

GABAergic neurogliaform cells represent local sources of insulin in the cerebral cortex

Abbreviated title: insulin in neocortical neurogliaform interneurons

Gábor Molnár^{1*}, Nóra Faragó^{2*}, Ágnes K. Kocsis^{1*}, Márton Rózsa¹, Sándor Lovas¹,
Eszter Boldog¹, Rita Báldi¹, Éva Csajbók³, János Gardi³, László G. Puskás^{2,4} and Gábor
Tamás¹

¹*Research Group for Cortical Microcircuits of the Hungarian Academy of Sciences,
Department of Physiology, Anatomy and Neuroscience, University of Szeged, Közép
fasor 52, Szeged, H-6726, Hungary*

²*Laboratory of Functional Genomics, Department of Genetics, Biological Research
Center, Hungarian Academy of Sciences, Temesvári krt. 62, H-6726, Szeged, Hungary*

³*Endocrinology Unit, 1st Department of Internal Medicine, University of Szeged,
Hungary, Korányi fasor 8, Szeged, H-6720 Hungary*

⁴*Avidin Ltd., Szeged, Alsó kikötő sor 11, Szeged, H-6726, Hungary*

*These authors contributed equally to this work.

Corresponding author:

Gábor Tamás

Research Group for Cortical Microcircuits of the Hungarian Academy of Sciences,
Department of Physiology, Anatomy and Neuroscience, University of Szeged
Közép fasor 52., Szeged, H-6726 Hungary,

Tel.: +36 62 544 851, Fax: +36 62 544 291

e-mail: gtamas@bio.u-szeged.hu

Number of pages: 19

Number of figures: 3

Number of words in the Abstract: 125

Number of words in the Introduction: 281

Number of words in the Discussion: 502

Total number of words: 4493

The authors declare no conflict of interest.

Acknowledgements

The authors thank B. Lambolez, B. Cauli and J. Rossier for training G.T. in harvesting cytoplasm of neurons, L. Schaffer for donating S961 and E. Tóth for reconstructions.

Abstract

Concentrations of insulin in the brain are several folds higher than blood plasma levels. Insulin in the brain regulates the metabolism, molecular composition and cognitive performance of microcircuits, reduces food intake and cerebral insulin levels are altered in diabetes, aging, obesity and Alzheimer's disease. Released by pancreatic beta cells, insulin passes the blood brain barrier, but sources of locally released insulin still remain unclear. We find that insulin is strongly expressed in GABAergic neurogliaform cells in the cerebral cortex of the rat detected by single cell digital polymerase chain reaction. Focal application of glucose or glibenclamide to neurogliaform cells mimics the excitation suppressing effect of external insulin on local microcircuits via insulin receptors. Thus, neurogliaform cells might link GABAergic and insulinergic action in cortical microcircuits.

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 (Havrankova et al., 1978b). Insulin regulates the metabolism, molecular composition and cognitive performance of microcircuits (Wan et al., 1997; Beattie et al., 2000) with specific alterations in diabetes, aging, obesity and Alzheimer's disease (Gasparini et al., 2002; Porte et al., 2005). Since this first report suggesting the presence of both pancreatic and locally synthesized insulin in the brain (Havrankova et al., 1978b), 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 (Margolis and Altszuler, 1967) and studies finding correlation between steady-state endogenous insulin levels in the plasma and cerebrospinal fluid (Woods and Porte, 1977) suggesting that insulin enters CNS through the blood-brain barrier by a saturable transport system (Banks et al., 1997). 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 (Havrankova et al., 1979; Baskin et al., 1985; Gasparini et al., 2002; Pilcher, 2006) and by *in situ* hybridization and immunocytochemical studies detecting insulin mRNA in developing and adult neurons and neuronal progenitor cells (Dorn et al., 1983; Devaskar et al., 1994; Kuwabara et al., 2011, Mehran et al. 2012) but the identity of neurons expressing insulin in terms of functional cell classes is not clear. We determined the number of insulin mRNAs in various cell classes in the cerebral cortex and tested if insulin can be released in the local microcircuit.

Materials and Methods

Electrophysiology and Imaging. All procedures were performed with the approval of the University of Szeged and in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. Male wistar rats (P22-35) were anaesthetized by intraperitoneal injection of ketamine (30mg/kg) and xylazine (10mg/kg), and following decapitation, coronal slices (350 μ m) were prepared from the somatosensory cortex. Slice preparation and recordings were performed as described

(Olah et al. 2009). Micropipettes (5-7M Ω) were filled with (in mM) 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na₂, 10 HEPES, 10 creatine phosphate and 8 biocytin (pH 7.25; 300mOsm); in low extracellular glucose concentrations the intracellular solution did not contain ATP-Mg, GTP-Na₂ and creatine phosphate. Signals were filtered at 5 kHz, digitized at 10 kHz and analyzed with PULSE software (HEKA). Voltage clamp protocols were applied according to (Zawar et al., 1999). 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 $-73.3\pm 3\text{mV}$ 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) and events were reviewed after automatic detection. Threshold was set to 3pA, onset time limit was set to 2ms, which defines the maximum interval from the baseline to the deflection reaches the threshold. Peak time limit was set to 3ms. 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 \pm S.D. throughout, n values refer to the number of neurons, statistical test are defined for each paradigm.

Histology. Visualization of biocytin and correlated light- and electron microscopy was performed as described earlier (Olah et al., 2009). Three-dimensional light microscopic reconstructions were carried out using NeuroLucida (MicroBrightfield) with 100x objective.

Single cell harvesting. At the end of electrophysiological recordings, the intracellular content was aspirated into the recording pipette by application of a gentle negative pressure while maintaining the tight seal. The pipette was then delicately removed to allow outside-out patch formation, and the content of the pipette (2 μ l) was expelled into a low-adsorption test tube (Axygen). Sample was snap-frozen in liquid nitrogen and stored or immediately used for RT.

First-strand cDNA synthesis. RT was carried out in two steps. First step was done for 5 min at 65°C in a total reaction volume of 5 μ l, containing 2 μ l intracellular solution with the cytoplasmic content of the neuron, 0.3 μ l reverse primer (Bioneer), 0.3 μ l 10mM dNTPs (Life Technologies, Foster City, CA), 1 μ l 5X first-strand buffer, 0.3 μ l of 0.1mol/L DTT, 0.3 μ l of RNase inhibitor (Life Technologies) and 100U of reverse transcriptase (Superscript III; Invitrogen). RT primers were designed using *CLC Main Workbench* (CLC Bio, Aarhus, Denmark) software and the sequences were the following: *rps18*: 5'-ATTAACAGCAAAGGCCCA-3'; *ins2*: 5'-TTTATTCATTGCAGAGGGG-3'. Second step of the reaction was carried out at 50°C for 1 h and then the reaction was stopped by heating at 70°C for 15 min. The RT reaction mix was used in PCR amplification.

Single cell QRT-PCR. Reactions were carried out after preamplification of cDNA in a total volume of 20 μ l (5 μ l RT product, 1 μ l of Taqman® primer (*rps18*: Rn01428913_gH; *ins2*: Rn01774648_g1), 10 μ l TaqMan® PreAmp Master Mix (Life Technologies) and 4.5 μ l nuclease-free water) in MyGenie 32 Thermal Block (Bioneer) using protocols as described (Faragó et al., 2013). 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.

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'-cccatgtcccgcgcg-3' (16) FOR2: 5'-gtggaggaccacaagtg-3' (18) REV1: 5'-tgccaaggtctgaaggtcac-3' (20) REV2: 5'-ttctgccgggccacctcc-3' (18).

Digital PCR. For digital PCR analysis, in case of *rps18* 2.5 μ l RT mixture or in case of *ins2* 5 μ l RT reaction mixture, 2 μ l *Taqman*[®] Assays, 10 μ l *OpenArray*[®] Digital PCR Master Mix (Life Technologies) and nuclease free water (2-4.5 μ l) were mixed in a total volume of 20 μ l. The mixture was distributed on an *OpenArray*[®] plate, cycled on an *OpenArray*[®] NT cycler and analysed using the Biotrove *OpenArray*[®] Digital PCR Software (version 1.0). as described (Faragó et al., 2013). For our dPCR protocol amplification reactions with C_T confidence values below 100 as well as reactions having C_T values below 23 and over 33 were considered primer dimers or background signals, respectively, and excluded from the dataset.

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 2pg/tube. BCA protein assay kit (Pierce) was used for detecting total protein content.

Results

We tested whether different neocortical neuron types, all of them identified by whole cell recordings and subsequent light microscopic assessment (Fig.1A), express the mRNA of the *ins2* gene encoding preproinsulin in the rat (Twigger et al., 2007). After electrophysiological and anatomical identification of cell types based on characterization of membrane and firing properties (Fig.1A), 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.1C). 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 (Farago et al., 2013) (Fig.1C). In high extracellular glucose concentration (10mM) 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 (2.4mM) and

hypoglycemia (0.5mM)(Silver and Erecinska, 1994) 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(Twigger et al., 2007) 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. 1C). 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. 1C).

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 (100nM) taking into account extra- and intracellular space ratios (0.18) and the $\sim 140\mu\text{m}$ diffusion into the slice pushing local concentrations down to a few nanomolar at our recording sites (Havrankova et al., 1978a, Nicholson and Sykova, 1998). Insulin reversibly decreased the frequency (from $13.0\pm 9.4\text{Hz}$ to $7.3\pm 5.5\text{Hz}$, $n=16$, $p<0.001$, Wilcoxon-test, Fig. 2A) and amplitude (from $12.1\pm 8.13\text{pA}$ to $10.1\pm 6.28\text{pA}$, $n=15$, $p<0.005$) of spontaneous EPSCs arriving to neocortical neurons in hypoglycaemia (0.5mM) and

application of the specific insulin receptor antagonist S961 (20nM)(Schaffer et al., 2008) prevented the effect ($12.2\pm 8.6\text{Hz}$ and $12.5\pm 9.47\text{pA}$). To test whether neurogliaform cells could mimic the reversible effect of externally added insulin, we performed simultaneous paired recordings in hypoglycaemic (0.5mM) conditions and puffed hyperglycaemic extracellular solution (10mM) 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. 2B). Relative to control, the frequency ($9.0\pm 8.3\text{Hz}$) of spontaneous EPSCs decreased following hyperglycaemic puffs to neurogliaform cells to $2.4\pm 1.6\text{Hz}$ (n=10, $p<0.004$, Wilcoxon-test). When applying S961 before local hyperglycaemia on neurogliaform cells, the frequency of spontaneous EPSCs remained unchanged ($8.7\pm 2.9\text{Hz}$ versus $8.6\pm 2.2\text{Hz}$, n=7, $p>0.47$). The effect of glucose puffs to neurogliaform cells was dependent on Y kinase signalling (Spicarova and Palecek, 2010) as shown by experiments in which lavendustin ($5\mu\text{M}$) intracellularly applied in neighboring pyramidal cells prevented the glucose-induced decrease in sEPSC frequency and amplitude ($6.67\pm 5.84\text{Hz}$ vs. $7.12\pm 5.76\text{Hz}$, n=5, $p=0.78$ and $12.50\pm 4.45\text{ pA}$ vs. $12.92\pm 3.16\text{ pA}$, $p=0.44$; Fig. 2C). 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 $4.61\pm 3.72\text{ pA}$ (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. 2D) suggesting a postsynaptic site of action. Thus, local hyperglycaemia on neurogliaform cells triggered insulin receptor mediated responses in the microcircuit mimicking the effect of external insulin.

In a final series of experiments, we addressed 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 (Taniguchi et al., 2006), we confirmed the presence of K_{ATP} channels in neurogliaform cells using protocols established for cortical interneurons (Zawar et al., 1999). Relative to control conditions having a partially suppressed activity of K_{ATP} channels due to hypoglycaemia (0.5mM) in the external solution (Taniguchi et al., 2006), glibenclamide (20 μ M) 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 \pm 2.9mV) close to the potassium equilibrium potential (Fig. 3A). In addition, bath-applied glibenclamide (20 μ M) 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 \pm 0.4% $\Delta F/F_0$, $p < 0.01$, Wilcoxon-test, Fig. 3B). Glibenclamide (20 μ M) puffs to the soma of neurogliaform cells in hypoglycaemia (0.5mM) decreased the frequency of spontaneous EPSCs arriving to simultaneously recorded neighbouring pyramidal cells (n=5) and fast spiking basket cells (n=5) (Fig. 3C, from 11.3 \pm 7.3Hz to 6.1 \pm 5.3Hz and S961 (20nM) reversed the effect to 9.2 \pm 6.2Hz (n=11, $p < 0.001$, Wilcoxon-test, Fig. 3C). When applying S961 before glibenclamide, the frequency of spontaneous EPSCs remained unchanged (8.5 \pm 7.8Hz versus 9.7 \pm 10.0Hz, n=9, $p > 0.47$, Fig. 3C). Moreover, intracellular application of BAPTA (4mM) in the neurogliaform cells targeted by glibenclamide also prevented changes in the frequency of spontaneous EPSCs (7.2 \pm 2.6Hz versus 6.8 \pm 2.7Hz, n=9, $p > 0.30$, Fig. 3C) confirming that the effect of glibenclamide was Ca^{2+} dependent. Neurogliaform cells potentially target $GABA_B$ receptors (Olah et al., 2009; Fuentealba et al., 2010) but

GABA_B blockade with CGP35348 (40 μ M) did not prevent the suppressing effect of glibenclamide on spontaneous EPSC frequencies (10.4 \pm 2.8Hz versus 8.5 \pm 3.4Hz, 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 \pm 4.5Hz versus 9.1 \pm 3.8Hz, p=0.76) or fast spiking interneurons (n=9, 7.4 \pm 2.8Hz versus 7.1 \pm 2.7Hz, p=0.65, Fig. 3C) in hypoglycaemia (0.5mM). Finally, we added glibenclamide (20 μ M) to hypoglycaemic (0.5mM) external solution of neocortical brain slices for 30 minutes and detected increased insulin levels with radioimmunoassay (80.8 \pm 17.5 pg/mg protein, n=10) in slices at the end of treatment compared to controls without glibenclamide (60.4 \pm 21.7 pg/mg protein, n=10, p<0.04, Mann-Whitney test, Fig. 3D). 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 10mM glucose showed increased insulin content relative to hypoglycaemia (75.4 \pm 14.1 and 104.2 \pm 26.9 pg/mg protein, n=10, p<0.05 and p<0.01, respectively) confirming local insulin synthesis.

Discussion

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 (Ludwig et al., 2002). 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 ± 0.6 mV, $n=8$, $p < 0.04$) 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 regulates the metabolism, molecular composition and cognitive performance of microcircuits (Wan et al., 1997; Biessels et al., 1998) and application of external insulin into the cerebrospinal fluid was found to decrease food intake (Woods et al., 1979; Porte et al., 2005). Cerebral insulin levels are altered in diabetes, aging, obesity and Alzheimer's disease (Havrankova et al., 1979; Baskin et al., 1985; Gasparini et al., 2002; Porte et al., 2005; Pilcher, 2006, Mehran et al. 2012). A decline in cognitive functions was proven in type II diabetic patients (Elias et al., 1997) and intranasal application of insulin, which does not alter plasma levels but reaches the cerebrospinal fluid (Born et al., 2002) was found to be promising in preventing the progression of cognitive impairment (Craft et al., 2012). Thus, potential local sources of insulin might

have a modulatory effect on neighboring neural microcircuits in health and disease. Damage to cortical insulin producing neurons could partially explain lower cerebral levels of the hormone in obesity, ageing and Alzheimer's disease(Havrankova et al., 1979; Gasparini et al., 2002) and potentially contribute to the onset of these conditions. Insulin receptors are abundant in the cerebral cortex(Havrankova et al., 1978a; Porte et al., 2005), but the short half-life might prevent neocortical insulin in reaching relatively distant brain areas known to mediate insulin receptor dependent processes in energy homeostasis and feeding(Porte et al., 2005). Locally however, neuronal insulin might fine tune the passage of glucose through the endothelium of local blood vessels expressing insulin sensitive glucose transporters(Magistretti et al., 1999; Porte et al., 2005). Neurogliaform cells use an action potential dependent form of GABAergic volume transmission(Olah et al., 2009) which can interact with insulin in regulating the efficacy of synapses(Wan et al., 1997; Beattie et al., 2000). Insulin recruits additional GABAA receptors to synaptic (Wan et al. 1997) and possibly extrasynaptic membrane compartments of pyramidal neurons shunting excitatory inputs and leading to decreased firing and a reduced frequency of sEPSCs. Neurogliaform cells hyperpolarize the membrane potential of cells expressing GABAergic receptors in their neighbourhood and the insulin receptor dependent effects of non-spiking neurogliaform cells suggested here might complement the spike triggered GABAergic actions.

References

- Banks WA, Jaspan JB, Huang W, Kastin AJ (1997) Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin. *Peptides* 18:1423-1429.

- Baskin DG, Stein LJ, Ikeda H, Woods SC, Figlewicz DP, Porte D, Jr., Greenwood MR, Dorsa DM (1985) Genetically obese Zucker rats have abnormally low brain insulin content. *Life Sci* 36:627-633.
- Beattie EC, Carroll RC, Yu X, Morishita W, Yasuda H, von Zastrow M, Malenka RC (2000) Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nat Neurosci* 3:1291-1300.
- Biessels GJ, Kamal A, Urban IJ, Spruijt BM, Erkelens DW, Gispen WH (1998) Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment. *Brain Res* 800:125-135.
- Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL (2002) Sniffing neuropeptides: a transnasal approach to the human brain. *Nat Neurosci* 5:514-516.
- Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, Arbuckle M, Callaghan M, Tsai E, Plymate SR, Green PS, Leverenz J, Cross D, Gerton B (2012) Intranasal insulin therapy for Alzheimer disease and amnesic mild cognitive impairment: a pilot clinical trial. *Arch Neurol* 69:29-38.
- Devaskar SU, Giddings SJ, Rajakumar PA, Carnaghi LR, Menon RK, Zahm DS (1994) Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J Biol Chem* 269:8445-8454.
- Dorn A, Bernstein HG, Rinne A, Ziegler M, Hahn HJ, Ansorge S (1983) Insulin- and glucagonlike peptides in the brain. *Anat Rec* 207:69-77.
- Elias PK, Elias MF, D'Agostino RB, Cupples LA, Wilson PW, Silbershatz H, Wolf PA (1997) NIDDM and blood pressure as risk factors for poor cognitive performance. The Framingham Study. *Diabetes Care* 20:1388-1395.
- Farago N, Kocsis AK, Lovas S, Molnar G, Boldog E, Rozsa M, Szemenyei V, Vamos E, Nagy LI, Tamas G, Puskas LG (2013) Digital PCR to determine the number of transcripts from single neurons after patch-clamp recording. *BioTechniques* 54:327-336.
- Fuentealba P, Klausberger T, Karayannis T, Suen WY, Huck J, Tomioka R, Rockland K, Capogna M, Studer M, Morales M, Somogyi P (2010) Expression of COUP-TFII nuclear receptor in restricted GABAergic neuronal populations in the adult rat hippocampus. *J Neurosci* 30:1595-1609.
- Gasparini L, Netzer WJ, Greengard P, Xu H (2002) Does insulin dysfunction play a role in Alzheimer's disease? *Trends Pharmacol Sci* 23:288-293.
- Havrankova J, Roth J, Brownstein M (1978a) Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* 272:827-829.
- Havrankova J, Roth J, Brownstein MJ (1979) Concentrations of insulin and insulin receptors in the brain are independent of peripheral insulin levels. Studies of obese and streptozotocin-treated rodents. *J Clin Invest* 64:636-642.
- Havrankova J, Schmechel D, Roth J, Brownstein M (1978b) Identification of insulin in rat brain. *Proc Natl Acad Sci U S A* 75:5737-5741.
- Kuwabara T, Kagalwala MN, Onuma Y, Ito Y, Warashina M, Terashima K, Sanosaka T, Nakashima K, Gage FH, Asashima M (2011) Insulin biosynthesis in neuronal progenitors derived from adult hippocampus and the olfactory bulb. *EMBO Mol Med*.
- Ludwig M, Sabatier N, Bull PM, Landgraf R, Dayanithi G, Leng G (2002) Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites. *Nature* 418:85-89.

- Magistretti PJ, Pellerin L, Rothman DL, Shulman RG (1999) Energy on demand. *Science* 283:496-497.
- Margolis RU, Altszuler N (1967) Insulin in the cerebrospinal fluid. *Nature* 215:1375-1376.
- Mehran AE, Templeman NM, Brigidi GS, Lim GE, Chu KY, Hu X, Botezelli JD, Asadi A, Hoffman BG, Kieffer TJ, Bamji SX, Clee SM, Johnson JD (2012) Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. *Cell metabolism* 16:723-737.
- Nicholson C, Sykova E (1998) Extracellular space structure revealed by diffusion analysis. *Trends in neurosciences* 21:207-215
- Olah S, Fule M, Komlosi G, Varga C, Baldi R, Barzo P, Tamas G (2009) Regulation of cortical microcircuits by unitary GABA-mediated volume transmission. *Nature* 461:1278-1281.
- Pilcher H (2006) Alzheimer's disease could be "type 3 diabetes". *Lancet Neurol* 5:388-389.
- Porte D, Jr., Baskin DG, Schwartz MW (2005) Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from *C. elegans* to humans. *Diabetes* 54:1264-1276.
- Schaffer L, Brand CL, Hansen BF, Ribel U, Shaw AC, Slaaby R, Sturis J (2008) A novel high-affinity peptide antagonist to the insulin receptor. *Biochem Biophys Res Commun* 376:380-383.
- Silver IA, Erecinska M (1994) Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. *J Neurosci* 14:5068-5076.
- Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85-96.
- Twigger SN, Shimoyama M, Bromberg S, Kwitek AE, Jacob HJ (2007) The Rat Genome Database, update 2007--easing the path from disease to data and back again. *Nucleic Acids Res* 35:D658-662.
- Wan Q, Xiong ZG, Man HY, Ackerley CA, Braunton J, Lu WY, Becker LE, MacDonald JF, Wang YT (1997) Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. *Nature* 388:686-690.
- Woods SC, Porte D, Jr. (1977) Relationship between plasma and cerebrospinal fluid insulin levels of dogs. *Am J Physiol* 233:E331-334.
- Woods SC, Lotter EC, McKay LD, Porte D, Jr. (1979) Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* 282:503-505.
- Zawar C, Plant TD, Schirra C, Konnerth A, Neumcke B (1999) Cell-type specific expression of ATP-sensitive potassium channels in the rat hippocampus. *J Physiol* 514 (Pt 2):327-341.

Legends

Figure 1. 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 (10mM) or low (0.5mM; 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 hyperglycaemic 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 2. 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 nM) and the specific insulin receptor antagonist S961 (20 nM) reversed the effect. **(B)** Mimicking the effect of insulin shown in (a), local application of hyperglycaemic extracellular solution containing 10mM glucose (gluc) to neurogliaform cells (NGFCs) identified

electrophysiologically and anatomically decreased the frequency of spontaneous EPSCs arriving to neighbouring neurons recorded in hypoglycaemic (0.5mM) conditions and S961 (20 nM) 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 (5 μ M) 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 3. 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 (20 μ M). **(B)** A neurogliaform cell identified by its firing pattern (top) responds to bath applied glibenclamide (20 μ M) 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 (0.5mM) show that glibenclamide (20 μ M) 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 hypoglycaemia during glibenclamide (glib) application and normo- or hyperglycaemia.



