

Research article

Insulin enhances GABA_A receptor-mediated inhibitory currents in rat central amygdala neurons

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ABSTRACT

Insulin, a pancreatic hormone, can access the central nervous system, activate insulin receptors distributed in selective brain regions and affect various cellular functions such as neurotransmission. We have previously shown that physiologically relevant concentration of insulin potentiates the GABA_A receptor-mediated tonic inhibition and reduces excitability of rat hippocampal CA1 neurons. The central nucleus of the amygdala (CeA) comprises heterogeneous neuronal populations that can respond to hormonal stimulus. Using quantitative PCR and immunofluorescent labeling, we report that the mRNA and protein of the insulin receptor are abundantly expressed in the rat CeA. The insulin receptor mRNA is also detected in the CeA from post-mortem human brain samples. Furthermore, our whole-cell patch-clamp recordings show that the application of insulin (5 and 50 nM) selectively enhances the frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) in rat CeA neurons. Our findings reveal that GABAergic synaptic transmission is a target in the CeA for insulin receptor signaling that may underlie insulin modulation of emotion- and feeding-related behaviors.

1. Introduction

The pancreatic hormone, insulin, enters the central nervous system via several putative routes [1]. It binds to the insulin receptor widely distributed in brain regions such as hypothalamus and hippocampus, and exerts diverse actions including regulation of energy homeostasis and cognitive functions [2,3]. Several lines of emerging evidence indicate that defective brain insulin signaling leads to increased food intake, hypothermia, hypothalamic hypogonadism and blunted response to hypoglycemia [4–6]. In addition, brain insulin resistance has been associated with cognitive impairments in type 2 diabetes, neurodegenerative diseases and mood disorders [7–9].

A key brain region connecting emotion/mood with control of food intake is the amygdala. The amygdala is located in the mid temporal lobe and best known for regulating emotional reactivity, such as fear and anxiety [10]. Furthermore, recent findings suggest that the amygdala integrates sensory and hormonal stimuli, interacts with various cortical and subcortical regions including hypothalamus, striatum, hippocampus and cortex, modulating feeding behavior [11]. Indeed, the amygdala expresses receptors for several metabolic hormones including insulin and leptin [12–14]. In addition, application of different metabolic hormones to the amygdala in animals affects food intake and results in anxiety-like behaviors [14,15]. Functional magnetic resonance imaging (fMRI) studies in humans have also shown the

modulation of amygdala activities after administration of hormones such as ghrelin [16,17]. However, the neuronal pathways underlying these effects are not fully understood.

The central nucleus of the amygdala (CeA) is the main output station of the amygdala and sends out efferent projections to various brain areas to coordinate physiological and behavioral responses [18,19]. Injection of insulin directly to the CeA produces potent anorectic effect and short-term high fat diet can induce amygdala insulin resistance in rats [15]. However, how insulin regulates the neural transmission in the CeA has been largely unexplored. The predominant neuronal type in the CeA is GABAergic (γ -aminobutyric acid) projection neurons that receive both glutamatergic and GABAergic innervations from other subdivisions of amygdala as well as other brain areas [20]. CeA projection neurons express GABA_A receptors (GABA_ARs) that mediate phasic and tonic inhibition regulating neuronal excitability and neuronal networks [21,22]. GABA-activated phasic inhibition involves fast spontaneous postsynaptic currents (sIPSCs), whereas tonic inhibition is carried by persistent inhibitory currents via activation of high-affinity extrasynaptic GABA_ARs. It has been shown that acute ethanol exposure modulates both GABA_AR mediated phasic and tonic inhibition in a subpopulation of CeA neurons in mice [22]. In hippocampal CA1 neurons, insulin enhances both synaptic and tonic currents by increasing the membrane insertion of synaptic GABA_AR and turning on extrasynaptic GABA_AR, respectively [23,24]. Since insulin exerts distinct

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actions in different brain regions, it is intriguing to investigate if insulin modulates these two types of GABA-activated neuronal inhibition mechanisms in CeA neurons.

In the current study we first sought to examine the mRNA expression and protein distribution of the insulin receptor in the CeA and further explored the effect of insulin on GABA_AR-mediated neuronal inhibition in rat CeA neurons.

2. Materials and methods

2.1. Animals

Wistar rats aged 16–22 days and 2-month old C57BL/6 mice were used in all experiments. All animal experiments were carried out in accordance with the local ethical guidelines and protocols approved by the Uppsala Animal Ethical Board (Uppsala, Sweden).

2.2. Rat brain slice preparation

Animals were decapitated and brains were rapidly removed and immersed into an ice-cold artificial cerebrospinal fluid (aCSF) comprising (in mM): 124 NaCl, 3 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 26 NaHCO₃, 2.5 Na₂HPO₄, and 10 glucose, 300 mOsm, pH 7.3–7.4 when bubbled with 95% O₂ and 5% CO₂. Coronal brain sections, 400 μm thick, were prepared with a vibratome (Leica VT1200, Leica, Germany) in the ice-cold aCSF. Slices were recovered in the same aCSF at 34 °C for at least half an hour and then kept at room temperature (20–22 °C) until used in experiments.

2.3. Reverse-transcription quantitative PCR (RT-qPCR)

The central amygdala and hippocampus were isolated from rat coronal brain sections. Post-mortem human brain samples from the CeA and hippocampal dentate gyrus were obtained at the New South Wales Tissue Resource Center (TRC), University of Sydney, Australia (<http://sydney.edu.au/medicine/pathology/trc/index.php>) [25,26]. Total RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Germany) and quantified with Nanodrop (Thermo Scientific, USA). Total RNA was further reverse transcribed to cDNA using SuperScript II reverse transcriptase (Thermo Scientific, USA) in a 20 μl reaction. RT negative control was also run in parallel by omitting reverse transcriptase in the RT reaction in order to examine the possible genomic DNA contamination in the extracted RNA sample. Real-time quantitative PCR was performed using ABI PRISM 7900HT Sequence Detection System (Thermo Scientific, USA) as previously described [25]. Cycle threshold (Ct) values were determined by the SDS2.4 and RQ Manager 1.2 softwares provided with the instrument. The melting curve was also examined to confirm the specificity of the PCR product. The predesigned KiCqStart primer pairs were synthesized by Sigma-Aldrich, for rat insulin receptor (from 5' to 3', forward: ATTATTGTCTCAAAGGGCTG, reverse: GATTGTGTTTTGAGAATCG), human insulin receptor (forward: CCTTGAAATTGGGAAGTACT, reverse: GGTTGTGTTGTCTCCAGTC) and reference genes rat *Gapdh* (Glyceraldehyde-3-Phosphate Dehydrogenase, forward: GCCAGCCTCGTCTCATAGACA, reverse: TGGTAACCAGGCGTCCGATA), human *PGK1* (phosphoglycerate kinase 1, forward: AGGGAAAAGATGCTTCTGGG, reverse: AAGTG AAGCTCGAAAGCTTCTAT), human *TBP* (TATA-binding protein, forward: GAGCTGTGATGTGAAGTTCC, reverse: TCTGGGTTTGATCATTCTGTAG). The expression of insulin receptor gene was normalized to the expression of the reference gene(s) and calculated with DataAssist V2.0 using the 2^{-ΔCt} method. The PCR product was further run on a 2% agarose gel stained with SYBR Gold dye.

2.4. Immunofluorescence staining and confocal microscopy

Rat and mouse coronal brain sections (1–2 mm thick), were

prepared using Vibratome (Leica VT1200, Leica, Germany) as described above and fixed in 4% paraformaldehyde for 1 h on ice. Sections were washed twice with 0.1 M phosphate buffer (PB, pH 7.4) and immersed in 30% sucrose overnight. Cryosections of 14 μm of thickness were prepared using a cryostat (Leica, Germany), washed with 0.1 M phosphate buffered saline (PBS, pH 7.4) and blocked in a blocking solution containing 10% normal donkey serum, 0.2% bovine serum albumin and 0.2% Triton X-100 for 1 h at room temperature. Cryosections were further incubated with the primary antibody rabbit anti-insulin receptor (IRβ C19, 1:100, Santa Cruz, sc-711, Germany) overnight at 4 °C followed by incubation with donkey anti-rabbit Alexa-fluor 488 antibody (1:500, Jackson Laboratory, USA) for 1 h at room temperature. The images were acquired using LSM700 confocal microscopy (Zeiss, Germany). Negative controls included omission of primary antibody incubation or pre-blocking the primary antibody with blocking peptides (1:5) before applying to the sections. Both controls resulted in no detectable staining. The anti-insulin receptor antibody (IRβ C19) is an affinity purified rabbit polyclonal antibody raised against peptide sequence mapping at the C-terminus of human insulin receptor β subunit (NCBI accession number P06213), which is also conserved in rat and mouse. The epitope does not show sequence similarity with human insulin-like growth factor 2 receptor (IGF2R, NCBI accession number NP_00867). This antibody does not cross-react with insulin-like growth factor 1 receptor (IGF1R) [27,28]. The specificity of IRβ C19 antibody has been validated by Western blot and immunofluorescence staining in mice that lack insulin receptor expression in the brain (e.g. NIRKO mice) [29].

2.5. Whole-cell patch-clamp recording and analysis

Whole-cell voltage-clamp recordings were performed at room temperature as previously described [24,30]. All drugs including insulin and GABA_AR antagonist bicuculline methbromide were purchased from Sigma-Aldrich (Germany). Insulin stock solution was prepared by dissolving insulin powder in diluted hydrochloric acid. Bicuculline methbromide was dissolved in water as a stock solution. All stock solutions were further diluted in aCSF containing kynurenic acid (3 mM) to the final working concentration. The aCSF containing kynurenic acid (3 mM) plus other drugs equilibrated with 95% O₂ and 5% CO₂ was continuously perfused to the brain slices in the recording chamber during experiments. The pipette solution contains (in mM): 140 CsCl, 1 CaCl₂, 3 EGTA, 0.5 KCl, 1 MgCl₂, 2 ATP-Mg, 0.3 GTP-Na, 5 QX-314 bromide, and 10 TES (pH 7.25 adjusted with CsOH). All recorded analog signals were low-pass filtered at 2 kHz using an Axopatch 200 B amplifier and digitized on-line at 10 kHz using a Digidata 1440A and pClamp 10 software (Molecular Device, USA). Recordings were done at –60 mV and rejected for analysis when the access resistance has changed more than 20%. The frequency, inter-event interval and average amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed with MiniAnalysis 6.0.3 (Synaptosoft, USA). The amplitude of tonic current was measured as the difference in the holding current level before and after drug application.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Data were presented as mean ± standard error of the mean (SEM). Unpaired *t*-test was used to compare mRNA expression levels in central amygdala and hippocampal regions. A Kolmogorov-Smirnov test was used to statistically compare cumulative values of sIPSC amplitudes and inter-event intervals before and after insulin application for individual cells. The frequency and average amplitude of sIPSCs were statistically analyzed using paired *t*-test for the comparison between mean values before and after insulin application. The significance level was set at *p* < 0.05.

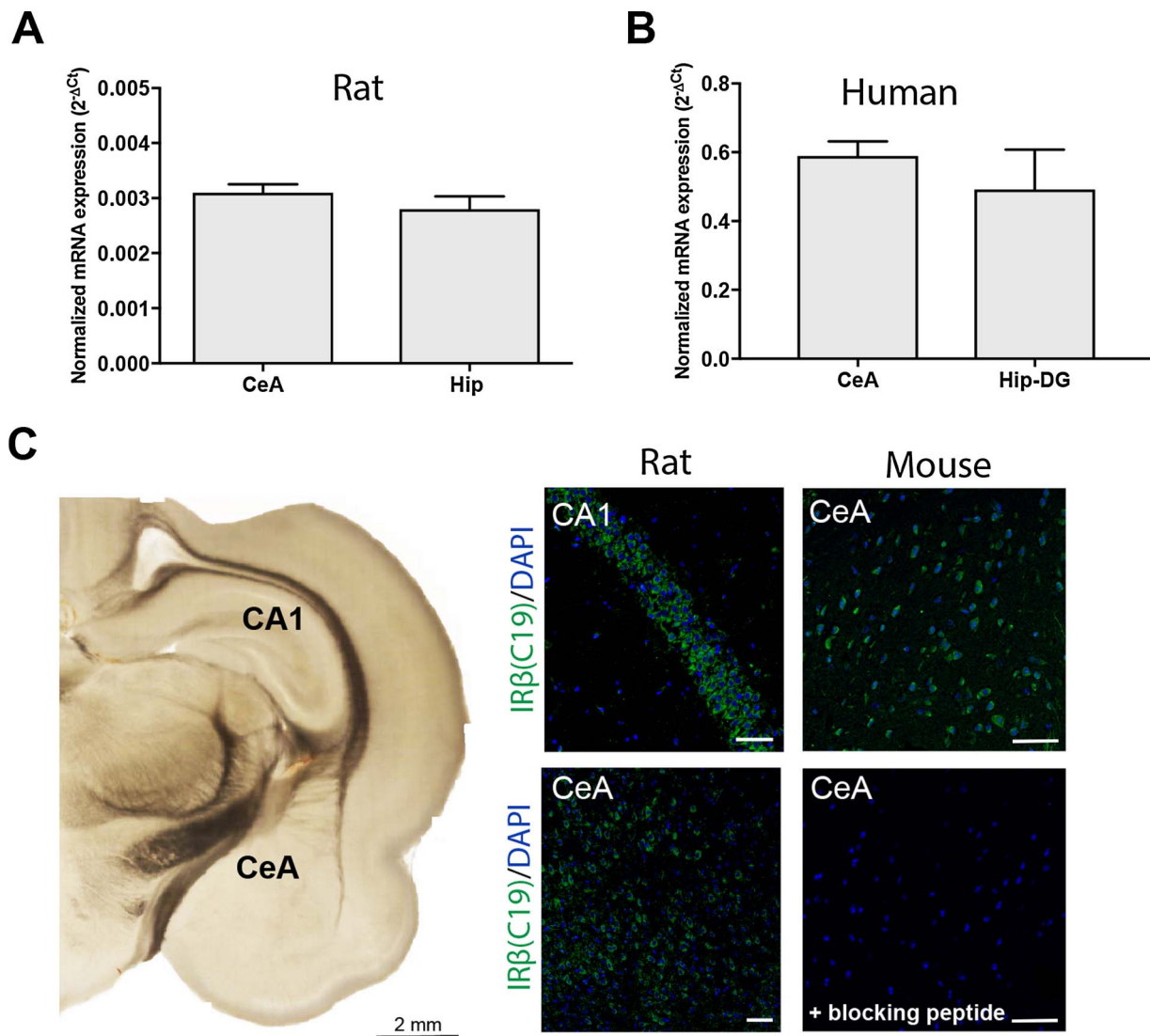


Fig. 1. Expression of insulin receptor mRNA and protein in rat, mouse and human central amygdala. (A) The relative expression of insulin receptor mRNA in rat central amygdala (CeA) and hippocampus (Hip) was measured by RT-qPCR, normalized to the expression level of the reference gene *Gapdh* and presented as mean \pm SEM ($n = 3$, unpaired *t*-test, two tailed $p = 0.3395$). (B) The relative expression of insulin receptor mRNA in human CeA ($n = 9$) and hippocampal dentate gyrus (Hip-DG) ($n = 15$) was normalized to the expression levels of the reference genes *PGK1* and *TBP*, and presented as mean \pm SEM (unpaired *t*-test, two tailed $p = 0.5338$). (C) The anatomic location of hippocampal CA1 and central amygdala (CeA) was illustrated in a bright-field image from a rat brain section. Insulin receptor-like immunoreactivity (IR β C19, green) was detected by immunofluorescent staining in rat CeA neurons and hippocampal CA1 pyramidal neurons as well as mouse CeA neurons. Pre-blocking IR β C19 with blocking peptide (1:5) totally abolished insulin receptor-like immunoreactivity in mouse CeA neurons. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, blue). White scale bar = 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Expression of insulin receptor mRNA and protein in the central amygdala neurons

To determine whether insulin receptor mRNA is present in the central amygdala, the hippocampus, that is known to abundantly express the insulin receptor, was used as an internal positive control. RT-qPCR was performed with insulin receptor specific primer pairs in samples from rat hippocampus and central amygdala. The presence of insulin receptor mRNA in the rat central amygdala was confirmed by the same peak in the melting curve and same size of band in the agarose gel as detected from hippocampal samples (data not shown). There was no significant difference in the expression level of insulin receptor mRNA between central amygdala and hippocampal samples (Fig. 1A). We also detected the expression of insulin receptor mRNA in the central amygdala and hippocampal dentate gyrus from post-mortem human brain samples (Fig. 1B).

We further examined the localization of insulin receptor in the rat and mouse central amygdala. Brain sections that were incubated with an antibody against the insulin receptor showed abundant fluorescent staining throughout the central amygdala region, as well as in hippocampal CA1 pyramidal neurons that were used as an additional positive control of antibody specificity (Fig. 1C). A high percentage of neuronal cell bodies in the central amygdala displayed insulin receptor-like immunoreactivity, although the precise numbers were not evaluated quantitatively. Pre-blocking insulin receptor antibody (IR β C19) with blocking peptide (1:5) abolished insulin receptor-like immunoreactivity in mouse CeA neurons.

3.2. Insulin enhances GABA-activated spontaneous inhibitory postsynaptic currents (sIPSCs) in rat central amygdala neurons

We have previously shown that insulin at physiologically relevant concentration selectively enhances GABA_AR-mediated neuronal inhibition in rat hippocampal CA1 neurons [24]. Here we examined the

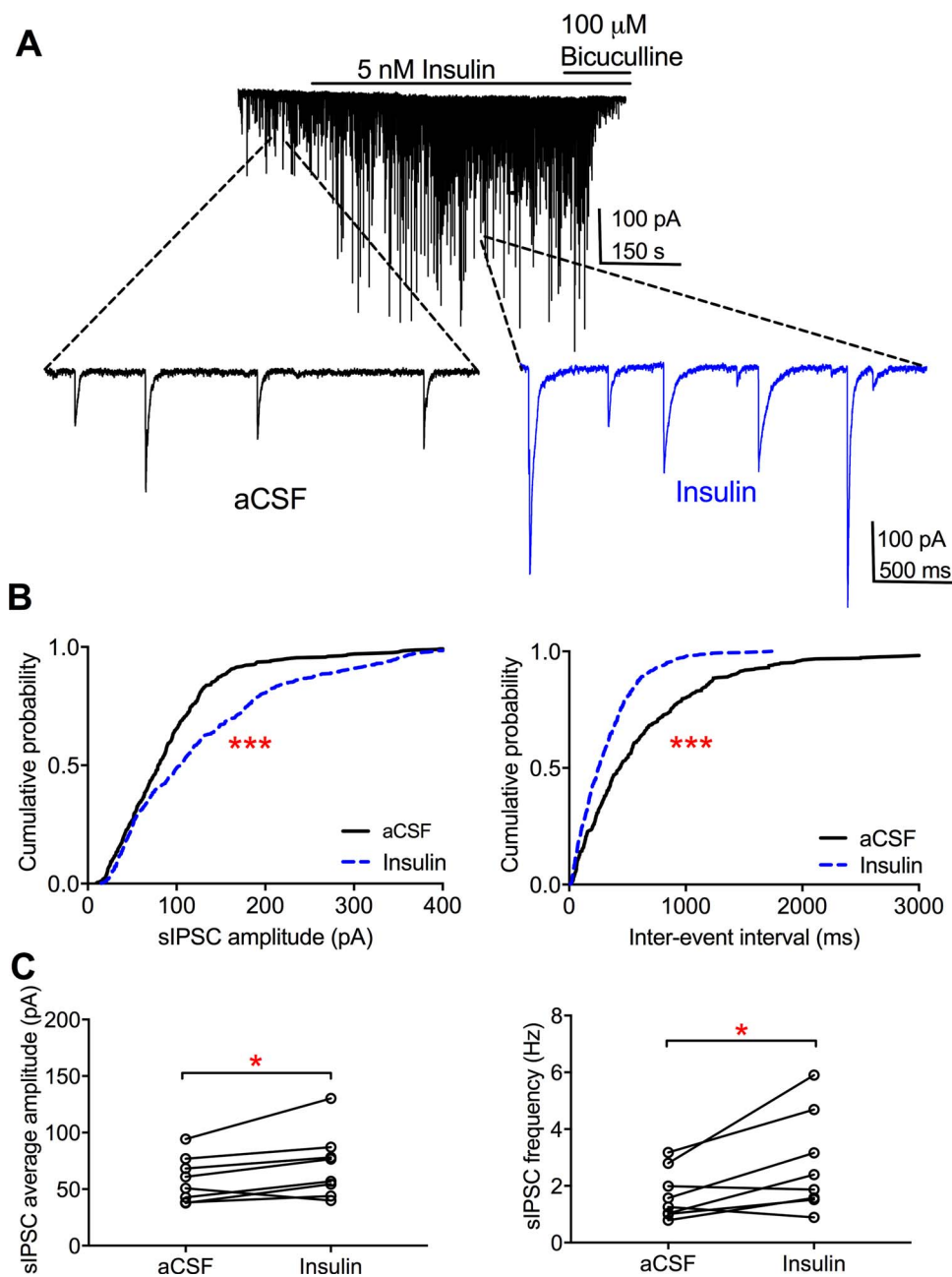


Fig. 2. Insulin enhances GABA_AR-mediated sIPSCs in rat central amygdala neurons. (A) A representative whole-cell patch-clamp recording demonstrating the effect of 5 nM insulin on sIPSCs in a central amygdala neuron (upper panel). Horizontal bars above the current recordings indicate the drug application periods. Enlargement of selected regions (aCSF vs. insulin) showed sIPSCs on a fast time scale (lower panel). (B) Cumulative probability distributions of sIPSC amplitudes (left panel) and inter-event intervals (right panel) for the same neuron as in (A) that were obtained from 2-min recording segments in each condition (aCSF vs. insulin). The right shift of the cumulative distribution curve indicates an increase of sIPSC amplitude (Kolmogorov-Smirnov test, ****p* < 0.001) and interval (Kolmogorov-Smirnov test, ****p* < 0.001), respectively. (C) Insulin (5 and 50 nM) significantly increased the frequency (left panel) and average amplitude (right panel) of sIPSCs in rat central amygdala neurons (*n* = 8, paired *t*-test, **p* < 0.05). Each connecting line indicates an individual cell.

effect of insulin on GABA-activated inhibitory currents in rat central amygdala neurons. The GABA_AR-mediated whole-cell currents were recorded in rat brain slices perfused with aCSF containing kynurenic acid. Insulin (5 or 50 nM) was applied to the recorded neuron and at the end of each experiment the GABA_AR specific antagonist bicuculline (100 μM) was added to inhibit the GABA-activated currents.

Representative current traces from a central amygdala neuron show that the application of 5 nM insulin enhanced the sIPSCs (Fig. 2A). The amplitude of sIPSCs detected in 2-min recordings was significantly increased during insulin application as compared to that in aCSF control condition in the same neuron (Fig. 2B), whereas inter-sIPSC event intervals were decreased (related to sIPSC frequency increase) in the presence of insulin (Fig. 2B). The application of 50 nM insulin to central amygdala neurons produced similar enhancement of sIPSC amplitudes and frequency as observed for 5 nM. We, therefore, combined the data at both concentrations for analysis. The potentiation of sIPSCs by insulin usually took 5–10 min after the insulin application to reach the maximal effect. The mean sIPSC frequency increased from

1.7 ± 0.31 Hz in aCSF to 2.75 ± 0.61 Hz during insulin application (Fig. 2C). In addition, the average amplitude of sIPSCs increased in the presence of insulin from 58.8 ± 7.2 to 70.9 ± 10.4 pA (Fig. 2C, *n* = 8, paired *t*-test, *p* < 0.05). We also examined the effect of insulin on the GABA_AR-mediated tonic current in the neurons. There was no significant change of tonic current amplitude before and after insulin application (aCSF, 1.28 ± 0.19 pA; insulin, 2.62 ± 1.37 pA, *n* = 4, *p* = 0.40). The above results suggest that insulin selectively enhances GABA_AR-mediated synaptic currents but not tonic currents in the central amygdala neurons.

4. Discussion

In the present study we first validated the central nucleus of the amygdala as a target for insulin by demonstrating the presence of the insulin receptor mRNA and protein in the rat CeA neurons. We further performed whole-cell patch-clamp studies on CeA neurons in acute rat brain slices and found that relevant concentration of insulin selectively

enhanced GABA_AR-mediated synaptic currents but had no apparent effects on tonic currents in CeA neurons. Thus, the GABAergic synapse could be an important target for insulin action in the CeA.

The multiple actions of insulin in the brain are mediated via the insulin receptors that are abundantly expressed in many brain regions including the hypothalamus and hippocampus. In recent years, the role of hippocampal insulin receptor signaling in cognitive function has been extensively investigated in both human and animal studies [31,32]. Thus, we have used rat hippocampal tissue as an internal control for the detection of insulin receptor in the CeA. Our quantitative PCR results demonstrate the expression level of insulin receptor mRNA is similar in rat hippocampus and CeA, which is consistent with findings from an early study using *in vitro* receptor autoradiography assay [33]. A subsequent immunohistochemical study revealed widespread expression of insulin receptor in all regions of rat amygdala [13]. Our immunofluorescent staining data further confirmed the presence of dense insulin receptor-like immunoreactivities in the soma of the majority of CeA neurons. There are two major types of neurons with different morphological and electrophysiological properties, which are homogeneously distributed throughout the rat CeA [34]. If insulin receptors are expressed in only one type or both types of CeA neurons remains a matter for future investigation.

Insulin is produced by pancreatic β cells, released to the circulation and crosses the blood-brain-barrier via an insulin receptor-mediated transport process in the brain endothelial cells [35]. An enzyme-linked immunosorbent assay (ELISA) of insulin has shown the insulin concentration in the adult rat brain and hippocampus is around 1 nM [36]. Thus, a range of relevant concentrations (1–50 nM) of insulin has been used in *in vitro* electrophysiological experiments on brain slices [24,37]. Higher insulin concentrations can result in desensitization of insulin receptors or down-regulation of subsequent signaling pathway or unspecific activation of IGF1R. Our data from whole-cell patch-clamp recordings indicate that the activation of insulin receptor by 5 or 50 nM insulin selectively enhances GABA-activated synaptic but not tonic currents in rat CeA neurons. A previous electrophysiological study has shown the application of 500 nM insulin increases the amplitude of GABA_AR-mediated miniature inhibitory postsynaptic currents (mIPSCs) in mouse hippocampal neurons indicating the increase of the number of postsynaptic GABA_A receptors [23]. Our results in rat CeA further confirmed the potentiation effect of insulin on GABA-activated synaptic currents. We have previously found prominent potentiation of GABA_AR-mediated tonic currents in hippocampal CA1 pyramidal neurons from rat brain slices incubated with 1 nM insulin [24]. In contrast, acute application of insulin (5 or 50 nM) does not appear to affect GABA-activated tonic currents in rat CeA neurons as shown in the current study. A number of factors, from insulin concentration, neuronal type, local microcircuitry to brain subregions, could contribute to the different results as described above.

Insulin modulates GABAergic neurotransmission through different mechanisms, including presynaptic release of GABA, the phosphorylation state or trafficking of GABA_AR from/to the cell membrane [38,39]. Therefore, it is of interest to further investigate if insulin-enhancing effect on sIPSCs in rat CeA is caused by pre- or post-synaptic mechanism or both. It has been well documented that receptors for various neuropeptides and hormones are distributed in the CeA and the activation of these receptors could modulate the neurotransmission within the CeA microcircuitry [18]. For example, corticotropin releasing factor (CRF) acts on CRF receptor-1 (CRF1)-expressing neurons and enhances GABA release in the CeA of rats [40]. These CRF1-containing neurons exhibit intrinsic α 1 subunit containing GABA_AR-mediated tonic conductance [22]. In contrast, we have not detected GABA-activated tonic current in the CeA neurons even when insulin was applied, which indicates they are less likely to be CRF1-expressing neurons.

5. Conclusion

The CeA is a heterogeneous structure and has extensive afferent and efferent connections within the amygdala as well as other brain regions such as hypothalamus, striatum and hippocampus. Animal behavioral studies suggest that the potential role of CeA response to hormone stimulus is related to emotion-related memory and regulation of food intake under physiological and pathophysiological conditions [14,15]. Our findings further reveal that the GABAergic synaptic transmission is a target for insulin in the CeA. However, future studies are needed to characterize the specific hormone-responding neuronal populations in the CeA and their participation in circuitry governing the distinct behaviors.

Conflicts of interests

None.

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References

- [1] S.M. Gray, R.I. Meijer, E.J. Barrett, Insulin regulates brain function, but how does it get there? *Diabetes* 63 (2014) 3992–3997.
- [2] A.M. Fernandez, I. Torres-Aleman, The many faces of insulin-like peptide signalling in the brain, *Nat. Rev. Neurosci.* 13 (2012) 225–239.
- [3] A. Kleinridders, H.A. Ferris, W. Cai, C.R. Kahn, Insulin action in brain regulates systemic metabolism and brain function, *Diabetes* 63 (2014) 2232–2243.
- [4] J.C. Bruning, D. Gautam, D.J. Burks, J. Gillette, M. Schubert, P.C. Orban, R. Klein, W. Krone, D. Muller-Wieland, C.R. Kahn, Role of brain insulin receptor in control of body weight and reproduction, *Science* 289 (2000) 2122–2125.
- [5] K.A. Diggs-Andrews, X. Zhang, Z. Song, D. Daphna-Iken, V.H. Routh, S.J. Fisher, Brain insulin action regulates hypothalamic glucose sensing and the counter-regulatory response to hypoglycemia, *Diabetes* 59 (2010) 2271–2280.
- [6] M. Sanchez-Alavez, O. Osborn, I.V. Tabarean, K.H. Holmberg, J. Eberwine, C.R. Kahn, T. Bartfai, Insulin-like growth factor 1-mediated hyperthermia involves anterior hypothalamic insulin receptors, *J. Biol. Chem.* 286 (2011) 14983–14990.
- [7] C. Moran, R. Beare, T.G. Phan, D.G. Bruce, M.L. Callisaya, V. Srikanth, I. Alzheimer's Disease Neuroimaging, Type 2 diabetes mellitus and biomarkers of neurodegeneration, *Neurology* 85 (2015) 1123–1130.
- [8] I. Sebastiao, E. Candeias, M.S. Santos, C.R. de Oliveira, P.I. Moreira, A.I. Duarte, Insulin as a bridge between type 2 diabetes and Alzheimer disease – how anti-diabetics could be a solution for dementia, *Front. Endocrinol. (Lausanne)* 5 (2014) 110.
- [9] A.N. Sharma, K.M. Elased, T.L. Garrett, J.B. Lucot, Neurobehavioral deficits in db/db diabetic mice, *Physiol. Behav.* 101 (2010) 381–388.
- [10] S. Duvarci, D. Pare, Amygdala microcircuits controlling learned fear, *Neuron* 82 (2014) 966–980.
- [11] M.F. Areias, P.O. Prada, Mechanisms of insulin resistance in the amygdala: influences on food intake, *Behav. Brain Res.* 282 (2015) 209–217.
- [12] B. Burguera, M.E. Couce, J. Long, J. Lamsam, K. Laakso, M.D. Jensen, J.E. Parisi, R.V. Lloyd, The long form of the leptin receptor (OB-Rb) is widely expressed in the human brain, *Neuroendocrinology* 71 (2000) 187–195.
- [13] J. Unger, T.H. McNeill, R.T. Moxley 3rd, M. White, A. Moss, J.N. Livingston, Distribution of insulin receptor-like immunoreactivity in the rat forebrain, *Neuroscience* 31 (1989) 143–157.
- [14] M. Alvarez-Crespo, K.P. Skibicka, I. Farkas, C.S. Molnar, E. Egecioglu, E. Hrabovszky, Z. Liposits, S.L. Dickson, The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence, *PLoS One* 7 (2012) e46321.
- [15] S. Boghossian, K. Lemmon, M. Park, D.A. York, High-fat diets induce a rapid loss of the insulin anorectic response in the amygdala, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297 (2009) R1302–R1311.
- [16] S. Malik, F. McGlone, D. Bedrossian, A. Dagher, Ghrelin modulates brain activity in areas that control appetitive behavior, *Cell Metab.* 7 (2008) 400–409.
- [17] P. Kirsch, C. Esslinger, Q. Chen, D. Mier, S. Lis, S. Siddhanti, H. Gruppe, V.S. Mattay, B. Gallhofer, A. Meyer-Lindenberg, Oxytocin modulates neural circuitry for social cognition and fear in humans, *J. Neurosci.* 25 (2005) 11489–11493.
- [18] N.W. Gilpin, M.A. Herman, M. Roberto, The central amygdala as an integrative hub

- for anxiety and alcohol use disorders, *Biol. Psychiatry* 77 (2015) 859–869.
- [19] A. Pitkänen, Connectivity of the rat amygdaloid complex. in: J.P. Aggleton (Ed.), *The Amygdala*, Oxford University Press, New York, 2000, pp. 31–115.
- [20] P. Sah, E.S. Faber, M. Lopez De Armentia, J. Power, The amygdaloid complex: anatomy and physiology, *Physiol. Rev.* 83 (2003) 803–834.
- [21] A. Semyanov, M.C. Walker, D.M. Kullmann, R.A. Silver, Tonic active GABA A receptors: modulating gain and maintaining the tone, *Trends Neurosci.* 27 (2004) 262–269.
- [22] M.A. Herman, C. Contet, N.J. Justice, W. Vale, M. Roberto, Novel subunit-specific tonic GABA currents and differential effects of ethanol in the central amygdala of CRF receptor-1 reporter mice, *J. Neurosci.* 33 (2013) 3284–3298.
- [23] Q. Wan, Z.G. Xiong, H.Y. Man, C.A. Ackerley, J. Braunton, W.Y. Lu, L.E. Becker, J.F. MacDonald, Y.T. Wang, Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin, *Nature* 388 (1997) 686–690.
- [24] Z. Jin, Y. Jin, S. Kumar-Mendu, E. Degerman, L. Groop, B. Birnir, Insulin reduces neuronal excitability by turning on GABA(A) channels that generate tonic current, *PLoS One* 6 (2011) e16188.
- [25] Z. Jin, I. Bazov, O. Kononenko, E.R. Korpi, G. Bakalkin, B. Birnir, Selective changes of GABA(A) channel subunit mRNAs in the hippocampus and orbitofrontal cortex but not in prefrontal cortex of human alcoholics, *Front. Cell. Neurosci.* 5 (2011) 30.
- [26] Z. Jin, A.K. Bhandage, I. Bazov, O. Kononenko, G. Bakalkin, E.R. Korpi, B. Birnir, Expression of specific ionotropic glutamate and GABA-A receptor subunits is decreased in central amygdala of alcoholics, *Front. Cell. Neurosci.* 8 (2014) 288.
- [27] A. Entingh-Pearsall, C.R. Kahn, Differential roles of the insulin and insulin-like growth factor-I (IGF-I) receptors in response to insulin and IGF-I, *J. Biol. Chem.* 279 (2004) 38016–38024.
- [28] S. Baudler, J. Baumgartl, B. Hampel, T. Buch, A. Waisman, C.M. Snapper, W. Krone, J.C. Bruning, Insulin-like growth factor-1 controls type 2T cell-independent B cell response, *J. Immunol.* 174 (2005) 5516–5525.
- [29] T.J. Dixon-Salazar, L. Fourgeaud, C.M. Tyler, J.R. Poole, J.J. Park, L.M. Boulanger, MHC class I limits hippocampal synapse density by inhibiting neuronal insulin receptor signaling, *J. Neurosci.* 34 (2014) 11844–11856.
- [30] S.V. Korol, Z. Jin, O. Babateen, B. Birnir, GLP-1 and exendin-4 transiently enhance GABAA receptor-mediated synaptic and tonic currents in rat hippocampal CA3 pyramidal neurons, *Diabetes* 64 (2015) 79–89.
- [31] C.A. Grillo, G.G. Piroli, R.C. Lawrence, S.A. Wrihten, A.J. Green, S.P. Wilson, R.R. Sakai, S.J. Kelly, M.A. Wilson, D.D. Mott, L.P. Reagan, Hippocampal insulin resistance impairs spatial learning and synaptic plasticity, *Diabetes* 64 (2015) 3927–3936.
- [32] G.J. Biessels, L.P. Reagan, Hippocampal insulin resistance and cognitive dysfunction, *Nat. Rev. Neurosci.* 16 (2015) 660–671.
- [33] J. Havrankova, J. Roth, M. Brownstein, Insulin receptors are widely distributed in the central nervous system of the rat, *Nature* 272 (1978) 827–829.
- [34] M.C. Schiess, P.M. Callahan, H. Zheng, Characterization of the electrophysiological and morphological properties of rat central amygdala neurons in vitro, *J. Neurosci. Res.* 58 (1999) 663–673.
- [35] S.M. Gray, K.W. Aylor, E.J. Barrett, Unravelling the regulation of insulin transport across the brain endothelial cell, *Diabetologia* 60 (2017) 1512–1521.
- [36] T. Kuwabara, M.N. Kagalwala, Y. Onuma, Y. Ito, M. Warashina, K. Terashima, T. Sanosaka, K. Nakashima, F.H. Gage, M. Asashima, Insulin biosynthesis in neuronal progenitors derived from adult hippocampus and the olfactory bulb, *EMBO Mol. Med.* 3 (2011) 742–754.
- [37] M.A. Stouffer, C.A. Woods, J.C. Patel, C.R. Lee, P. Witkovsky, L. Bao, R.P. Machold, K.T. Jones, S.C. de Vaca, M.E. Reith, K.D. Carr, M.E. Rice, Insulin enhances striatal dopamine release by activating cholinergic interneurons and thereby signals reward, *Nat. Commun.* 6 (2015) 8543.
- [38] Q. Wang, L. Liu, L. Pei, W. Ju, G. Ahmadian, J. Lu, Y. Wang, F. Liu, Y.T. Wang, Control of synaptic strength, a novel function of Akt, *Neuron* 38 (2003) 915–928.
- [39] S.L. Chiu, H.T. Cline, Insulin receptor signaling in the development of neuronal structure and function, *Neural Dev.* 5 (2010) 7.
- [40] M. Roberto, M.T. Cruz, N.W. Gilpin, V. Sabino, P. Schweitzer, M. Bajo, P. Cottone, S.G. Madamba, D.G. Stouffer, E.P. Zorrilla, G.F. Koob, G.R. Siggins, L.H. Parsons, Corticotropin releasing factor-induced amygdala gamma-aminobutyric Acid release plays a key role in alcohol dependence, *Biol. Psychiatry* 67 (2010) 831–839.