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Whole genome expression profiling associates activation of unfolded protein response with impaired production and release of epinephrine after recurrent hypoglycemia

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Abstract

Recurrent hypoglycemia can occur as a major complication of insulin replacement therapy, limiting the long-term health benefits of intense glycemic control in type 1 and advanced type 2 diabetic patients. It impairs the normal counter-regulatory hormonal and behavioral responses to glucose deprivation, a phenomenon known as hypoglycemia associated autonomic failure (HAAF). The molecular mechanisms leading to defective counter-regulation are not completely understood. We hypothesized that both neuronal (excessive cholinergic signaling between the splanchnic nerve fibers and the adrenal medulla) and humoral factors contribute to the impaired epinephrine production and release in HAAF. To gain further insight into the molecular mechanism(s) mediating the blunted epinephrine responses following recurrent hypoglycemia, we utilized a global gene expression profiling approach. We characterized the transcriptomes during recurrent (defective counter-regulation model) and acute hypoglycemia (normal counter-regulation group) in the adrenal medulla of normal Sprague-Dawley rats. Based on comparison analysis of differentially expressed genes, a set of unique genes that are activated only at specific time points after recurrent hypoglycemia were revealed. A complementary bioinformatics analysis of the functional category, pathway, and integrated network indicated activation of the unfolded protein response. Furthermore, at least three additional pathways/interaction networks altered in the adrenal medulla following recurrent hypoglycemia were identified, which may contribute to the impaired epinephrine secretion in HAAF: greatly increased neuropeptide signaling (proenkephalin, neuropeptide Y, galanin); altered ion homeostasis (Na⁺, K⁺, Ca²⁺) and downregulation of genes involved in Ca²⁺-dependent exocytosis of secretory vesicles. Given the pleiotropic effects of the unfolded protein response in different organs, involved in maintaining glucose homeostasis, these findings uncover broader general mechanisms that arise...
following recurrent hypoglycemia which may afford clinicians an opportunity to modulate the magnitude of HAAF syndrome.

Introduction

Profound or recurrent hypoglycemia (RH) leads to increased morbidity and mortality with acute and chronic symptomatology and is a major public health problem in diabetic patients ([1], [2], [3]). Normally, counter-regulatory hormones (i.e. glucagon, epinephrine, cortisol, and growth hormone) oppose the actions of excessive insulin to reverse falling or dangerously low plasma glucose levels. Recurrent insulin-induced hypoglycemia blunts the counter-regulatory response (CRR) and the patient becomes unaware of the danger, a condition known as Hypoglycemia Associated Autonomic Failure or HAAF ([4], [5]). Interestingly, HAAF can be induced in healthy subjects, is observed in newborn infants with congenital hyperinsulinism, and can be demonstrated in animal models indicating that it represents a maladaptive physiologic response rather than a disease consequence of diabetes ([6], [7], [8], [9], [10], [11]).

The central nervous system mechanisms that contribute to HAAF have been extensively studied in humans and in animal models. Increasing evidence indicates their dominant role in the detection of hypoglycemia and initiation of CRR ([12], [13], [14], [15], [5], [16], [17], [18], [19], [20]). However, peripheral components of the sympathoadrenal system are also directly affected by RH ([21,22,23,24,25]). The molecular fundamentals underlying the defective counter-regulation remain elusive ([13]).

We have recently shown that the initial rise in tyrosine hydroxylase (TH; the rate limiting enzyme in the catecholamine biosynthesis) gene transcription, phosphorylation of the TH enzyme and induction of protein kinase A (PKA) activity in the adrenal medulla (AM) of normal Sprague-Dawley (SD) rats are similar whether measured during an acute single episode of hypoglycemia or during RH (an animal model of HAAF, [10]). This is consistent with normally functioning signaling between the CNS and the peripheral components of the sympathoadrenal system ([25]). However, the adrenal medulla’s cellular capacity to synthesize catecholamines was impaired in animals experiencing RH. This was evident by lower levels of adrenal TH mRNA attributed to enhanced degradation ([26], [27]) as well as accelerated inactivation of TH enzyme and lack of accumulation of TH protein at later time points following RH; all of which alone or collectively could affect the accumulation of epinephrine for release and indeed, correlated with attenuated epinephrine blood levels ([11], [25]). Given that the release and production of catecholamines in response to trans-synaptic stimulation is sustained for prolonged periods of time in situ ([28,29], [24]), but not during HAAF in vivo, additional regulatory factors or processes must exist in the intact animal to account for this ([30], [27], [26], [31]). To ascertain the widest possibilities of gene networks that may be involved in these mechanisms, we performed a whole genome expression analysis of adrenal medullary responses.

Materials and methods

Animals

Adult, male Sprague—Dawley rats weighing 285-320g with jugular vein (JV) and carotid artery (CA) catheter implants were purchased from Harlan Laboratories, Inc. Indianapolis, IN. Animals were individually housed in temperature (22˚C) and humidity-controlled rooms and allowed access to food (regular rat chow, Agway Prolab 3,000; Syracuse, NY) and water ad
libitum unless otherwise specified. The animals were acclimated to the animal facility and to handling for 3–4 days before experiments. Animal care and experimentation conformed to the Public Health Service Guide for Care and Use of Laboratory Animals and American Veterinary Medical Association Panel on Euthanasia Guidelines, and were approved by the Institutional Animal Care and Use Committee at New York Medical College.

**Antecedent treatments.** The animals were randomly assigned to one of two experimental groups: sham-treated (recurrent saline injections twice daily at 9 am and 1 pm, 2RS) and insulin-treated (subjected to recurrent insulin-induced hypoglycemia twice daily, 2RH, see Fig 1). All treatments were for three consecutive days followed by a hyperinsulinemic-hypoglycemic glucose clamp on day 4. Antecedent hypoglycemic episodes were induced by intraperitoneal (i.p.) injection of regular human insulin (Humulin R, Eli Lilly, Indianapolis, IN) at a dose of 2 IU/kg body weight ([10], [11], [25]). Food was removed in all groups for 3 h after each insulin or saline injection. Blood glucose was monitored from tail nick samples using a handheld glucometer (AlphaTrak, Abbott Laboratories, Chicago, IL) every 30 minutes throughout each episode of hypoglycemia in 2RH group in order to achieve nadir glucose levels between 40 and 50 mg/dL (2.22–2.77 mmol/L).

Animals from the 2RS group were also subjected to the same treatments, except for the saline injections instead of insulin to avoid differences in any additional stress exposure (Fig 2). Hypoglycemia was terminated by providing the animals with solid food. Only one injection was given in the morning on the third day and the animals were fasted overnight before the glucose clamp studies began on the fourth day. The weight of each animal was monitored on a daily basis to ensure wellbeing and achievement of comparable nutrition.

**Hyperinsulinemic-hypoglycemic clamp.** On the day of the experiment, the vascular catheters were connected to extension sets to minimize the stress from blood sampling via CA and dextrose/insulin infusion via JV ([25]). Animals were rested at least 2 hours before baseline sampling in order to let them recover from handling stress. A constant infusion of regular human insulin (50 mIU/kg/min; Eli Lilly) and a variable 20% dextrose infusion were started at 0' and plasma glucose levels were monitored every 5 min (GM9 Analyzer, Analox Instruments Ltd, London, UK) to guide dextrose infusion. Plasma glucose levels were lowered to 45 mg/dL.

**Fig 1. Experimental design.** The schematic represents the basic protocol and the time line of the experiments. Before the antecedent treatments, a period of habituation was planned to minimize any external influences from travel and new housing. Body weight was monitored on a daily basis for each animal during the experiments.

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(2.5mmol/L) and maintained at this target until the end of the clamp study (Fig 3, [25], [11]). Blood samples were collected at 30 min intervals throughout the study, plasma was separated immediately and stored for subsequent hormonal analyses. Following each sample collection, the erythrocytes were resuspended in an equivalent volume of artificial plasma and infused back into the animal through the carotid artery to prevent volume depletion and anemia ([32], [11]). The animals (n = 6 per time point per group) were sacrificed either before (at 0 time point—baseline) or during (30’ and 60’ after achieving the target glucose levels) the clamp procedure with an overdose of sodium pentobarbital, followed by decapitation (Fig 1). The adrenal medullae were dissected and immediately frozen on dry ice. The tissues were stored at -80˚C until further analyses.

Hormone analyses

Plasma hormone concentrations (glucagon and insulin) were determined using commercially available radioimmunoassay kits from Linco Research, St. Charles, MO and Diagnostic Products, Los Angeles, CA ([33]). For measurement of catecholamine concentration, serum samples were analyzed using a competitive enzyme immunoassay (Rocky Mountain Diagnostics, Colorado Springs, CO) as described ([34], [11], [25]).

Western blot analyses

Total protein extracts were prepared from each right adrenal medulla sample as described ([35]). Protein lysates were separated on 10% SDS-PAGE, electrophotolsted onto a nitrocellulose membrane (BioRad; Hercules, CA) and incubated with validated primary antibodies overnight (anti-Grp78 rabbit polyclonal antibody, dilution 1:1000 from GeneTex, cat. # GTX113340, Irvine CA; rabbit polyclonal anti-Derlin1- dilution 1:5000, ThermoFisher Scientific cat. # PA1-16598, Rockford, IL; rabbit polyclonal anti-TH—dilution 1:4000, from Novus Biologicals.

Fig 2. Blood glucose levels during the antecedent treatments on days 1–3. The values (mg/dL) are expressed as mean ± SE, n = 6.

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cat. # NB300-109, Littleton, CO). After incubation with secondary antibody (Goat Anti-Rabbit IgG, from Pierce, Rockford IL; diluted 1:30000) the immune reaction was visualized by enhanced chemiluminescent substrate from Pierce, utilizing a horseradish peroxidase label and Kodak XAR-5 film, as described by the manufacturer. Blots were re-probed with primary antibody for housekeeping protein GAPDH (a rabbit polyclonal antibody from Sigma, St. Louis, MO, product number G9545) to confirm equal loading. The blots were exposed to autoradiography and the X-ray films were scanned and quantified with BioRad Quantity One software. For quantification, we always used a signal in the linear range. The ratios of immunoreactivity were calculated for each sample and the results are presented as fold induction compared to the corresponding control group on the same Western blot ([25]).

Whole genome expression profiling

Total RNA was isolated from each left adrenal medulla sample by using RNA STAT-60 (Tel-Test, Inc, Friendswood, TX) and further purified as per Affymetrix guidelines. Pooled samples from each experimental group and each time point (n = 6 each) were subjected to microarray analyses using Rat Genome 230 2.0 array, Affymetrix (analyses performed at Yale Center for Genome Analysis, New Haven, CT). Microarray data was imported to Partek software for normalization by GCRMA methods, quantification of gene expression, and statistics. Genes showing altered expression with fold change >2 or < -2 were exported for functional annotation, pathway and comparison analysis by MetaCore (Thomson Reuter). Genes that are unique to each experiment group at different time point, and genes that overlap between any comparing groups were identified. The expression levels of selected genes were confirmed by real time RT-PCR analyses as described before ([25]). Raw and quantile-normalized
microarray data and an associated project metadata file are available through the NCBI-GEO repository, access number GSE82145.

Statistics

One-way analysis of variance (ANOVA) or repeated measures ANOVA, followed by a Newman-Keuls post hoc analysis were used as appropriate. Statistics were performed using Sigma STAT/Plot software version 12 (Sigma, San Jose, CA). All data were expressed as means ± SE, \( P \leq 0.05 \).

Results

Plasma glucose and hormone levels

The experimental design of the current study is illustrated on Fig 1. RH was produced by twice daily i.p. injections of insulin (2IU/kg) for 3 days as we described before ([11], [25]). The target blood glucose levels during the antecedent insulin treatments were 45–50 mg/dL in the 2RH experimental group (see time course of blood glucose decline on Fig 2). There were no significant differences in blood glucose levels between individual animals (n = 6) in the 2RH group at any time point tested. Control animals received saline (2RS group). After overnight fast all animals (from both, 2RH and 2RS groups) were subjected to hyperinsulinemic-hypoglycemic clamp on day 4. A set of animals from both experimental groups were sacrificed at baseline (before insulin infusion, 0 time point). Once target plasma glucose levels between 40–45 mg/dL for each individual animal were achieved (0’ time point), a set of animals from each study group were sacrificed 30 (time point 30’) and 60 minutes later (time point 60’ on Fig 1) and tissues were dissected for analyses as described in the methods section of the manuscript. There were no significant differences in the plasma glucose levels between individual animals from each group and between the groups at each time point studied during the clamp (Fig 3). Also, plasma insulin concentrations were measured and found not significantly different between 2RH and 2RS groups at baseline and at the end of the clamp (data not shown), ensuring that the rats were exposed to the same glucose and insulin stimuli and only differed in the antecedent history between the comparison groups. Baseline serum concentrations of epinephrine and glucagon were also measured prior to the initiation of the clamp on day 4 (Fig 4A and 4B) and no significant difference was found between both experimental groups. Both epinephrine and glucagon levels rose significantly as insulin infusion began in both groups. However, the magnitude of the rise was significantly attenuated in 2RH group as expected, thus confirming proper implementation of the HAAF model. Corticosterone responses were also evaluated, which increased significantly from the baseline values during the clamp in both groups and did not display significant differences between the groups (results not shown).

Differential gene expression profiles triggered by acute hypoglycemia and RH

To identify potential cellular targets during acute hypoglycemia and RH, we used microarray technology allowing genome-wide simultaneous measuring of changes in gene expression. Total RNA was isolated from individual AM samples, pooled from 6 animals for each specific time point/experimental group and subjected to microarray analysis as described in methods. Differentially expressed genes (DEGs) were identified and ranked according to significant up-regulation or down-regulation of two fold or more (\( ^* P \leq 0.05 \)). Fold-change criteria combined with a minimum \( P \)-value cut off derived from an appropriate \( t \)-test is considered a straightforward method for identifying DEGs ([36]).
At baseline (0 time point—before insulin infusion on day 4, see Fig 1) a total of 167 genes were significantly altered in the 2RH group (vs. 2RS group): from them, 72 were up-regulated and 95 were down-regulated (top 10 genes for each category listed on Table 1, for complete list of DEGs see S1 Table). During the hyperinsulinemic-hypoglycemic clamp, the number of genes affected by RH increased. 30 min after achieving the target plasma glucose levels total of

![Fig 4. Plasma hormonal responses during the hypoglycemic clamp.](https://example.com)

**A** Epinephrine (ng/ml) and B) glucagon (pg/ml) responses in twice daily recurrently hypoglycemic (2RH) rats and in the corresponding saline group (2RS). Data are summarized from three independent experiments, n = 6 animals per group. Values are shown as mean ± SE, *p < 0.05 or **p < 0.002 vs. corresponding control at given time point.

At baseline (0 time point) a total of 167 genes were significantly altered in the 2RH group (vs. 2RS group): from them, 72 were up-regulated and 95 were down-regulated (top 10 genes for each category listed on Table 1, for complete list of DEGs see S1 Table). During the hyperinsulinemic-hypoglycemic clamp, the number of genes affected by RH increased. 30 min after achieving the target plasma glucose levels total of

| Gene Title                                      | Gene Symbol | RefSeq Transcript ID | Fold change |
|-------------------------------------------------|-------------|---------------------|-------------|
| Neurotensin                                     | Nts         | NM_001102381        | 106.364     |
| Proenkephalin                                   | Penk        | NM_017139           | 29.329      |
| Galanin/GMAP prepropeptide                      | Gal         | NM_033237           | 15.726      |
| Neuromedin                                       | Nmu         | NM_022239           | 11.486      |
| Lipocalin 2                                     | Lcn2        | NM_130741           | 9.038       |
| TIMP metallopeptidase inhibitor 1               | Timp1       | NM_053819           | 8.067       |
| VGF nerve growth factor inducible               | Vgf         | NM_030997           | 7.745       |
| Prolactin releasing hormone                     | Prlh        | NM_022222           | 6.515       |
| Pro-neuropeptide Y-like                         | NPY         | NM_012614           | 5.201       |
| Myosin heavy chain B                            | MyhB        | NM_00100485         | 5.089       |
| Neurotrophic tyrosine kinase receptor type 1    | Ntrk1       | NM_021589           | 4.15        |
| Cleavage and polyadenylation specific factor 4  | Cpsf4       | NM_001012351        | 3.528       |
| Bradykinin receptor B2                          | Brkrb2      | NM_001270713        | 3.465       |
| Kelch-like family member 8                      | Khl8        | NM_00105995         | 3.378       |
| Centrosomal protein 95kDa                       | Cep95       | NM_001013862        | 3.378       |
| Sodium channel, voltage gated type III beta     | Scn3        | NM_130907           | 3.359       |
| Diphthamide biosynthesis 1                      | Dph1        | NM_00105809         | 3.088       |
| Ubiquitin specific peptidase                    | Usp3        | NM_001025424        | 3.077       |
| cAMP response element modulator                 | Crem        | NM_001110860        | 3.017       |
| Hyaluronan and proteoglycan link protein 4      | Hapln4      | NM_001108398        | 2.977       |

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728 transcripts were significantly altered, 402 up-regulated and 326 down-regulated (2RH30 vs. 2RH0). At 60 min these numbers were 933, 515 and 418 respectively; Table 2, S2 Table. In animals exposed to acute hypoglycemia (2RS group) a total of 1,122 transcripts were found significantly altered at the 60’ min time point, 620 up-regulated and 502 down-regulated (2RS60 vs 2RS0).

We also performed a comparative analysis between the differentially expressed genes in adrenal medulla during acute hypoglycemia and RH. While a variety of comparisons can be drawn from the data set, our aim was to detect changes unique to RH at different time points. The results from two of the comparisons are illustrated on Fig 5. A relatively small number of DEGs was identified to be overlapping (common) between the animals exposed to acute hypoglycemia (2RS) and RH (2RH) (25, listed on Table 3, S1 Table). Several genes were up-regulated at both baseline (before insulin infusion on day 4) and 60 min after achieving the target glucose levels during the hyperinsulinemic-hypoglycemic clamp. A total of 16 were down-regulated and the expression of one gene (Get4, Golgi to ER traffic protein 4) was induced at the 0 time point and inhibited at the 60 min time point (shown in bold on the Table 3). A total of 167 genes were identified as unique for the 2RH0 group (animals with previous history of RH) at baseline, both induced (72) and suppressed (95) compared to the 2RS0 experimental group. At the 60 min time point the number of unique genes in the 2RH60 group (vs. maximal response group 2RS60) increased to a total of 213 (51 up-regulated and 162 down-regulated, respectively), see S1 Table.

The comparison between RH samples taken at different time points before and during the clamp revealed a total of a 404 genes affected at 30 min (241 induced and 163 down-regulated) and these numbers further increased to 520 at 60 min (Fig 5B). From them, 273 genes were common (overlapping) for all conditions, and 130 genes were unique for 2RH30 experimental
Fig 5. Venn diagrams of differentially expressed genes. A) Comparative analysis of DEGs in the 2RH0 and 2RH60 groups vs their respective saline controls (2RS0 and 2RS60); B) Comparative analysis of DEGs in 2RH groups—2RH30 and 2RH60 vs 2RHO respectively.

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Table 3. List of common DEGs during acute hypoglycemia and RH.

| Gene Title                                      | Gene Symbol | 2RH0/2RS0 | 2RH60/2RS60 |
|-----------------------------------------------|-------------|-----------|-------------|
| anillin                                       | Anin        | 4.36      | 3.19        |
| Chemokine (CXC) ligand 10                     | Cxcl10      | 3.02      | 5.25        |
| gamma-2a immunoglobulin heavy chain           | IgG-2a      | 4.69      | 11.33       |
| lipocalin 2                                    | Lcn2        | 9.038     | 3.43        |
| RT1 class 1a, locus A2                         | RT1-A2      | 2.62      | 3.78        |
| transcription elongation factor B, polypeptide 1 | Tceb1      | 2.31      | 2.69        |
| bradykinin receptor B2                         | Bdkrb2      | 3.46      | 2.29        |
| Calcyon neuron-specific vesicular protein      | Caly        | 2.66      | 3.24        |
| caspase 6                                      | Casp6       | 2.36      | 2.25        |
| diphthamide biosynthesis 1                     | Dph1        | 3.09      | 2.83        |
| ELAV like RNA binding protein 1                | ElavL1      | 2.03      | 2.13        |
| glutamyl-prolyl-tRNA synthase                  | Eprs        | 2.37      | 2.11        |
| Goldgi to ER traffic protein 4                 | Get4        | 2.1       | 2.46        |
| Guanine nucleotide binding protein α activating polypeptide 0 | Gnao1 | 2.48 | 2.53 |
| hyaluronan and proteoglycan link protein 4     | Hapln4      | 2.98      | 2.17        |
| neurotrophic tyrosine kinase receptor type 1   | Ntrk1       | 4.15      | 2.17        |
| parkinson protein 2, E3 ubiquitin ligase       | Park2       | 2.42      | 2.84        |
| regulator of G-protein signaling 4            | Rgs4        | 2.29      | 3.72        |
| SAP domain containing ribonucleoprotein        | Sarnp       | 2.44      | 2.3         |
| solute carrier family (Na/K/Ca exchanger), 2   | Slc24       | 2.02      | 2.13        |
| solute carrier family 6 (neurotransmitter transporter), 4 | Slc6a4 | 2.03 | 2.17 |
| tetraspanin                                   | Tspan18     | 2.04      | 2.02        |

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group and 247 for the 2RH60 group, suggesting dynamic changes in the adrenal transcriptome during the hyperinsulinemic clamp.

Enrichment analysis of differentially expressed genes

To define which biological processes are switched on or off during RH, we performed gene enrichment analysis in four different functional ontologies: canonical pathway maps, process networks, disease categories and gene ontologies (GO) using MetaCore™. This allowed us to analyze functionally related genes (for example, genes belonging to a specific biochemical process) as a whole. The results for unique DEGs in the RH group are shown on Fig 6. Among the top 10 process networks (2RH0 vs.2RS0) were neuropeptide signaling and endoplasmic reticulum (ER) stress pathways (Fig 6A). RH predominantly affected genes involved in cell adhesion, synaptic contact, calcium transport, transmission of nerve impulse and synaptic vesicle exocytosis. Most of them were significantly down-regulated compared to the maximal response group 2RS60 (animals exposed acute hypoglycemia, see distribution by process networks for unique genes shown on Fig 6B).

We also followed the dynamic changes in adrenal gene expression during the hyperinsulinemic-hypoglycemic clamp. The results for 2RH group are illustrated on Fig 7 (distribution by process networks for overlapping genes in the 2RH group, comparison on Fig 5B). Several key process categories were affected by RH including protein folding in normal condition, apoptosis/ apoptotic nucleus and signaling/leptin signaling. Interestingly, number of genes associated with the unfolded protein response (UPR) were induced at different time points of RH. Among these were mRNAs encoding Derlin-1, Cebpb, (member of the CHOP family of proteins) as well as several chaperons (such as members of the heat shock protein family- Hsp22, Hsp70, Hsp40; as well as Tor1A and Tcp1). Fig 8 summarizes UPR pathways with the genes detected with altered expression in our analysis (shown in bold).

RH induces the expression of Grp78—Master initiator of UPR

A significantly increased amounts of glucose regulated protein (GRP) 78 protein over baseline expression has become an established indicator and marker for the presence of cellular ER stress (rev. in [38]). To confirm the induction of UPR in RH we performed western blot analysis as described in methods section (Fig 9). Grp78 was not detectable at any time point during acute hypoglycemia (2RS group). However, in the 2RH group, the presence of GRP78 was evident at baseline and increased >2–3 fold during the clamp (at 30 and 60 min), consistent with the induction of the UPR. In addition, Derlin-1 protein, functional component of the ER-associated degradation (ERAD) pathway for misfolded luminal proteins [39], was detectable in all protein lysates on the same blots. Notably, the relative Derlin-1 immunoreactivity was significantly increased in 2RH0 group, followed by decline to baseline levels (in 2RH60), consistent with the microarray data.

Discussion

In the current study we discovered a novel association between activation of UPR signaling following recurrent glucoprivation and the defective counter-regulatory response that was evident even in otherwise normal rats. For the first time our whole genome expression profiling approach illustrated DEGs in the adrenal medulla of rats that were unique to the exposure to RH. We showed that among the RH-activated networks are ER stress and UPR, including up-regulation of UPR-related chaperones (Grp78 master initiator of UPR and other members of the Hsp70 family; DnaJb2, Hsp22, Tcp and Tor1). We also identified up-regulation of transcription factors (ATF4, Cebpb, Cebpd, CREB3L1, CREB3L3, and HSF1) and proteins
involved in ERAD (Derlin-1, ERP5, SIAH, E3 ligases; Figs 7 and 8). This is the first report of the potential role of UPR in TH biology or during HAAF. We speculate that the induction of UPR-related general inhibition of translation and potentiated decay of ER-localized mRNAs together with the disturbed Ca\(^{2+}\) homeostasis and suppression of the regulated secretory
pathway appear to contribute significantly to the altered regulation and diminution of adrenal catecholamine production and release in HAAF.

A prior exposure to RH results in prolonged changes in adrenal gene expression profiles

Through gene expression profiling, a total of 1,122 differentially-expressed genes were identified in response to acute hypoglycemia in the current study (genes altered two-fold or more at 60 min time point vs 0 time point on day 4 – Fig 1, 2RS60 vs 2RS0). This number was similar (1,100 DEGs) for the animals exposed to RH (2RH60 vs 2RS0) confirming that in both cases there was a significant transcriptional re-programming of adrenal chromaffin cells. Notably, several genes remained affected even 20 hrs after the last antecedent episode of RH (2RH0 vs 2RS0, Figs 5A and 6A, for complete list of unique genes see S1 Table). Among the top score process networks identified for unique genes in the 2RH group at baseline were apoptosis, neuropeptide signaling and interestingly, ER stress (Fig 6A).

The mRNAs encoding both, Derlin-1 and ERP5 (also named Protein disulfide isomerase (EC 5.3.4.1), PDIA6) were significantly upregulated in the 2RH0 group, compared to the 2RS0 (Fig 6A and S1 Table). The ER not only provides mechanisms to facilitate folding of newly synthesized secretory and membrane proteins, but also harbors molecular machineries that eliminate proteins that fail to fold or assemble correctly ([40]). We confirmed the significant upregulation of Derlin-1 protein during RH by Western blot (Fig 9). While altered expression of Derlin-1 was not associated with HAAF so far, physiological or pathological changes in Derlin-1 expression levels have been shown to affect glucose-stimulated insulin secretion by altering the surface expression of ATP-sensitive potassium channels ([39]).
ERP5 is known to catalyze formation, reduction, and isomerization of disulfide bonds in proteins, and to play a role in folding of disulfide-bonded proteins ([41]). It reacts with substrates that are known to associate with Grp78, including those targeted for ER-associated degradation ([42]). In this regard, we also identified an increase in Grp78 immunoreactivity in the 2RH group, evident at baseline and gradually enhanced during the clamp portion of the experiment (Fig 9), thus confirming the induction of ER stress in the AM of animals exposed to RH.

In many cell types glucose uptake occurs by facilitated diffusion and is affected by blood glucose concentration ([43]). Our results support the notion that in addition to the well described CNS-mediated trans-synaptic effects of hypoglycemia, deranged glucose fluxes in central and peripheral tissues, including AM caused by antecedent glucoprivation, may disturb cell homeostasis inducing ER stress and UPR.

Another set of genes, encoding several neuropeptides (including PENK, NPY and galanin) which are co-released with catecholamines in neuronal activity-dependent manner ([44]), were also significantly increased in the 2RH0 group (Table 1, Fig 6A, S1 Table). Our data are consistent with previously reported long lasting overexpression of several neuropeptides in response to stress (including acute hypoglycemia, [45], [46], [47]). These elevated neuropeptides can be logically assigned relevant to the defective CRR in HAAF: opioids exert an almost universal suppressive paracrine effect on the secretion of classical neurotransmitters,
neuropeptides, and hormones in neurons, adrenal chromaffin and other cells ([48]). Furthermore, opioid receptor blockage has been shown to prevent HAAF ([49,50]). NPY is also known to exert a negative feedback loop on TH expression and both, NPY ([51], [52], [53]) and galanin [54], [55] can control adrenal secretory capacity. Recently NPY expression was found required for fasting-induced autonomic synaptic plasticity at the preganglionic-chromaffin cell synapse ([56]).

Unique genes and process networks/pathways induced during the hyperinsulinemic—hypoglycemic clamp in animals, previously exposed to RH

In the current study a total of 213 adrenal genes were identified as unique for the 2RH60 group (vs. 2RS60 – maximal response to hypoglycemia group). From them, 51 were significantly induced and 162 were suppressed (Fig 5A). The majority of the down regulated genes belonged to process networks involved in synaptic contact/cell adhesion (Scd2, Syt, NRNX, SNAP25), calcium transport (PKD2, CALM, NOL3, Ca$^{2+}$-ATPase 2), transmission of nerve impulse, and exocytosis (NET, Rab3, Syt, SNAP25) (Fig 6B). These include genes encoding synaptotagmins—synaptic vesicle membrane proteins abundant in nerve and some endocrine cells proposed to function as calcium sensors in the regulation of neurotransmitter release and hormone secretion ([57, 58]). More specifically, Syt1 is recognized as a Ca$^{2+}$ sensor for fast synchronous neurotransmitter release in forebrain neurons and chromaffin cells ([59, 60]) and Syt7 as a major Ca$^{2+}$ sensor for exocytosis in chromaffin cells [61,62]. Recently it was found that Syt1 and Syt7 play an essential overlapping role in maintaining the readily-releasable pool of vesicles, in addition to their gene-specific function as Ca$^{2+}$ sensors and fusion clamps [61].

![Western blot analysis confirms activation of UPR following RH](image-url)
Ca-binding synaptotagmins are involved in both, early synaptic vesicle docking to the presynaptic membrane (via interaction with neurexin beta or SNAP25) and the late steps of synaptic vesicle fusion with the presynaptic membrane ([63]). Those genes were also found down regulated in our study. Our results suggest that the observed down regulation of multiple genes involved in Ca$^{2+}$ transport, synaptic contact and exocytosis and the developing of ER stress/activation of the UPR response in adrenal medulla of repeatedly hypoglycemic animals may be related to the reduced epinephrine secretion during HAAF.

It is worth mentioning that the gene encoding protein phosphatase 2 regulatory subunit B (PP2A) is among the few unique genes significantly induced in the 2RH60 group in our study (Fig 6B, S1 Table). PP2A is one of the four major Ser/Thr phosphatases with diverse function in the cell (rev. in [64]) and its B subunit might modulate substrate selectivity and catalytic activity. Interestingly the increased expression of PP2A (known to dephosphorylate TH enzyme in vitro—[65], [66]) correlated with the decline in phosphorylated TH enzyme at later time points in the 2RH group ([25]). Thus we speculate that ER stress and activation of UPR may account for the posttranscriptional regulation of TH previously reported by us during HAAF where both, TH mRNA longevity and TH translation are reduced (due to Ire1-dependent decay of ER membrane-associated mRNAs [67] and global inhibition of translation induced by UPR activation [68]). They would also promote faster inactivation of TH enzyme (either via PP2A mediated dephosphorylation at Ser40 or proteasomal degradation by the ERAD [69]). In this regard, one earlier study has demonstrated that a fraction of TH enzyme is an integral component of bovine chromaffin granule membranes ([70]) and could thus serve a purpose in coordinating TH activity and catecholamine release.

A potential contribution of adrenal ubiquitin proteasome system and ER stress in blunting the sympathoadrenal responses in HAAF has not been reported before. Accumulating evidence indicates that ER stress-mediated cell dysfunction and death is involved in the pathogenesis of human chronic disorders including metabolic diseases (obesity and Type 2-diabetes) and neurodegeneration ([38]), and UPR has been a growing subject of extensive investigations as a potential therapeutic target ([71], [72], [73], [74], [75], [76], [77], [78], [79], [80], [81]). Although the possible impact of chronic adrenal ER stress in the development of HAAF has not been tested previously, it has been shown that acute hypoglycemia in rodents increases several biochemical markers of the UPR and glucose production in the liver (not in the kidneys or the pancreas [82]).

Evidence for activation of adrenal UPR during subsequent episode of hypoglycemia in RH animals

Pathway analysis indicated that “Protein folding” was the most significantly enriched item in the distribution by process network for common genes (2RH30 vs.2RH0 and 2RH60 vs. 2RH0, Fig 7, comparison shown on Fig 5B) and “Response to unfolded proteins” was in the top 5 scored networks. A gene set for “Protein folding and response to unfolded proteins” includes molecular chaperons, members of the 70 kDa heat shock protein family (HSP70, HSPA1A, HSPA1B), 40kDa heat shock protein family (DNAJB2), small heat shock proteins (HSP22), heat shock factor 1 (HSF1) and BCL2-associated athanogene 3 (BAG3 – co-chaperone shown to regulate formation of SNARE complex and insulin secretion in beta cells [83]), indicating that activation of UPR may be a significant aspect of the altered responses to hypoglycemia and defective CRR in HAAF.

Diverse physiological or pathological challenges can provoke ER stress and activate the set of intracellular signaling pathways termed the UPR ([75]). Beneficial outputs of UPR restore homeostasis and normal ER functions, while destructive outputs trigger programmed cell
Prior to this report, activation of UPR in the adrenal medulla has not been described in HAAF, yet it has been implicated in the proper functioning and survival of pancreatic islet beta cells in Type 1 and Type 2 diabetes ([77], [86]). Accumulation of unfolded or misfolded proteins in the ER causes the dissociation of Grp78 chaperone from the three widely expressed ER transmembrane sensors: protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme-1 (IRE1a), eliciting the UPR. Combinatorial signals from IRE1a, PERK, and ATF6 initially trigger transcriptional programs that up-regulate genes encoding many ER chaperones, oxidoreductases, and ERAD components ([38]). The UPR also imposes a transient translational block during ER stress and promotes decay of ER-localized mRNAs ([87]) in a stress-dependent manner, thereby concentrating available resources to allow preexisting proteins to fold before new ones are made (rev. in [88], see Fig 8). Stress levels in the ER are reflected in the degree of activation of IRE1a, PERK, and ATF6; therefore, these upstream sensors are centrally poised to receive and transmit the information needed by the cells to commit in a dichotomous manner either toward adaptation or self-destruction.

Limitations of design. Our study was not designed to address causality in our integrative view of the altered stress responses following RH. The cause and effect relationships between ER stress, UPR and the attenuated epinephrine responses in HAAF will be tested in validation experiments in the future. In addition, in our microarray analysis data we selected relatively high p-value cut off (two-fold and more) and the actual number of genes with biologically significant changes in gene expression could be higher. It should be also mentioned that our observation and the interpretation of the results are based on few time points studied—before and during the exposure to acute hypoglycemia and RH. Given the highly dynamic changes in variety of homeostatic processes we may have missed some events. Moreover, we measured the mRNA levels, not the protein or its function.

Conclusions

We used microarray experiments to uncover genes regulated by acute hypoglycemia and RH in the adrenal medulla of normal SD rats. Overall, the reported observation underscores a physiologically important role for ER stress and the UPR to alter the peripheral sympathoadrenal components of the stress response to hypoglycemia. To date, no mechanisms linking the attenuated adrenal epinephrine response in HAAF to ER stress and UPR have been described. Although we do not provide direct evidence to support this hypothesis, we propose that the epinephrine responses to hypoglycemia result from a complex and dynamic interplay between centrally mediated trans-synaptic stimulation of catecholamine synthesis and release, as well as the opposing effects of cellular ER stress and the activation of UPR caused by glucoprivation. Further functional studies are necessary to confirm these correlations and validate the biological significance potentially leading to the useful therapeutic targets for many metabolic disorders.

Supporting information

S1 Table. Comparison analysis of DEGs during acute hypoglycemia and RH. DEGs were identified for 0 time point (before) and 60 min time point (during the hypoglycemic clamp) for 2RS and 2RH experimental groups. Overlapping genes regulated at all conditions and genes unique for each time point (2RH0 compared to 2RS0 and 2RH60 compared to 2RS60) are listed on separate spreadsheets. (XLSX)
S2 Table. Comparison analysis of DEGs at different time points during RH. DEGs were identified before (at 0 time point) and during (at 30 and 60 min) the hypoglycemic clamp in the 2RH experimental group. DEGs common for all conditions, and genes unique for 2RH30 (vs. 2RH0) and for 2RH60 (vs. 2RH0) are listed on separate spreadsheets. (XLSX)

S1 Text. Abbreviations for Figs 6 and 7. (DOCX)

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References
1. (1997) Hypoglycemia in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group. Diabetes 46: 271–286. PMID: 900705
2. Cryer PE (2011) Death during intensive glycemic therapy of diabetes: mechanisms and implications. Am J Med 124: 993–996. doi: 10.1016/j.amjmed.2011.08.008 PMID: 22017775
3. Seaquist ER, Anderson J, Childs B, Cryer P, Dagogo-Jack S, Fish L et al. (2013) Hypoglycemia and diabetes: a report of a workgroup of the American Diabetes Association and the Endocrine Society. J Clin Endocrinol Metab 98: 1845–1859. doi: 10.1210/jc.2012-4127 PMID: 23589524
4. Cryer PE (2015) Hypoglycemia-Associated Autonomic Failure in Diabetes: Maladaptive, Adaptive, or Both? Diabetes 64: 2322–2323. doi: 10.2337/db15-0331 PMID: 26106190
5. Davis SN, Tate D, Hedrington MS (2014) Mechanisms of hypoglycemia and exercise-associated autonomic dysfunction. Trans Am Clin Climatol Assoc 125: 281–291; discussion 291–282. PMID: 25125745
6. Christesen HT, Brugsgaard K, Hussain K (2012) Recurrent spontaneous hypoglycaemia causes loss of neurogenic and neuroglycopaenic signs in infants with congenital hyperinsulinism. Clin Endocrinol (Oxf) 76: 548–554.
7. Davis SN, Mann S, Galassetti P, Neill RA, Tate D, Ertl AC et al. (2000) Effects of differing durations of antecedent hypoglycemia on counterregulatory responses to subsequent hypoglycemia in normal humans. Diabetes 49: 1897–1903. PMID: 11076457
8. Davis SN, Tate D (2001) Effects of morning hypoglycemia on neuroendocrine and metabolic responses to subsequent afternoon hypoglycemia in normal man. J Clin Endocrinol Metab 86: 2043–2050. doi: 10.1210/jcem.86.5.7495 PMID: 11344204
9. Heller SR, Cryer PE (1991) Reduced neuroendocrine and symptomatic responses to subsequent hypoglycemia after 1 episode of hypoglycemia in nondiabetic humans. Diabetes 40: 223–226. PMID: 1991573
10. Shum K, Inouye K, Chan O, Mathoo J, Bilinski D, Matthews SG et al. (2001) Effects of antecedent hypoglycemia, hyperinsulinemia, and excess corticosterone on hypoglycemic counterregulation. Am J Physiol Endocrinol Metab 281: 455–465. PMID: 11500300
11. LaGamma EF, Kirtok N, Chan O, Nankova BB (2014) Partial blockade of nicotinic acetylcholine receptors improves the counterregulatory response to hypoglycemia in recurrently hypoglycemic rats. Am J Physiol Endocrinol Metab.
12. Raju B, Cryer PE (2005) Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin-deficient diabetes: documentation of the intraislet insulin hypothesis in humans. Diabetes 54: 757–764. PMID: 15734853
13. Cryer PE (2013) Mechanisms of hypoglycemia-associated autonomic failure in diabetes. N Engl J Med 369: 362–372. doi: 10.1056/NEJMra1215228 PMID: 23883381
14. Cryer PE (2008) Hypoglycemia: still the limiting factor in the glycemic management of diabetes. Endocrinology 149: 750–756. doi: 10.1210/en.2005-1247 PMID: 18996798
15. Beall C, Ashford ML, McCrimmon RJ (2012) The physiology and pathophysiology of the neural control of the counterregulatory response. Front Neurosci 6: 38. doi: 10.3389/fnins.2014.00038 PMID: 24616659
16. Tesfaye N, Seaquist ER (2010) Neuroendocrine responses to hypoglycemia. Ann N Y Acad Sci 1212: 12–28. doi: 10.1111/j.1749-6632.2010.05820.x PMID: 21039590
17. Verberne AJ, Sabetghadam A, Korim WS (2014) Neural pathways that control the glucose counterregulatory response. Front Neurosci 8: 38. doi: 10.3389/fnins.2014.00038 PMID: 24616659
18. McCrimmon RJ (2012) Update in the CNS response to hypoglycemia. J Clin Endocrinol Metab 97: 1–8. doi: 10.1210/jc.2011-1927 PMID: 22223763
19. Sprague JE, Arbelaez AM (2011) Glucose counterregulatory responses to hypoglycemia. Pediatr Endocrinol Rev 8: 463–473; quiz 474–465. PMID: 2278644
20. Parekh B (2009) Mechanisms of the blunting of the sympatho-adrenal response: a theory. Curr Diab Rev 5: 79–91. doi: 10.2174/157339909789238839 PMID: 19442093
21. Sivitz WI, Herlein JA, Morgan DA, Fink BD, Phillips BG, Haynes WG (2001) Effect of acute and antecedent hypoglycemia on sympathetic neural activity and catecholamine responsiveness in normal rats. Diabetes 50: 1119–1125. PMID: 11344147
22. Herlein JA, Morgan DA, Phillips BG, Haynes WG, Sivitz WI (2006) Antecedent hypoglycemia, catecholamine depletion, and subsequent sympathetic neural responses. Endocrinology 147: 2781–2788. doi: 10.1210/en.2005-1247 PMID: 16527844
23. De Galan BE, Tack CJ, Willemsen JJ, Sweep CG, Smits P, Lenders JW (2004) Plasma metanephrine levels are decreased in type 1 diabetic patients with a severely impaired epinephrine response to hypoglycemia, indicating reduced adrenomedullary stores of epinephrine. J Clin Endocrinol Metab 89: 2057–2061. doi: 10.1210/jc.2003-031289 PMID: 15126521
24. Orban BO, Routh VH, Levin BE, Berlin JR (2015) Direct effects of recurrent hypoglycemia on adrenal catecholamine release. Diab Vasc Dis Res 12: 2–12. doi: 10.1177/1479164114549755 PMID: 25268022
25. Kudrick N, Chan O, La Gamma EF, Kim JL, Tank AW, Sterling C et al. (2015) Posttranscriptional regulation of adrenal TH gene expression contributes to the maladaptive responses triggered by insulin-induced recurrent hypoglycemia. Physiol Rep 3.
26. Parab S, Nankova BB, La Gamma EF (2007) Differential regulation of the tyrosine hydroxylase and enkephalin neuropeptide transmitter genes in rat PC12 cells by short chain fatty acids: Concentration-dependent effects on transcription and RNA stability. Brain Res 1132: 42–50. doi: 10.1016/j.brainres.2006.11.013 PMID: 17174279
27. Aranyi T, Sarkis C, Berrard S, Sardin K, Siron V, Khalifallah O et al. (2007) Sodium butyrate modifies the stabilizing complexes of tyrosine hydroxylase mRNA. Biochem Biophys Res Commun 359: 15–19. doi: 10.1016/j.bbrc.2007.05.025 PMID: 17524356
28. Wakade AR (1988) Noncholinerger transmitter(s) maintains secretion of catecholamines from rat adrenal medulla for several hours of continuous stimulation of splanchic neurons. J Neurochem 50: 1302–1308. PMID: 2894411
29. Wakade AR, Wakade TD, Malhotra RK (1988) Restoration of catecholamine content of previously depleted adrenal medulla in vitro: importance of synthesis in maintaining the catecholamine stores. J Neurochem 51: 820–829. PMID: 2900877
30. Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW et al. (2002) Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. Proc Natl Acad Sci U S A 99: 461–466. doi: 10.1073/pnas.012608999 PMID: 11756684

31. Wong DL, Tank AW (2007) Stress-induced catecholaminergic function: transcriptional and post-transcriptional control. Stress 10: 121–130. doi: 10.1080/10253890701393529 PMID: 17514580

32. McDermott JC, Hubter A, Tan MH, Benon A (1989) The use of a cell-free perfusate in the perfused rat hindquarter: methodological concerns. Can J Physiol Pharmacol 67: 1450–1454. PMID: 2627868

33. Chan O, Zhu W, Ding Y, McCormon RJ, Sherwin RS (2006) Blockade of GABA(A) receptors in the ventromedial hypothalamus further stimulates glucagon and sympathoadrenal but not the hypothalamo-pituitary-adrenal response to hypoglycemia. Diabetes 55: 1080–1087. PMID: 16567532

34. Turcanu DS, Kirtok N, Eibl C, Guendisch D, LaGamma EF, Nankova BB (2012) Nicotinic receptor partial agonists alter catecholamine homeostasis and response to nicotine in PC12 cells. Neurosci Lett 516: 212–216. doi: 10.1016/j.neulet.2012.03.088 PMID: 22503726

35. Bobrovskaya L, Damanhuri HA, Ong LK, Schneider JJ, Dickson PW, Dunkley PR et al. (2010) Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: an in vivo analysis. Neurochem Int 57: 162–167. doi: 10.1016/j.neuci.2010.05.009 PMID: 20493917

36. Guo L, Lobenhofer EK, Wang C, Shippy R, Harris SC, Zhang L et al. (2006) Rat toxicogenomic study reveals analytical consistency across microarray platforms. Nat Biotechnol 24: 1162–1169. PMID: 17061323

37. Tsaytler P, Harding HP, Ron D, Bertolotti A (2011) Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. Science 332: 91–94. doi: 10.1126/science.1201396 PMID: 21385720

38. Schonthal AH (2012) Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. Scientifica (Cairo) 2012: 857516.

39. Wang F, Olson EM, Shyng SL (2012) Role of Derlin-1 protein in proteostasis regulation of ATP-sensitive potassium channels. J Biol Chem 287: 10482–10493. doi: 10.1074/jbc.M111.312223 PMID: 22311976

40. Hegde RS, Ploegh HL (2010) Quality and quantity control at the endoplasmic reticulum. Curr Opin Cell Biol 22: 437–446. doi: 10.1016/j.cceb.2010.05.005 PMID: 20570125

41. Meunier L, Usherwood YK, Chung KT, Hendershot LM (2002) A subset of chaperones and folding enzymes form multicomponent complexes in endoplasmic reticulum to bind nascent proteins. Mol Biol Cell 13: 4456–4469. doi: 10.1091/mbc.E02-05-0311 PMID: 12475965

42. Jessop CE, Watkins RH, Simmons JJ, Tasab M, Bulleid NJ (2009) Protein disulfide isomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. J Cell Sci 122: 4287–4295. doi: 10.1242/jcs.059154 PMID: 19887585

43. Svitiz WI, Yorek MA (2010) Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. Antioxid Redox Signal 12: 537–577. doi: 10.1089/ars.2009.2531 PMID: 19650713

44. Podvin S, Bundey R, Toneff T, Ziegler M, Hook V (2015) Profiles of secreted neuropeptides and catecholamines illustrate similarities and differences in response to stimulation by distinct secretagogues. Mol Cell Neurosci 68: 177–185. doi: 10.1016/j.mcn.2015.06.008 PMID: 26092702

45. Anouar Y, Eiden LE (1995) Rapid and long-lasting increase in galanin mRNA levels in rat adrenal medulla following insulin-induced reflex splanchnic nerve stimulation. Neuroendocrinology 62: 611–618. PMID: 8751287

46. Fischer-Colbrie R, Eskay RL, Eiden LE, Maas D (1992) Transsynaptic regulation of galanin, neuropeptide, and substance P in the adrenal medulla: combinatorial control by second-messenger signaling pathways. J Neurochem 59: 780–783. PMID: 1378491

47. Kanamatsu T, Unsworth CD, Diliberto EJ Jr., Viveros OH, Hong JS (1986) Reflex splanchnic nerve stimulation increases levels of proenkephalin A mRNA and proenkephalin A-related peptides in the rat adrenal medulla. Proc Natl Acad Sci U S A 83: 9245–9249. PMID: 3538020

48. Dermitzaki E, Gravanis A, Venhaki M, Stournaras C, Margioris AN (2001) Opioids suppress basal and nicotine-induced catecholamine secretion via a stabilizing effect on actin filaments. Endocrinology 142: 2022–2031. doi: 10.1210/endo.142.5.8133 PMID: 1116769

49. Milman S, Leu J, Shamoon H, Vele S, Gabriely I (2012) Opioid receptor blockade prevents exercise-associated autonomic failure in humans. Diabetes 61: 1609–1615. doi: 10.2337/db11-1622 PMID: 22526212

50. Vele S, Milman S, Shamoon H, Gabriely I (2011) Opioid receptor blockade improves hypoglycemia-associated autonomic failure in type 1 diabetes mellitus. J Clin Endocrinol Metab 96: 3424–3431. doi: 10.1210/jc.2011-1723 PMID: 21917871
51. Whim MD (2006) Near simultaneous release of classical and peptide cotransmitters from chromaffin cells. J Neurosci 26: 6637–6642. doi: 10.1523/JNEUROSCI.5100-05.2006 PMID: 16775152

52. Cavadas C, Cefai D, Rosmaninho-Salgado J, Vieira-Coelho MA, Moura E, Busso N et al. (2006) Deletion of the neuropeptide Y (NPY) Y1 receptor gene reveals a regulatory role of NPY on catecholamine synthesis and secretion. Proc Natl Acad Sci U S A 103: 10497–10502. doi: 10.1073/pnas.0600913103 PMID: 16798884

53. Wang Q, Wang M, Whim MD (2013) Neuropeptide Y gates a stress-induced, long-lasting plasticity in the sympathetic nervous system. J Neurosci 33: 12705–12717. doi: 10.1523/JNEUROSCI.3132-12.2013 PMID: 23904607

54. Andreis PG, Tortorella C, Ziolkowska A, Spinazzi R, Malendowicz LK, Neri G et al. (2007) Evidence for a paracrine role of endogenous adrenomedullary galanin in the regulation of glucocorticoid secretion in the rat adrenal gland. Int J Mol Med 19: 511–515. PMID: 17273801

55. Lang R, Gundlach AL, Holmes FE, Hobson SA, Wynick D, Hokfelt T et al. (2015) Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. Pharmacol Rev 67: 118–175. doi: 10.1124/pr.112.006536 PMID: 25428932

56. Wang M, Wang Q, Whim MD (2016) Fasting induces a form of autonomic synaptic plasticity that prevents hypoglycemia. Proc Natl Acad Sci U S A.

57. Zimmerberg J, Akimov SA, Frolov V (2006) Synaptotagmin: fusogenic role for calcium sensor? Nat Struct Mol Biol 13: 301–303. doi: 10.1038/nsmb0406-301 PMID: 16715046

58. Chapman ER (2002) Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? Nat Rev Mol Cell Biol 3: 498–508. doi: 10.1038/nrm855 PMID: 12094216

59. Schonn JS, Maximov A, Lao Y, Sudhof TC, Sorensen JB (2008) Synaptotagmin-1 and -7 are functionally overlapping Ca2+ sensors for exocytosis in adrenal chromaffin cells. Proc Natl Acad Sci U S A 105: 3998–4003. doi: 10.1073/pnas.0712373105 PMID: 18308932

60. Gustavsson N, Han W (2009) Calcium-sensing beyond neurotransmitters: functions of synaptotagmins in neuroendocrine and endocrine secretion. Biosci Rep 29: 245–259. doi: 10.4102/BSR20090031 PMID: 19500075

61. Bacaj T, Wu D, Burre J, Malenka RC, Liu X, Sudhof TC (2015) Synaptotagmin-1 and -7 Are Redundantly Essential for Maintaining the Capacity of the Readily-Releasable Pool of Synaptic Vesicles. PLoS Biol 13: e1002267. doi: 10.1371/journal.pbio.1002267 PMID: 26437117

62. Bacaj T, Wu D, Yang X, Morishita W, Zhou P, Xu W et al. (2013) Synaptotagmin-1 and synaptotagmin-7 trigger synchronous and asynchronous phases of neurotransmitter release. Neuron 80: 947–959. doi: 10.1016/j.neuron.2013.10.026 PMID: 24267651

63. Jackman SL, Turecek J, Belinsky JE, Regehr WG (2016) The calcium sensor synaptotagmin 7 is required for synaptic facilitation. Nature 529: 88–91. doi: 10.1038/nature16507 PMID: 26738595

64. Kurimchak A, Grana X (2015) PP2A: more than a reset switch to activate pRB proteins during the cell cycle and in response to signaling cues. Cell Cycle 14: 18–30. doi: 10.4161/cellcycle.15384101.2014.985069 PMID: 25483052

65. Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem 91: 1025–1043. doi: 10.1111/j.1471-4159.2004.02797.x PMID: 15569247

66. Saraf A, Oberg EA, Strack S (2010) Molecular determinants for PP2A substrate specificity: charged residues mediate dephosphorylation of tyrosine hydroxylase by the PP2A/B’ regulatory subunit. Biochemistry 49: 986–995. doi: 10.1021/bi902160t PMID: 20017541

67. Gaddam D, Stevens N, Hollien J (2013) Comparison of mRNA localization and regulation during endoplasmic reticulum stress in Drosophila cells. Mol Biol Cell 24: 14–20. doi: 10.1091/mbc.E12-06-0491 PMID: 23135994

68. Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13: 89–102. doi: 10.1038/nrm3270 PMID: 22251901

69. Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: the long road to destruction. Nat Cell Biol 7: 766–772. doi: 10.1038/rcb0805-766 PMID: 16056268

70. Kuhn DM, Arthur R Jr., Yoon H, Sankaran K (1990) Tyrosine hydroxylase in secretory granules from bovine adrenal medulla. Evidence for an integral membrane form. J Biol Chem 265: 5780–5786. PMID: 1969407

71. Balasubramanyam M, Lenin R, Monickaraj F (2010) Endoplasmic reticulum stress in diabetes: New insights of clinical relevance. Indian J Clin Biochem 25: 111–118. doi: 10.1007/s12291-010-0022-1 PMID: 23105896
72. Engin F, Yermalovich A, Nguyen T, Hummasti S, Fu W, Eizirik DL et al. (2013) Restoration of the unfolded protein response in pancreatic beta cells protects mice against type 1 diabetes. Sci Transl Med 5: 211ra156. doi: 10.1126/scitranslmed.3006534 PMID: 24225943
73. Engin F, Nguyen T, Yermalovich A, Hotamisligil GS (2014) Aberrant islet unfolded protein response in type 2 diabetes. Sci Rep 4: 4054. doi: 10.1038/srep04054 PMID: 24514745
74. de la Cadena SG, Hernandez-Fonseca K, Camacho-Arroyo I, Massieu L (2014) Glucose deprivation induces reticulum stress by the PERK pathway and caspase-7- and calpain-mediated caspase-12 activation. Apoptosis 19: 414–427. doi: 10.1007/s10495-013-0930-7 PMID: 24185830
75. Chaudhari N, Talwar P, Parimisetty A, Lefebvre d'Helencourt C, Ravanan P (2014) A molecular web: endoplasmic reticulum stress, inflammation, and oxidative stress. Front Cell Neurosci 8: 213. doi: 10.3389/fncel.2014.00213 PMID: 25120434
76. Salvado L, Palomer X, Barroso E, Vazquez-Carrera M (2015) Targeting endoplasmic reticulum stress in insulin resistance. Trends Endocrinol Metab 26: 438–448. doi: 10.1016/j.tem.2015.05.007 PMID: 26078196
77. Brozzi F, Eizirik DL (2016) ER stress and the decline and fail of pancreatic beta cells in type 1 diabetes. Ups J Med Sci: 1–7.
78. Mihailidou C, Chatzistamou I, Papavassiliou AG, Kiaris H (2015) Regulation of P21 during diabetes-associated stress of the endoplasmic reticulum. Endocr Relat Cancer 22: 217–228. doi: 10.1530/ERC-15-0018 PMID: 25670031
79. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E et al. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 306: 457–461. doi: 10.1126/science.1103160 PMID: 15486293
80. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO et al. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 313: 1137–1140. doi: 10.1126/science.1128294 PMID: 16931765
81. Schonthal AH (2013) Pharmacological targeting of endoplasmic reticulum stress signaling in cancer. Biochem Pharmacol 85: 653–666. doi: 10.1016/j.bcp.2012.09.012 PMID: 23000914
82. Gonzales JC, Gentile CL, Pfaffenbach KT, Wei Y, Wang D, Pagliassotti MJ (2008) Chemical induction of the unfolded protein response in the liver increases glucose production and is activated during insulin-induced hypoglycaemia in rats. Diabetologia 51: 1920–1929. doi: 10.1007/s00125-008-1094-9 PMID: 18651128
83. Iorio V, Festa M, Rosati A, Hahne M, Tiberti C, Capunzo M et al. (2015) BAG3 regulates formation of the SNARE complex and insulin secretion. Cell Death Dis 6: e1684. doi: 10.1038/cddis.2015.53 PMID: 25766323
84. Wu J, Kaufman RJ (2006) From acute ER stress to physiological roles of the Unfolded Protein Response. Cell Death Differ 13: 374–384. doi: 10.1038/sj.cdd.4401840 PMID: 16397578
85. Benbrook DM, Long A (2012) Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. Exp Oncol 34: 286–297. PMID: 23070014
86. Halban PA, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ et al. (2014) beta-cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment. Diabetes Care 37: 1751–1758. doi: 10.2337/dc14-0396 PMID: 24812433
87. Hollen J, Weissman JS (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. Science 313: 104–107. doi: 10.1126/science.1129631 PMID: 16825573
88. Cao SS, Kaufman RJ (2012) Unfolded protein response. Curr Biol 22: R622–626. doi: 10.1016/j.cub.2012.07.004 PMID: 22917505