, especially during periods of intensive neuronal activity [84]. Moreover, a rapid increase in local glycolysis following insulin administration was found in the hippocampus and was suppressed in type 2 diabetes [85]. However, glucose levels drop significantly in the extracellular space during intense cognitive

operations and might not be rate-limiting for the metabolic supply of microcircuits because of astrocytic metabolic routes [86]. A consensus on neuronal glucose metabolism in relation to insulin is of particular interest for two reasons. First, neuronal ensembles in the hippocampus and the neocortex are engaged in increased high-frequency epochs of firing during memory formation or cognitive tasks and the extra metabolic demand created by intensive action potential generation might be met by alternative routes of supply. An unorthodox pathway of glucose supply during cognitive surges in energy demand was suggested by Emmanuel et al [84], who proposed that non-insulin-dependent GLUT1 and GLUT3 transport is sufficient for resting brain activity, while sustained cognitive activity induces the addition of insulin-signalled GLUT4 transport. Second, unlike in other organs, glucose is central for the energy metabolism of the brain and temporary or sustained changes in glucose supply could be crucial in differentiating the normal and pathological functions of neural circuits. Cognitive deficits are associated with insulin resistance and diabetes [87, 88] and impaired insulin-dependent mechanisms for glucose uptake during tasks requiring extra supply might lead to deficient energy metabolism [84]. Along the same vein, 'type 3 diabetes' was suggested as an alternative term for Alzheimer's disease [34], based on observations showing reduced insulin expression and signalling mechanisms in the sporadic form of the disease [89].

In addition to the involvement in neuronal metabolism, the classic PI3/Akt and Ras/MAPK insulin-activated pathways are also important in neuron-specific cellular functions, such as the development of neuronal dendritic arbors [90] and the maintenance of excitatory synapses [91, 92]. These functions are crucial in regulating cellular processes of learning and memory and lead to long-term potentiation (LTP) or long-term depression (LTD) of synaptic efficacy through the removal of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors from postsynaptic specialisations for LTD [91, 92] and through shifting the stimulation frequency–response function for LTP [93]. Corroborating these results, a deficit of LTP is observed in models of type 1 diabetes, but results from experiments using type 2 diabetes model systems are not unequivocal [80]. Insulin is instrumental in modulating inhibitory mechanisms by moving additional γ-aminobutyric acid (GABA) receptors to inhibitory synapses [94] and extrasynaptic regions of the plasma membrane [95], providing mechanisms synergistic with the LTD induction detailed above for shifting the balance away from excitation in neural networks.

Neurogliaform interneurons, capable of releasing insulin in cortical microcircuits, are also GABAergic [96] and use GABA for volume transmission of widespread inhibition [73], thus these cells are ideally suited to synchronise the local actions of insulin and GABA. It is

not yet known which combination of neural afferents elicit insulin release from neurogliaform cells. However, it is reasonable to assume that strong excitatory inputs might contribute to the intracellular Ca^{2+} accumulation required for peptide release. One can speculate that insulin release could be synchronised to elevated overall activity in networks around neurogliaform neurons. This way transient local energy demand could be met by insulin release-driven additional glucose transport through insulin-dependent GLUT4, as suggested for epochs of intense hippocampal or cortical activity during cognitive processing [84]. At the same time, the overall excitation-suppressing activity of insulin released from neurogliaform cells is likely to be coupled with the synchronous release and inhibitory action of GABA from neurogliaform cells, which might curtail energy demand.

At the neuronal network level, insulin is an effective neuromodulatory peptide with an array of effects including control of food intake and body weight, regulation of the reproductive or hypothalamic–pituitary–gonadal axis, influencing neuronal survival and modulation of memory and cognitive processes [9–12]. Apart from the classic cellular signalling pathways, insulin also acts through alternative mechanisms in neurons. Insulin directly opens neuronal ATP-gated potassium (K_{ATP}) channels, resulting in suppressed firing [97], and this signalling route was suggested as a mechanism for modulating hippocampal memory performance [98]. Moreover, the action of insulin on neurons might interact with glucocorticoid signalling through opposite modulation of hippocampal GLUT4 [99] and by additional mechanisms expertly reviewed previously [80, 86].

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. Our analyses of transcriptional changes in identified neurons in response to variable glucose concentrations 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 sequencing techniques [100–104] 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 [54]. 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 [105], to increased presynaptic GABA release. Moreover, considering the effect of GLP-1 in enhancing synaptic and tonic inhibitory currents arriving at hippocampal pyramidal cells [106], 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 [73], 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 [73, 107]. 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 [73], and are further supported by insulin-triggered tonic inhibition through GABAA receptors [95]. It is not yet clear whether neurogliaform cells receive innervation from GLP-1-releasing neurons of the brainstem [54, 56, 57]; 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 [52, 53], and could modulate insulin release from neurogliaform cells.

Pancreatic beta cells and neurogliaform cells of the cerebral cortex

A related developmental lineage for pancreatic endocrine cells and neurons has been implicated [75], 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 [76]. 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) [76]; 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 [108, 109]. Synergistically with the basic helix-loop-helix transcription factor BETA2, ISL1 activates the insulin promoter in beta cells [110] and promotes pancreatic islet cell proliferation [111], and is required for the differentiation of interneurons in the spinal cord [112]. 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) [113] and our results in neurogliaform cells confirm the widespread expression of MAFB reported earlier in developing and differentiated neocortical interneurons [114]. 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 [55] 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 [95, 106]. GLP-1 is produced in the brainstem [50], 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 [50] 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 [51]) 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 [115–117] and human brain-slice experiments showing that neurogliaform cells provide widespread inhibition in prefrontal microcircuits [118]. 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 [119]. Given that GLP-1 receptor agonists promise therapeutic effectiveness against neurodegeneration in models of Alzheimer's, Huntington's and Parkinson's diseases [120, 121] 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 [11]; intranasal insulin delivery fails to increase plasma insulin levels significantly [28]. 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 following the spectacular results of Kuwabara et al [43] that raised the possibility of neural stem cells isolated from the adult brain functionally replacing beta cells in diabetic patients [122, 123]. The suggested workflow for autologous neural stem cell-based therapy for diabetes is, critically, based on the suggestion that insulin-expressing neural stem cells of the dentate gyrus or the olfactory bulb might find similar molecular niches for their survival and insulin-expressing ability in the brain as well as in the pancreas, and that this might involve Wnt3 and neurogenic differentiation factor 1 (NeuroD) [43, 123]. Neural stem cells can be isolated from rodent and human olfactory bulbs [43, 124] and rat cells can be transplanted directly into the pancreas of

diabetic rats [43], 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 [43]. 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 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 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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FIGURES AND LEGENDS

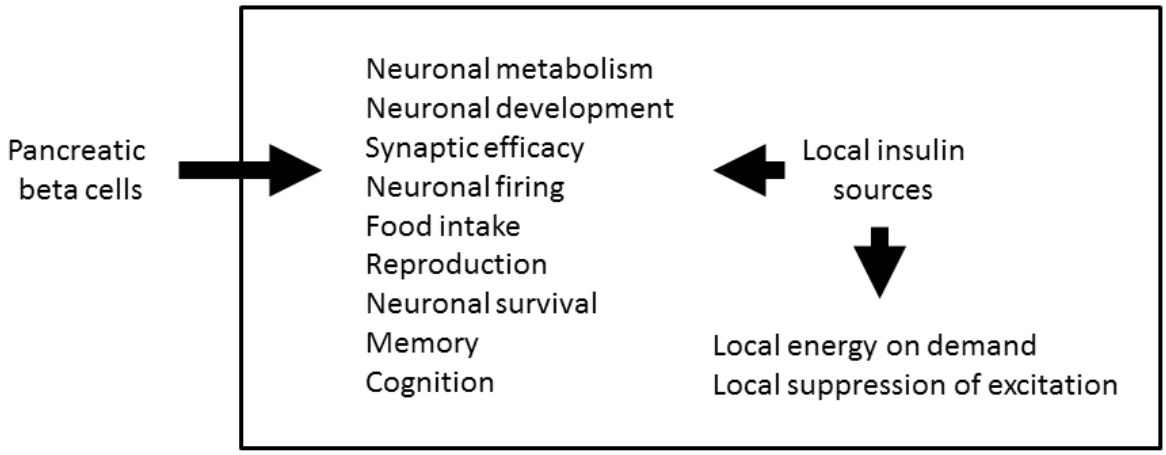

Figure 1. Potential central actions of insulin. The box represents the blood–brain barrier.

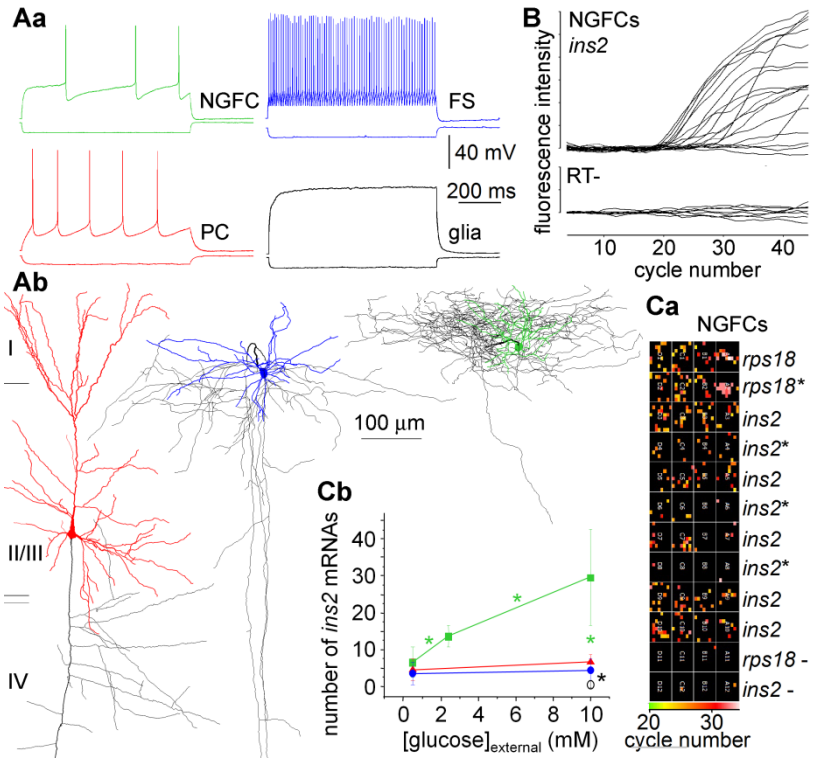

Figure 2. Cell type dependent insulin mRNA expression in the cerebral cortex. (**Aa**) Typical responses of a neurogliaform cell (NGFC), pyramidal cell (PC), fast spiking cell (FS) and a glial cell (Glia) to hyperpolarizing and depolarizing current pulses recorded before harvesting their cytoplasm. (**Ab**) Anatomical reconstructions of the cells shown in Aa, colors of dendrites correspond to firing patterns in Aa, axons are black. (**B**) Single cell quantitative RT-PCR results of the *Ins2* gene in neurogliaform cells (top) with negative controls (RT-, bottom). (**Ca**) Representative raw data from a single cell digital PCR array showing the *Rps18* house-

keeping gene and *Ins2* under high (10 mmol/l) or low (0.5 mmol/l; asterisks) extracellular glucose concentrations in neurogliaform cells (NGFCs). Results of negative controls for both genes (*Rps18* - and *Ins2* -; RT-) are also shown. Color coding indicates the cycle number at which reactions crossed threshold for detecting *Ins2* or *Rps18* in each nanowell. (**Cb**) The number of *Ins2* mRNAs in neurogliaform cells (green) increased significantly (asterisks) together with the extracellular glucose concentration from hypoglycaemic to euglycaemic and further to hypergylcaemic extracellular conditions. In contrast, the number of *Ins2* mRNAs remained stable, thus significantly lower in pyramidal (red) and fast spiking (blue) cells regardless of changes in glucose concentration. Copy numbers of *Ins2* in glial cells were smaller compared to other cell types tested.

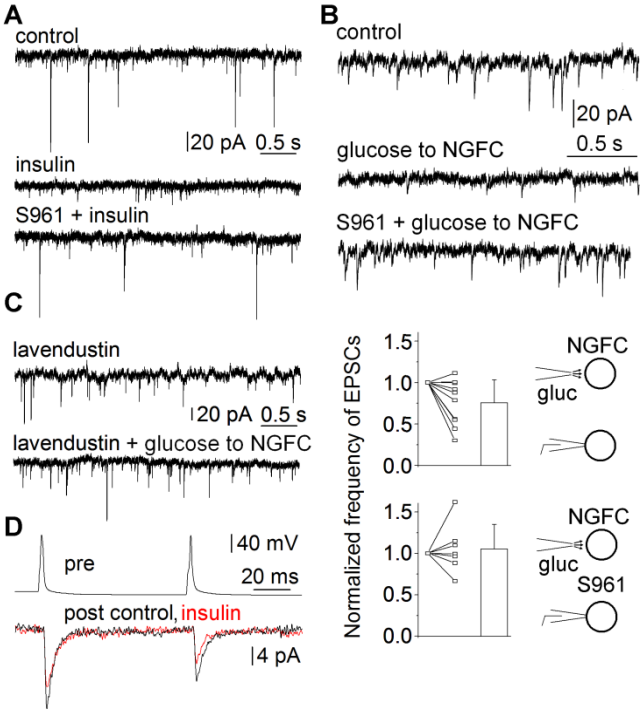

Figure 3. Neurogliaform cells mimic the action of external insulin via insulin receptors. (**A**) The frequency of spontaneous EPSCs arriving to neocortical neurons was decreased in response to physiological concentrations of insulin (100 nmol/l) and the specific insulin receptor antagonist S961 (20 nmol/l) reversed the effect. (**B**) Mimicking the effect of insulin shown in (a), local application of hyperglycaemic extracellular solution containing 10 mmol/l glucose (gluc) to neurogliaform cells (NGFCs) identified electrophysiologically and anatomically decreased the frequency of spontaneous EPSCs arriving to neighbouring neurons recorded in hypoglycaemic (0.5 mmol/l) conditions and S961 (20 nmol/l) also reversed the effect (top, individual experiment, bottom, population data). (**C**) The effect of

hyperglycaemic puffs to neurogliaform cells on spontaneous EPSCs in neighbouring pyramidal cells was blocked by lavendustin (5μM) intracellularly applied in the pyramidal cells. (**D**) Insulin suppresses the amplitude of unitary EPSCs between layer 2/3 pyramidal cells while leaving the paired pulse ratio unchanged.

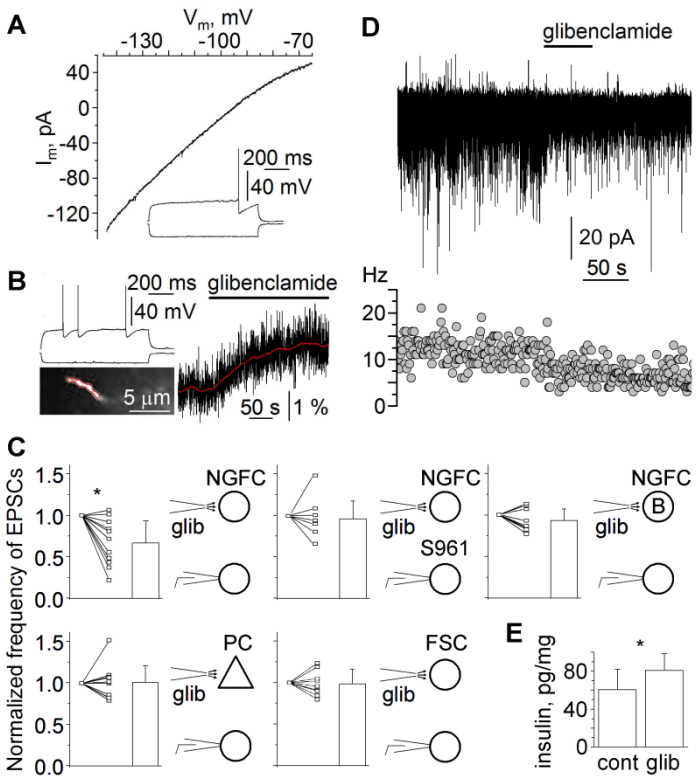

Figure 4. K_{ATP} channels and intracellular Ca^{2+} contribute to insulin receptor mediated action of neurogliaform cells. (**A**) Current-voltage (I-V) relationship of the glibenclamide sensitive component of currents recorded in a late spiking (inset) neurogliaform cell in response to ramping membrane potential from -145 to -65mV with and without glibenclamide (20μM). (**B**) A neurogliaform cell identified by its firing pattern (top) responds to bath applied glibenclamide (20μM) with an increase of the intracellular Ca^{2+} concentration detected by changes in OGB-1 fluorescence (right) in one of the dendrites (bottom, red border indicates imaged area). (**C**) Whole cell recordings performed in hypoglycemia (0.5 mmol/l) show that glibenclamide (20μM) delivered to neurogliaform cells (NGFC) significantly decreased the frequency of EPSCs in simultaneously monitored neighbouring neurons and this effect was blocked by the insulin receptor blocker S961 applied extracellularly and also by intracellular application of BAPTA (B) in the neurogliaform cell. In contrast, glibenclamide applied to pyramidal cells (PC) and fast spiking cells (FSC) caused no significant changes in the frequency of EPSCs in neighbouring neurons. (**D**) Time course of sEPSC amplitude (top) and

frequency (bottom) changes in a representative experiment as shown in C, top left panel. (**E**) Radioimmunoassay measurements in homogenates of neocortical slices showed significantly increased insulin levels relative to hypoglycemia during glibenclamide (glib) application and normo- or hyperglycemia.

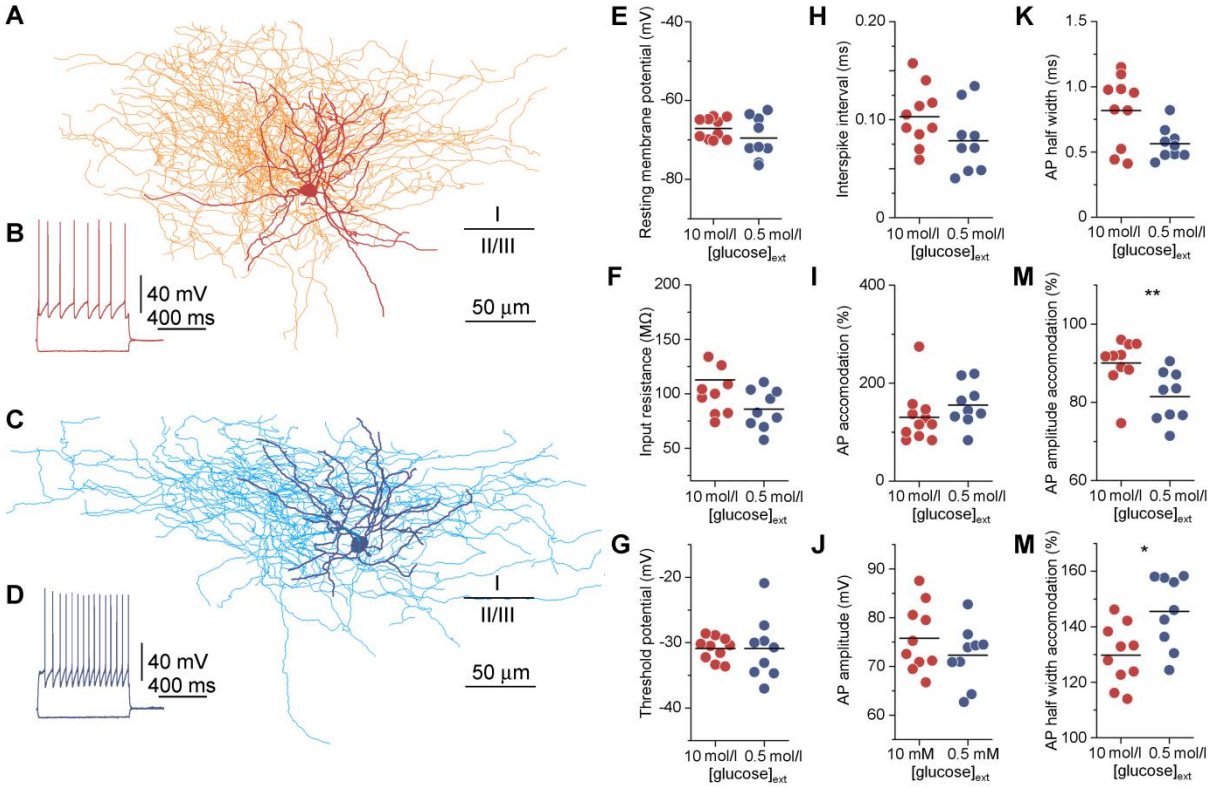

Figure 5. Anatomical and electrophysiological features of neurogliaform cells harvested for transcriptomic analysis. (**A**–**D**) Three-dimensional reconstructions (**A**,**C**) and somatically recorded firing patterns (**B**,**C**) of neurogliaform cells that were recorded by whole-cell patch-clamp and subsequently harvested for molecular analysis in brain slices of the rat frontal cortex. The neurogliaform cell in (**A**) was recorded as having hyperglycaemic external glucose concentration (10 mmol/l) in the artificial cerebrospinal fluid. This is standard in brain-slice experiments. Red, soma and dendrites; orange, axons. The neurogliaform cell in (**c)**was recorded in artificial cerebrospinal fluid containing 0.5 mmol/l glucose (similar to that reported during hypoglycemia in the brain [67]) blue, soma and dendrites; light blue, axons). Blue, soma and dendrites; light blue, axons. (**E**–**M**) Electrophysiological variables of neurogliaform cells measured in hyperglycemia (10 mmol/l external glucose [$glucose_{ext}$]; red) and hypoglycemia (0.5 mmol/l $glucose_{ext}$; blue). Basic membrane variables were not significantly different; however, the amplitude of action potentials (APs) decreased during a train at a significantly higher rate in neurogliaform cells recorded in hypoglycemia (**L**, $p <$

0.010) and the half width of their successive action potentials increased more rapidly compared with neurogliaform cells measured in hyperglycemia (**M**, $p < 0.019$). *$p < 0.05$.

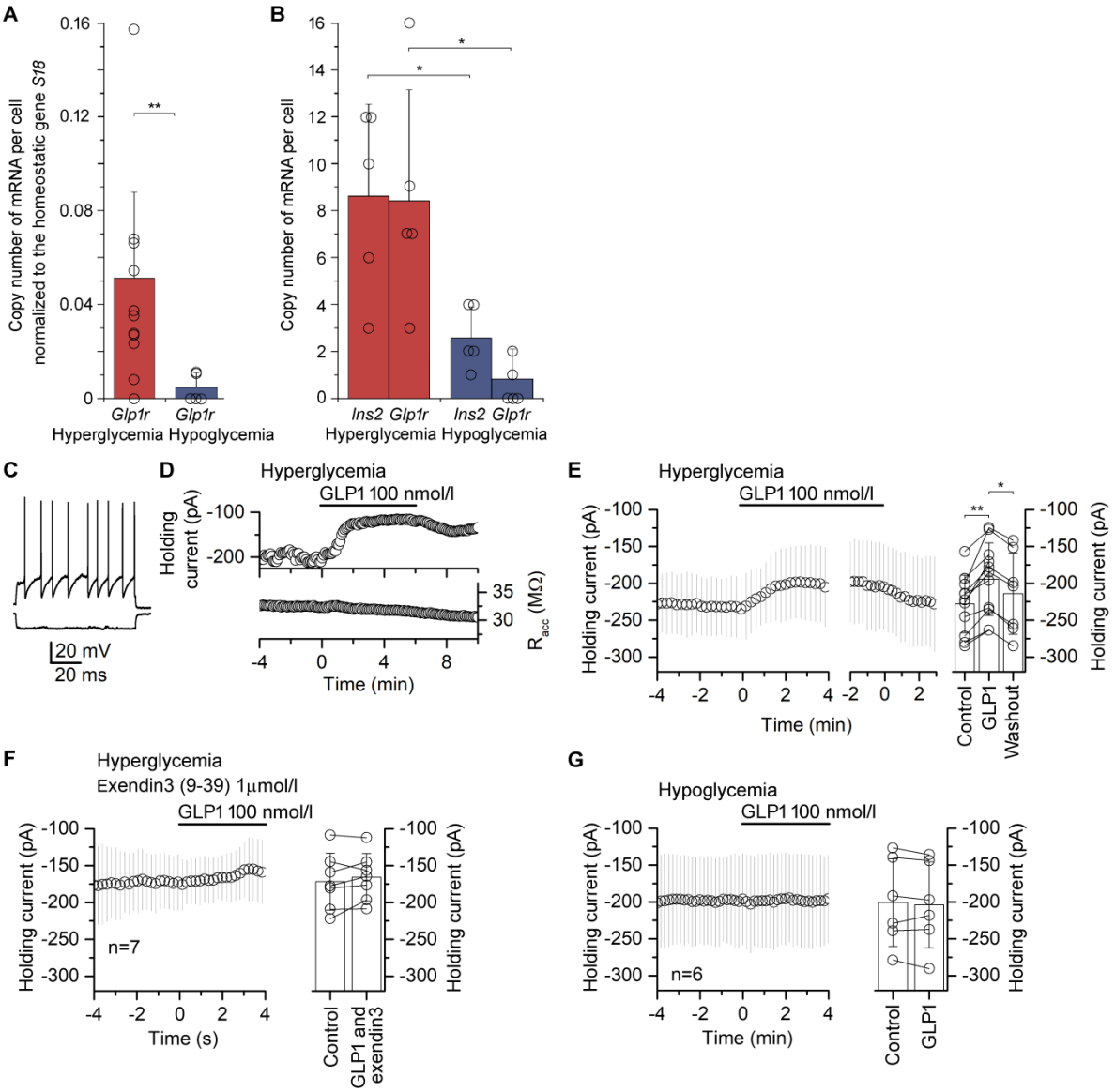

Figure 6. Functional expression of GLP-1 receptors in neurogliaform cells. (**A**) Expression of GLP-1 receptors in electrophysiologically and anatomically identified neurogliaform cells detected by single-cell digital PCR. Copy numbers of *Glp1r* mRNA were higher in hyperglycemia compared with hypoglycemia when normalised to copy numbers of the homeostatic *S18* gene ($p < 0.008$). (**B**) Co-expression of GLP-1 receptors and insulin in individual neurogliaform cells. Neurogliaform cells tested for co-expression under hyperglycaemic conditions contained higher numbers of mRNA of both *Ins2* and *Glp1r* compared with neurogliaform cells under hypoglycaemic conditions ($p < 0.037$ and $p < 0.016$, respectively). (**C**–**G**) Functional expression of GLP-1 on neurogliaform cells. (**C**,**D**) Example

of an experiment testing the effect of GLP-1 (100 nmol/l) on identified neurogliaform cells. (**C**) Firing pattern of a neurogliaform cell recorded as having a hyperglycaemic external glucose concentration (10 mmol/l) in the artificial cerebrospinal fluid. (**D**) The application of 100 nmol/l GLP-1 to the same neurogliaform cell as in (**C**) altered the current required for a –90 mV holding potential. (**E**) Population data confirmed a decrease in the holding current ($p < 0.003$) with GLP-1 treatment, which was reversible upon washout ($p < 0.022$). Four out of 11 experiments were terminated before washout because of unstable access resistance. (**F**) Pre-treatment with the GLP-1 receptor-specific antagonist exendin-3(9-39) (1 µmol/l) blocked the response to GLP-1 application in identified neurogliaform cells ($n = 7$). (**G**) Changes in the holding current in neurogliaform cells ($n = 6$) did not occur with the application of GLP-1 in hypoglycemia. *$p < 0.05$, **$p < 0.01$.

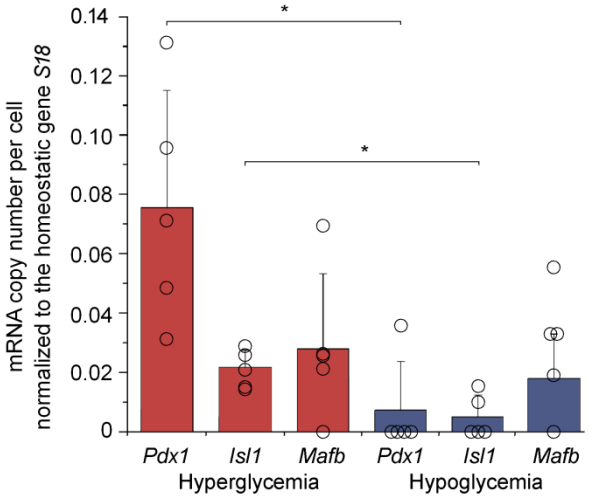

Figure 7. Single-cell digital PCR of neurogliaform cells confirms the expression of transcription factors important in beta cells. Changes in extracellular glucose concentrations modulated the copy number of *Isl1* and *Pdx1* mRNA in electrophysiologically and anatomically identified neurogliaform cells ($p < 0.037$ and $p < 0.016$, respectively). *$p < 0.05$.

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