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ORIGINAL ARTICLE

Effects of Hypoglycaemia on Neurotransmitter and Hormone Receptor Gene Expression in Laser-Dissected Arcuate Neuropeptide Y/Agouti-Related Peptide Neurones

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Arcuate neuropeptide Y (NPY)/agouti-related pepide (AgRP) neurones regulate energy homeostasis, and express the putative glucosensor, glucokinase (GCK). The present study performed multi-transcriptional profiling of these neurones to characterise NPY, AgRP and GCK gene expression during intermediate insulin-induced hypoglycaemia, and to determine whether these transcriptional responses acclimate to repeated insulin dosing. We also examined whether these neurones express insulin, glucocorticoid and oestrogen receptor gene transcripts, and whether the levels of these receptor mRNAs are modified by insulin-induced hypoglycaemia. Individual NPY-immunoreactive neurones were laser-microdissected from the caudal arcuate nucleus after single or serial dosing with neutral protamine Hagedorn insulin (NPH), and evaluated by quantitative real-time reverse transcriptase-polymerase chain reaction for the assessment of neurotransmitter and receptor gene expression. Mean NPY and AgRP mRNA in harvested NPY neurones was unchanged or augmented, respectively, by one NPH dose, although repeated NPH administration up-regulated NPY, whereas AgRP gene transcripts were down-regulated. NPH elicited divergent modifications in the ERα and ERβ mRNA content of sampled neurones. ERα transcripts were amplified by both acute and chronic NPH-induced hypoglycaemia, whereas ERβ gene expression was unaltered during a single bout, but suppressed during recurring hypoglycaemia. Glucocorticoid receptor (GR) mRNA levels were increased by a single insulin dose, but unaffected by serial NPH dosing. Insulin receptor-β chain (InsRb) gene transcripts were insensitive to acute NPH-induced hypoglycaemia, but repeated NPH inhibited this gene transcript. Neither acute nor recurring hypoglycaemia modified GCK mRNA levels in caudal hypothalamic arcuate nucleus (ARH) NPY/AgRP neurones, but baseline GCK transcription was suppressed by the latter. This evidence for the habituation of hypoglycaemic patterns of InsRb, GR and ERβ gene transcription to serial NPH dosing implies that such treatment may alter reactivity of caudal ARH NPY/AgRP neurones to receptor ligands, and supports the need to determine whether adaptive changes in neuronal sensitivity to insulin, corticosterone and/or oestrogen cause up- versus down-regulation of NPY and AgRP neurotransmission, respectively, by this caudal ARH subpopulation during chronic hypoglycaemia.

Key words: neutral protamine Hagedorn insulin, neuropeptide Y, laser catapult microdissection, insulin receptor, glucocorticoid receptor, estrogen receptor α, estrogen receptor β.

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The hypothalamic arcuate nucleus (ARH) participates in the control of energy homeostasis through integration of central and peripheral signals of metabolic status, including ambient nutrient levels (1–4). Several ARH neurotransmitters function as effectors of metabolic input to this structure, including the 36 amino acid anabolic peptide, neuropeptide Y (NPY). NPY exerts wide-ranging control over energy balance through the regulation of food intake, glucoregulatory hormone secretion (e.g. insulin, glucagon and corticosterone)

and reproductive neuroendocrine function (5–7). ARH NPYergic neurones co-express the orexigenic neurotransmitter, agouti-related peptide (AgRP) (8, 9), which functions as an endogenous antagonist of the anorectic neuropeptide, α-melanocyte-stimulating hormone, at MC3/4 receptors (10). Insulin is secreted by pancreatic β-cells during energy surfeit, and decreases blood glucose levels. Intracranial insulin administration decreases ARH NPY gene expression in the fed state (11) and reverses fasting-induced increases in NPY mRNA levels (12); the effects of this hormone on AgRP gene transcription remain unclear. Adrenal glucocorticoids are secreted in response to energy insufficiency and increase glycaemic profiles. NPY and AgRP gene transcripts are decreased by adrenalectomy (13) but are stimulated by the exogenous glucocorticoid, dexmethasone (14). At present, the issue of whether *in vivo* regulatory actions of insulin and glucocorticoids on ARH NPY/AgRP neurones occur by direct or indirect mechanisms remains controversial.

The ovarian steroid, oestrogen, exerts divergent, concentration-dependent effects on NPY; low physiological levels of oestradiol inhibit NPY gene expression and neurotransmission, whereas elevated plasma hormone levels stimulate these neurones (7, 15). Oestrogenic action on target cells is mediated, in part, by ligand-activated nuclear receptors that function as transcription factors (16). Two oestrogen receptor (ER) variants (e.g. ERα and ERβ) are expressed in the brain, including the ARH (17). These receptor subtypes share moderate homology of their ligand-binding domain and bind 17β-oestradiol with similar affinity (18), but their N-terminal domains differ significantly (19), a dichotomy that supports the possibility that activation of EBα and ERβ may elicit divergent cellular responses through differential effects at DNA AP-1 regulatory sites (20). Recent *in vitro* studies reveal that cloned NPY/AgRP-secreting murine hypothalamic cell lines express both ERα and ERβ (21), although it is not known whether ARH NPY/AgRP neurones express one or both ER variants *in vivo*.

Insulin-induced hypoglycaemia is an unremitting complication of conventional therapeutic management of insulin-dependent diabetes mellitus, which is commonly based on daily intermediate-release insulin injection (22). Our studies show that administration of the intermediate-acting insulin, neutral protamine Hagedorn insulin (NPH), elicits gender-specific alterations in ARH NPY mRNA levels and, in each sex, serial dosing with this formulation causes adaptation of hypoglycaemic patterns of NPY gene expression (23, 24). However, it is unclear whether ARH AgRP mRNA levels are modified by NPH-induced hypoglycaemia and, if so, whether AgRP transcriptional reactivity to this stimulus parallels or diverges, at the cellular level, from that of NPY. It is also not known if hypoglycaemic patterns of AgRP gene expression habituate to recurring NPH treatment. The low-affinity, high K_m hexokinase, glucokinase (GCK), is an intracellular glucose monitor in pancreatic β-cells, and is purported to fulfill a similar function in the brain (25). GCK is expressed in several neural structures that regulate metabolic stasis, including the ARH (26), where it is localised to NPY and pro-opiomelanocortin neurones (27). Recent studies show that whole-ARH ARH GCK gene expression adapts to chronic NPH administration in a sex-specific manner (28), although the impact of acute versus chronic induction of this metabolic stress on NPY cellular GCK mRNA content has not been investigated. Our studies show that precedent intermediate insulin-induced hypoglycaemia alters baseline hormone receptor [insulin receptor-β chain (InsRb), type II glucocorticoid receptor (GR), ERα and ERβ] gene expression in the whole ARH, as well as InsRb, GR and ER transcriptional responses to serial NPH dosing (29), although that work did not shed light on receptor gene activity in neurochemically-characterised cells, including NPY/AgRP neurones.

The current studies applied quantitative real-time reverse transcription-polymerase chain reaction (qPCR) to individual laser-microdissected ARH NPY/AgRP neurones from the caudal ARH to investigate the hypotheses: (i) NPY and AgRP gene responses to NPH deviate at the single-cell level; (ii) these neuropeptide gene transcription rates adapt, albeit differentially, to repeated NPH dosing; and (iii) habituation of neuropeptide transcriptional reactivity correlates with modified GCK gene responses to NPH. We also examined whether these cells co-express insulin, glucocorticoid and ER mRNAs, and whether expressed hormone receptor gene transcripts are sensitive to acute and chronic intermediate insulin-induced hypoglycaemia.

Materials and methods

Animals

Ten days before the experiment, adult female Sprague-Dawley rats (220–280 g body weight) were bilaterally ovariectomised (OVX), under ketamine/xylazine anesthesia (0.1 ml/100 g body weight i.p.; 90 mg ketamine:10 mg xylazine/ml; Henry Schein Inc., Melville, NY, USA), and implanted with s.c. silastic capsules (inner diameter 0.062 inches, outer diameter 0.125 inches, 10 mm/100 g body weight) filled with 30 μg of oestradiol benzoate/ml safflower oil, as previously described (30). This oestrogen replacement strategy results in plasma hormone levels that approximate basal physiological oestradiol concentrations in intact cycling female rats (31). The present studies utilised an experimental model for recurrent intermediate insulin-induced hypoglycaemia, based upon serial s.c. injection of NPH, aiming to replicate the route of delivery, frequency of administration, and duration of insulin action in the clinical setting (32, 33). This animal model is characterised by exacerbated hypoglycaemia, impaired glucagon and adrenomedullary catecholamine secretion, and attenuated central nervous system (CNS) neuronal transcriptional activation in male rats, but oestrogen-dependent uniformity of glycaemic and Fos protein responses to acute and recurring hypoglycaemia in females. On days 1–4 of the experiment, groups of animals (n = 5 per group) were injected s.c. at 11.00 h with NPH (12.5U/kg body weight) or vehicle according to the schedule (Table 1): group 1, vehicle on days 1–4 (VVVV); group 2, vehicle on days 1–3, followed by NPH on day 4 (VVVI); group 3, NPH on days 1–3, followed by and vehicle on day 4 (IIIV); group 4, NPH on days 1–4 (IIII).

Table 1. Experimental Design.

Treatment groups	Day 1	Day 2	Day 3	Day 4
Group 1: VVVV	Vehicle s.c.	Vehicle s.c.	Vehicle s.c.	Vehicle s.c.
Group 2: VVVI	Vehicle s.c.	Vehicle s.c.	Vehicle s.c.	NPH s.c.
Group 3: IIIV	NPH s.c.	NPH s.c.	NPH s.c.	Vehicle s.c.
Group 4: IIII	NPH s.c.	NPH s.c.	NPH s.c.	NPH s.c.

NPH, neutral protamine Hagedorn insulin.

Quick immunostaining of NPY neurones

Two hours after the final injections on day 4, the animals were sacrificed by decapitation. Glucose levels were measured in trunk blood with an Accu-Chek™ Advantage glucometer (Roche Diagnostics, Basal, Switzerland), and evaluated by one-way ANOVA and Duncan's multiple range test. The brains were snap frozen in cooled isopentane, and 10-μm thick serial frozen sections were cut through the hypothalamus at the level of the ARH. Sections were collected at approximately −3.2 mm relative to bregma, mounted on PALM polyethylene naphthalate membrane-coated slides (prod. no. 911724; Carl Zeiss, Microimaging Inc., Thornwood, NY, USA) and stored at −80 °C. After tissues were thawed to room temperature for 30 s, they were fixed with ice-cold acetone for 5 min, washed with 0.1 M Tris-buffered saline, 0.9% NaCl, pH 7.2 (TBS), then blocked with normal goat serum (Vectastain Elite ABC Rabbit IgG kit; prod. no. PK-6101; Vector Laboratories, Burlingame, CA, USA) diluted in TBS containing 0.05% Triton X-100 (TBX-TX), for 15 min. The sections were subsequently incubated for 30 min with a rabbit anti-NPY primary antiserum (prod. no.22940, 1 : 500; ImmunoStar Inc., Hudson, WI, USA) diluted in TBS-TX. The tissues were then sequentially incubated, for 25 min each, with biotinylated goat anti-rabbit antibodies and ABC reagent (Vectastain Elite ABC Rabbit IgG kit,) diluted in TBS. NPY-immunoreactive (-ir) neurones were visualised by incubation with Vector diaminobenzidine kit reagents (prod. no. PK-4100; Vector Laboratories) for approximately 3–5 min. All reagents were prepared in diethyl pyrocarbonate-treated water.

Laser catapult microdissection of NPY-ir neurones from the ventromedial region of the caudal ARH

A PALM UV-A microlaser (Carl Zeiss Microimaging Inc.) was utilised at 44 mV for automated photoablation, under × 20 objective, of a 4 μm wide zone peripheral to individual NPY-ir-positive neurone cell bodies. Only neurones that exhibited a visible intact nucleus and complete staining of the cytoplasmic compartment were selected for harvesting. After dissociation of individual immunoreactive neurones from surrounding tissue, each cell was ejected from the object plane, by a single subfocal laser pulse, directly into a separate 0.5 ml PALM Adhesive Cap (prod. no. 415101-4400-255; Carl Zeiss Microimaging, Inc.) containing 30 μl of ArrayPure Nano-Scale Lysis Solution with 5.0 μg of proteinase K (prod. no. MPS04050; Epicentre Biotechnol Inc., Madison, WI, USA).

Single-neurone RNA extraction and amplification

Total RNA was isolated from each of ten neurones collected per treatment group, two per rat, with ArrayPure Nano-Scale RNA Purification Kit reagents (Epicentre Biotechnol Inc.), in accordance with the manufacturer's instructions. Briefly, the cells were individually incubated in Lysis Solution for 15 min at 65–70 °C, and protein was precipitated by addition of 18 μl of MPC Protein Precipitation, followed by vortexing and centrifugation at 10 000 *g* for 7 min at 4 °C. Fify microlitres of isopropanol was added to the supernatant, followed by centrifugation at 10 000 *g* for 5 min at 4 °C. Contaminating DNA was removed from preparations: pellets were air-dried for 5 min, resuspended in 20 μl of DNase I solution containing 1 μl of RNase-Free DNase I and 40 μl of 1 × DNase buffer, then incubated for 10 min. After addition of 20 μl each of 2X Nano-Scale Lysis Solution and MPC Protein Precipitation Reagent, the mixtures were vortexed and centrifuged at 10 000 *g* for 5 min at 4 °C. After the addition of 50 ul isopropanol, the supernatants were centrifuged at 10 000 *g* for 5 min at 4 °C to pellet purified RNA. Final rinsing was carried out with 70% ethanol. Resulting pellets were air-dried for 5 min, and resuspended in 5 μl of RNase-Free water supplemented with 1 μl of ScriptGuard RNase inhibitor (Epicentre Biotechnol Inc.). All RNA samples were stored at −80 °C. Single-cell RNA samples were evaluated in a Bio-Rad Experion system (Bio-Rad, Hercules, CA, USA), using Experion RNA HighSens chips. An aliquot of 1–2 μl per cellular RNA sample was amplified with TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit materials (Epicentre Biotechnol Inc.), in accordance with the manufacturer's instructions: (i) Round one, first-strand cDNA synthesis: Poly(A) RNA was reverse-transcribed into first-strand cDNA by a T7-Oligo(dT) primer containing a phage T7 RNA polymerase promoter sequence at the 5′-end, catalysed by super-script III reverse transcriptase; (ii) Round one, second-strand cDNA synthesis: the RNA component of the generated cDNA: RNA hybrid was digested by RNase H into RNA fragments for priming of second-strand cDNA synthesis, aiming to generate double-stranded cDNA containing the T7 transcription promoter in an orientation appropriate for anti-sense RNA (aRNA) production; (iii) Round one, *in vitro* transcription: aRNA was generated from double-stranded cDNA produced in step 2; (iv) Round one, RNA purification: synthesised aRNA was purified by spin column chromatography; (v) Round two, first-strand cDNA synthesis: purified aRNA was reverse-transcribed to first-strand cDNA using random sequence hexamer primers, catalysed by superscript II reverse transcriptase; (vi) Round two, second-strand cDNA synthesis: the RNA component of the cDNA: RNA hybrid generated in the step 5 was digested by RNase H into small fragments, for priming of second-strand cDNA synthesis to yield double-stranded cDNA containing the T7 transcription promoter in appropriate orientation for aRNA production; (vii) *In vitro* transcription of aminoallyl-aRNA: double-stranded cDNA generated in step 6 was used to produce aminoallyl-aRNA, with partial substitution of the canonical UTP nucleotide by aminoallyl-UTP. Amplified RNA was evaluated by electrophoresis in a Bio-Rad Experion system.

Single-cell qPCR

Aminoallyl-aRNA (< 50 ng) from each laser-microdissected neurone was reverse-transcribed to cDNA with Sensiscript RT Kit reagents (prod. no., 205213; Qiagen Inc., Valencia, CA, USA), in accordance with the manufacturer's instructions. Each RT reaction mixture consisted of 2 μl of 10 × RT buffer, 2 μl of dNTP mix (final concentration: 5 mM), 2 μl of oligo-dT primer solution (final concentration: 10 uM), 1 μl of RNase inhibitor (final concentration: 10 U/μl), 1 μl of Sensiscript reverse transcriptase solution, and aRNA dissolved in sufficient RNase-free water to yield a total volume of 20 μl. Forward and reverse primers for target genes (Table 2) were designed with Beacon Designer 5 software (Premier Biosoft Intl., Palo Alto, CA, USA), and obtained from Genemed Synthesis, Inc. (San Francisco, CA, USA). PCR samples consisted of 2 μl of cDNA, 10.3 μl of RNase/DNase-free water, 0.2 μl of each primer, and 12.5 μl of i*Q* SYBR Green Supermix (prod. no. 170-8880; Bio-Rad). PCR reactions were performed in a Bio-Rad i*Q*™5 iCycler system, with cyclic parameters: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 1 min each (30 s at 95 °C; followed by 30 s at 50 °C for ERβ and InsRb, 30 s at 58 °C for GR and ERα, 30 s at 51 °C for NPY, and 30 s at 61 °C for AgRP). The housekeeping gene, GAPDH, was measured for normalisation of mRNA expression in each neurone. Annealing temperatures were optimised for each primer pair. In each real-time RT-PCR analysis, no-template and -RT controls were performed. Amplified PCR products were evaluated by agarose gel electrophoresis, and melt curves were generated and analysed to identify nonspecific products and primer-dimers. The data were analysed by the $2^{\Delta\Delta}$Ct method, and group means were evaluated by one-way ANOVA, followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Mean circulating glucose levels were similar in vehicle-injected oestrogen-implanted OVX female rats at +2 h on day four of the experiment [127.5 ± 5.3 mg/dl (VVV) versus 121.3 ± 4.6 mg/dl

Table 2. Polymerase Chain Reaction Primer Sequences.

Sequence name	Accession number	Primer sequence
NPY forward primer	NM_012614	5′-ATGCTAGGTAACAAACG-3′
NPY reverse primer		5′-ATGTAGTGTCGCAGAG-3′
AGRP forward primer	XM_001075738	5′-ACCTTGCTGCGACCTGTG-3′
AGRP reverse primer		5′-GGTTCGTGGTGCCAGTAC-3′
InsRb forward primer	M29014	5′-GCAGGAACTCTCGGAAG-3′
InsRb reverse primer		5′-GATGTCACGGAATCAATGG-3′
GR forward primer	NM_012576	5′-TTGGCGACAGAAGCAGTTGAG-3′
GR reverse primer	-NR3c1	5′-CTTGGGCACGGTGGTTTAGG-3′
ERα forward primer	NM_012689	5′-AAGCACAAAGCGTAGAG-3′
ERα reverse primer		5′-GGTTCAGCATCCAATAAGG-3′
ERβ forward primer	NM_012754	5′-AAAGCCAAGAGAAACGGTGGG-CAT-3′
ERβ reverse primer		5′-GCCAATCATGTGCACCAGTTCCT-3′
GCK forward primer	NM_012565	5′- GAGACTGTTGCCTTCATATAC-3′
GCK reverse primer		5′- GACCTGAGTGTTGGAGATG-3′
GAPDH forward primer	NM_017008	5′-ACAGCCGCATCTTCTTGTGC-3′
GAPDH reverse primer		5′-GCCTCACCCCATTTGATGTT-3′

AgRP, agouti-related pepide; ER, oestrogen receptor; GCK, glucokinase; GR, glucocorticoid receptor; InsRb, Insulin receptor-β chain; NPY, neuropeptide Y.

(IIIV)]. Injection of either one or the fourth of four doses of NPH on day 4 caused a significant decrease in blood glucose relative to controls [VVVI versus VVVV; IIII versus IIIV; glucose values were reduced to an equivalent level in VVVI [35.0 + 1.0 mg/dl] versus IIII groups [38.5 + 1.3 mg/dl].

The coronal map in Fig. 1(A) illustrates the approximate rostrocaudal level of the ARH (3.2 mm posterior to bregma) from which NPY neurones were harvested. The red rectangle in Fig. 1(A) depicts the medial hypothalamus, and is enlarged in Fig. 1(B). The red ovals in Fig. 1(B) depict the ventromedial ARH; NPY-ir neurones in this site were identified by a quick-immunostaining protocol involving rabbit polyclonal primary antibodies and Vector Elite ABC and substrate kit reagents. Figure 1(C) shows several representative NPY-ir-positive neurones (black arrows) in the red oval-enclosed ventromedial ARH; the yellow arrow points to the ependymal lining of the third ventricle (V3). Figure 1(D1) depicts the positioned path of the PALM microlaser, indicated by the red line, peripheral to two separate neurone cell bodies (white asterisks). The black asterisks in Fig. 1(D2) illustrate the spaces that remain after laser-catapulting of the asterisk-labelled neurones in Fig. 1(D1). It should be noted that the vacant area reflects the removal of an outer 4 μm-wide ablation ring, as well as the ejected cell, and that this method of microdissection causes minimal damage to surrounding tissue.

The data shown in Fig. 2(A) demonstrate that mean NPY mRNA levels in laser-microdissected caudal ARH NPY-ir-positive ARH neurones were not modified in response to a single NPH dose, but were significantly enhanced in response to serial insulin administration. By contrast, AgPR gene transcription in the same cells was increased during acute NPH-induced hypoglycaemia, but was suppressed in response to recurring hypoglycaemic bouts (Fig. 2B). As shown in Fig. 3(A), InsRb mRNA levels in NPY/AgRP neurones were resistant to acute NPH administration, but serial exposure to NPH resulted in augmented baseline transcription, as well as inhibitory transcriptional reactivity to recurring insulin treatment. GR gene transcripts in the harvested neurones were increased by a single insulin dose, but were unchanged after multiple injections (Fig. 3B). The present studies demonstrate that ARH NPY/AgRP neurones express genes that encode both ERα and ERβ, and that these mRNAs exhibited dissimilar responses to acute and chronic NPH administration. Figure 4(A) shows that ERα transcripts in sampled neurones were increased by both acute and chronic insulin administration. By contrast, ERβ mRNA levels were unchanged after one NPH injection, but were decreased in response to serial NPH dosing, relative to adaptive augmentation of basal gene transcription (Fig. 4B). The mean ratio of ERα : ERβ transcripts in NPY/AgRP neurones was significantly increased during acute and chronic NPH administration (Fig. 5), but was suppressed on day 4 prior to delivery of the final dose of insulin. The data shown in Fig. 6 demonstrate that NPY GCK gene transcription rates were unresponsive to both acute and chronic insulin administration, but that baseline gene expression was significantly diminished as a result of repeated exposure to NPH.

Discussion

The present studies utilised quantitative real-time RT-PCR to measure mRNAs encoding neurotransmitters, GCK, and insulin, glucocorticoid and oestrogen receptors, in caudal ARH NPY-ir neurones harvested from OVX, oestrogen-implanted female rats after single versus serial dosing with the intermediate-acting insulin, NPH. The results obtained show that genes encoding NPY and AgRP respond differently, at the single-cell level, to intermediate insulin-induced hypoglycaemia. Acute NPH injection did not modify NPY mRNA content, but increased AgRP transcripts in harvested neurones, whereas recurring NPH dosing either up- or down-regulated NPY and AgRP gene transcripts, respectively. The data further demonstrate that the GCK mRNA content of NPY/AgRP neurones is resistant to single or serial NPH-induced hypoglycaemia, but that basal GCK transcription rates decline after repeated induction of this metabolic stress. The present work provides unique evidence that ARH NPY/AgRP neurones contain InsRb, GR, ERα and ERβ gene transcripts, implying that these cells function as a common target for insulin, glucocorticoid and oestrogen actions on the brain. The discovery of chronic NPH-associated adjustments in basal and/or hypoglycaemic patterns of InsRb, GR and ERβ gene expression suggests that recurring hypoglycaemia may modify NPY/AgRP neuronal sensitivity to insulin, glucocorticoid and ERβ-specific oestrogen signalling. Taken together, these studies offer new insight on functional habituation of ARH NPY/AgRP neurones to intermediate insulin-induced hypoglycaemia, and support the need to investigate potential causal relationships between adaptive adjustments in glucose/hormone receptivity and NPY/AgRP neurotransmission.

The current evidence for the positive habituation of NPY gene transcription rates in microdissected caudal ARH NPY-ir neurones

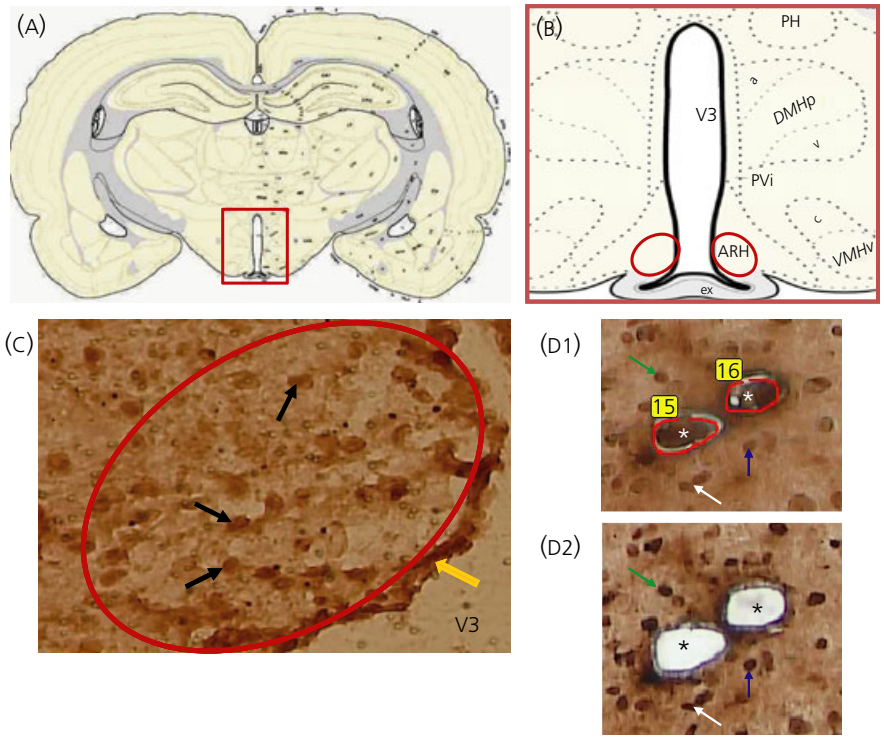

Fig. 1. Laser-catapult microdissection of hypothalamic arcuate nucleus (ARH) neuropeptide Y (NPY)-immunoreactive (-ir) neurones. (A) Approximate rostrocaudal level of the ARH at which NPY neurones were harvested; the red rectangle depicts the location of the ARH within medial hypothalamus. The red ovals in (B) illustrate the location of ventromedial ARH NPY-ir neurones that were harvested after quick-immunostaining. (C) Showing several representative NPY-ir-positive neurones (black arrows) in the red oval-enclosed ventromedial ARH; the yellow arrow points to the ependymal lining of the third ventricle (V3). (D1) The positioned path of the PALM microlaser, indicated by the red line, peripheral to two separate neurone cell bodies (white asterisks). The black asterisks in (D2) illustrate the spaces that remain after laser-catapulting of the asterisk-labelled neurones in (D1). It should be noted that the vacant area reflects the removal of an outer 4 μm-wide ablation ring, as well as the ejected cell, and that this method of microdissection causes minimal damage to surrounding tissue. DMHa,p,v, anterior, posterior, and ventral parts of the DMH; VMHc,vl, central and ventrolateral parts of the ventromedial hypothalamic nucleus; Meex, median eminence, external lamina; Pvi, intermediate periventricular hypothalamic nucleus; PH, posterior hypothalamic nucleus; V3, third ventricle.

to NPH-induced hypoglycaemia is consistent with previous studies demonstrating that whole-ARH NPY mRNA is up-regulated during serial dosing of oestrogen-replaced OVX female rats (23). AgRP transcripts in laser-harvested cells were increased during acute hypoglycaemia, but diminished during chronic induction of this metabolic stress; the data further demonstrate that baseline AgRP gene expression was elevated by recurring NPH administration. Although it is presumed that these measures of altered cellular NPY and AgRP mRNA content coincide with net changes in neurotransmitter production, it is acknowledged that neurochemical mRNA and peptide expression patterns may deviate in an as yet undetermined proportion of these neurones. At present, it is unclear whether NPY and AgRP are co-packaged within all or a subset of synaptic vesicles, or contained with separate vesicle populations in NPY/AgRP axon terminals. Further research is required to determine how divergent modifications in NPY and AgRP production during NPH-induced hypoglycaemia impact absolute and relative release rates of these molecules at central synapses. There is also a need to characterise the physiological and behavioral consequences of potential hypoglycaemia-associated changes in NPY and AgRP neurotransmission, such as effects on food intake (NPY and AgRP), reproductive neuroendocrine function (NPY), glucoregulatory hormone secretion (NPY) and mood disorders, including anxiety and depression (NPY (34–36)). It would be informative to determine whether amplification of baseline AgRP gene transcription after recovery from the third of three precedent NPH injections constitutes a 'rebound' from inhibitory effects of recurring insulin dosing, and to determine the functional impact of this adaptation on AgRP-sensitive functions at those post-recovery time points.

Microdissected NPY-ir neurones exhibited no change in GCK mRNA content during acute or chronic NPH-induced hypoglycaemia, but showed a net decline in basal GCK gene transcription during recurring insulin treatment. These results do not preclude the possibility that GCK protein production in these cells may be altered. Previous studies in our laboratory showed that both acute and chronic NPH dosing reduced counts of GCK-immunoreactive neurones in the caudal ARH of oestrogen-implanted OVX female rats, suggesting that, during acute hypoglycaemia, GCK protein expression in caudal NPY/AgRP neurones may be decreased, relative to baseline, despite the maintenance of gene transcription rates. Further studies are needed to investigate the impact of adaptive reductions in baseline GCK mRNA on glucose sensing functionality between bouts of recurring hypoglycaemia.

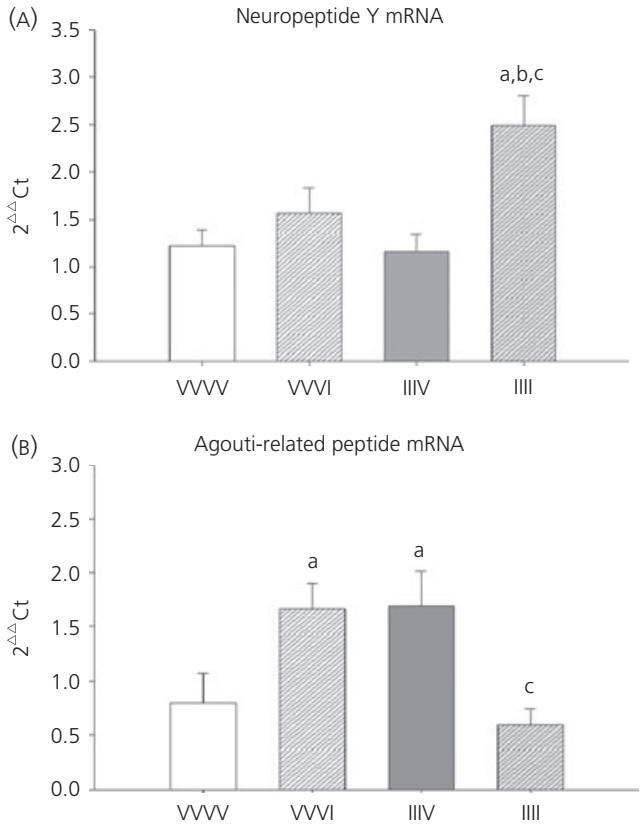

Fig. 2. Effects of single versus serial injection of neutral protamine Hagedorn insulin (NPH) on neuropeptide Y (NPY) (A) and agouti-related pepide (B) gene expression in laser-microdissected hypothalamic arcuate nucleus (ARH) NPY-immunoreactive (-ir) neurones. ARH NPY-ir neurones were harvested by laser-catapult microdissection from oestradiol-implanted ovariectomised female rats injected s.c. with: (i) four doses of vehicle, one dose per day (VVVV; solid white bar); (ii) vehicle on days 1–3, followed by NPH on day 4 (VVVI; diagonal-striped white bar); (iii) NPH on days 1–3, followed by vehicle on day 4 (IIIV; solid grey bar); or (iv) four doses of NPH, one dose per day (IIII; diagonal-striped grey bar). The data are the mean ± SEM mRNA levels, respectively, as calculated by the $2^{\Delta\Delta}$Ct method for n = 10 NPY-ir neurones. $^{a}P < 0.05$ compared to VVVV; $^{b}P < 0.05$ compared to VVVI; $^{c}P < 0.05$ compared to IIIV.

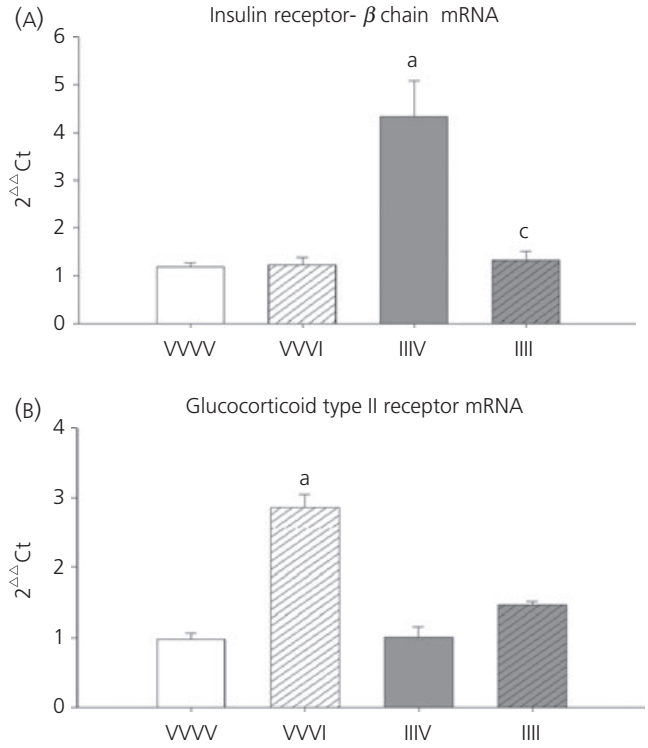

Fig. 3. Effects of single versus serial neutral protamine Hagedorn insulin (NPH) injection on insulin receptor-β chain (A) and type II glucocorticoid receptor (B) gene transcription in laser-microdissected hypothalamic arcuate nucleus (ARH) neuropeptide Y (NPY)-immuoreactive (-ir) neurones. Single ARH NPY-ir neurones were removed by laser-catapult microdissection from oestradiol-implanted ovariectomised female rats injected s.c. with: (i) four doses of vehicle, one dose per day (VVVV; solid white bar); (ii) vehicle on days 1–3, followed by NPH on day 4 (VVVI; diagonal-striped white bar); (iii) NPH on days 1–3, followed by vehicle on day 4 (IIIV; solid grey bar); or (iv) four doses of NPH, one dose per day (IIII; diagonal-striped grey bar). The data depict mean ± SEM mRNA levels , respectively, as calculated by the $2^{\Delta\Delta}$Ct method, for n = 10 NPY-ir neurones. $^{a}P < 0.05$ compared to VVVV; $^{b}P < 0.05$ compared to VVVI; $^{c}P < 0.05$ compared to IIIV.

The present evidence for co-expression of InsRb, GR, ERα and ERβ gene transcripts by caudal ARH NPY/AgRP neurones supports the concept that these cells serve as a mutual substrate for insulin, glucocorticoid, and oestrogen action *in vivo*, and that NPY/AgRP neurones may function to integrate regulatory effects of these hormones on ingestive, reproductive, glucoregulatory and other functions. The results obtained in the present study show that InsRb and GR gene expression levels habituated to insulin-induced hypoglycaemia through reductions in transcription rates; for example, InsRb mRNA levels were unaltered by acute NPH, but suppressed relative to baseline by chronic NPH, whereas GR gene transcription was stimulated by acute NPH, but unchanged from baseline during chronic NPH. These data suggest that the receptivity of ARH NPY/AgRP neurones to insulin and glucocorticoid input may be comparatively diminished during recurring versus acute hypoglycaemia; it remains to be determined whether serial insulin dosing causes a corresponding decline in cellular InsRb and GR receptor protein levels. There is also a need to evaluate the potential role of adapted cellular sensitivity to insulin and glucocorticoid signalling in the habituation of hypoglycaemic patterns of NPY and AgRP gene expression. Basal InsRb gene expression by this cell population was increased after recovery from the third insulin dose, and these results suggest that, in the nondiabetic model used in the present study, NPY neurones may exhibit augmented sensitivity to endogenous insulin, for an as yet undetermined length of time, between bouts of exogenous insulin-induced hypoglycaemia. Further research involving the implementation of hyperinsulinemic-euglycaemic clamps is required to discriminate between the effects of systemic hyperinsulinemia versus hypoglycaemia *per se* on neurotransmitter, InsRb and GR gene responses by NPY neurones to acute and recurring NPH treatment.

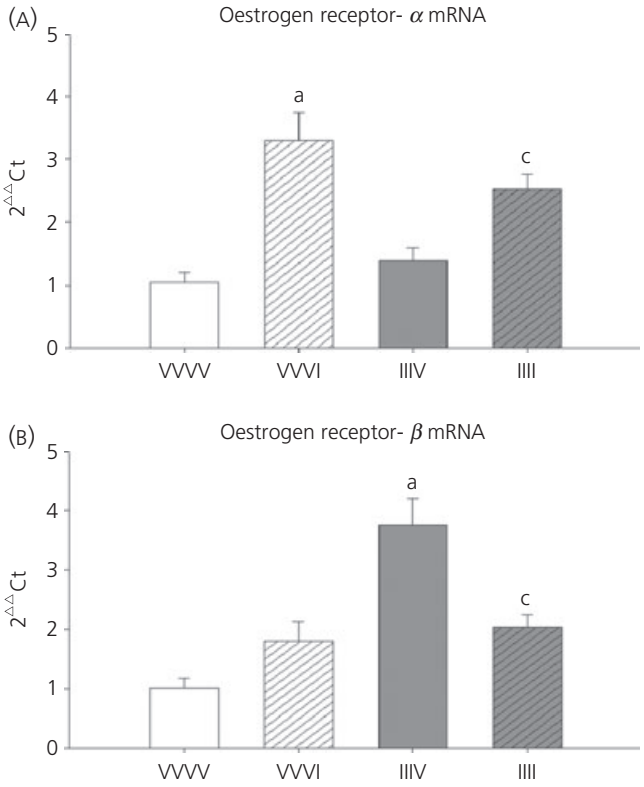

Fig. 4. Effects of single versus serial neutral protamine Hagedorn insulin (NPH) dosing on oestrogen receptor α (A) and oestrogen receptor β (B) gene transcription in laser-microdissected hypothalamic arcuate nucleus (ARH) neuropeptide Y (NPY)-immuoreactive (-ir) neurones. Individual ARH NPY-ir neurones were microdissected from oestradiol-implanted ovariectomised female rats injected s.c. with: (i) four doses of vehicle, one dose per day (VVVV; solid white bar); (ii) vehicle on days 1–3, followed by NPH on day 4 (VVVI; diagonal-striped white bar); (iii) NPH on days 1–3, followed by vehicle on day 4 (IIIV; solid grey bar); or (iv) four doses of NPH, one dose per day (IIII; diagonal-striped grey bar). The data depict mean ± SEM mRNA levels , respectively, as calculated by the $2^{\Delta\Delta}$Ct method for n = 10 NPY-ir neurones. $^{a}P < 0.05$ compared to VVVV; $^{b}P < 0.05$ compared to VVVI; $^{c}P < 0.05$ compared to IIIV.

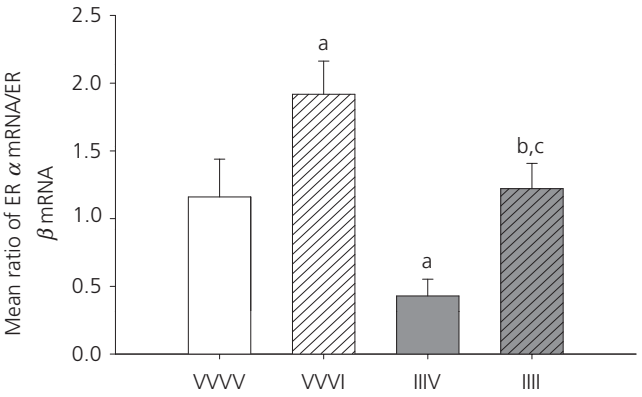

Fig. 5. Effects of acute versus chronic neutral protamine Hagedorn insulin (NPH) administration on mean ratio of oestrogen receptor (ER) α versus ERβ mRNA in laser-microdissected neuropeptide Y (NPY)/agouti-related pepide neurones. The data depict mean ratios of ERα : ERβ gene transcripts for harvested NPY-immunoreactive (-ir) neurones collected from oestradiol-implanted ovariectomised (OVX) female rats injected s.c. with: (i) four doses of vehicle, one dose per day (VVVV; solid white bar); (ii) vehicle on days 1–3, followed by NPH on day 4 (VVVI; diagonal-striped white bar); (iii) NPH on days 1–3, followed by vehicle on day 4 (IIIV; solid grey bar); or (iv) four doses of NPH, one dose per day (IIII; diagonal-striped grey bar). $^{a}P < 0.05$ compared to VVVV; $^{b}P < 0.05$ compared to VVVI; $^{c}P < 0.05$ compared to IIIV.

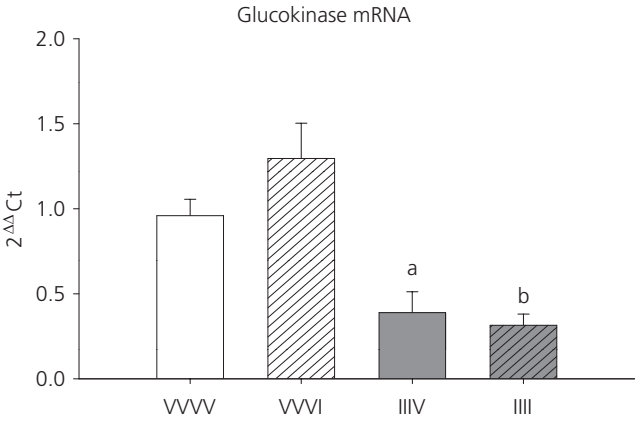

Fig. 6. Effects of single versus serial neutral protamine Hagedorn insulin (NPH) injection on glucokinase (GCK) gene transcription in laser-microdissected hypothalamic arcuate nucleus (ARH) neuropeptide Y (NPY)-immuoreactive (-ir) neurones. Individual ARH NPY-ir neurones were microdissected from oestradiol-implanted ovariectomised (OVX) female rats injected s.c. with: (i) four doses of vehicle, one dose per day (VVVV; solid white bar); (ii) vehicle on days 1–3, followed by NPH on day 4 (VVVI; diagonal-striped white bar); (iii) NPH on days 1–3, followed by vehicle on day 4 (IIIV; solid grey bar); or (iv) four doses of NPH, one dose per day (IIII; diagonal-striped grey bar). The data depict depict mean ± SEM GCK mRNA levels, respectively, as calculated by the $2^{\Delta\Delta}$Ct method for n = 10 NPY-ir neurones. $^{a}P < 0.05$ compared to VVVV; $^{b}P < 0.05$ compared to VVVI; $^{c}P < 0.05$ compared to IIIV.

In studies on adult male rats, we and others have shown that systemic insulin administration increases ARH NPY gene expression (24, 37), whereas other studies report that this mRNA is reduced after intracranial delivery of insulin (11). This disparity suggests that, in this sex, the collective effects of body-wide insulin actions, involving central and peripheral targets, elevate ARH NPY gene expression, whereas selective, unopposed effects of insulin on the brain exert an opposite (e.g. negative) influence on NPY transcription rates. Because we have not compared intracranial versus systemic insulin effects on ARH NPY gene transcription in female rats, we do not know whether CNS-specific or body-wide actions of insulin exert similar or opposite effects on NPY or AGRP mRNA levels in that sex.

The present study offers novel proof of coincident ERα and ERβ mRNA expression by caudal ARH NPY/AgRP neurones *in vivo*, and these findings extend the *in vitro* findings demonstrating that immortalised NPY- and AgRP-expressing hypothalamic cell lines express both receptor variants (21). Our results show that ERα transcriptional rates were stimulated by acute NPH-induced hypoglyca-

emia, a response that did not vary between original and recurring bouts of hypoglycaemia, whereas ERβ mRNA levels were unchanged by a single insulin injection, but diminished during repeated dosing. These data suggest that cellular sensitivity to ERα-mediated oestrogen stimulation may be amplified during acute and chronic hypoglycaemia, and that the adaptation of NPY/AgRP neurones to recurring hypoglycaemia involves a reduction in ERβ-specific hormonal input. The present finding that baseline ERβ gene expression is increased after serial insulin administration suggests that receptivity of NPY/AgRP neurones to ERβ-dependent oestrogenic signalling may be augmented as a possible secondary rebound response to the inhibition of this gene profile by recurring NPH treatment. An alternative explanation for concurrent increases in baseline AgRP, InsRβ and ERβ gene transcripts subsequent to precedent insulin dosing is the existence of a causal positive relationship between tranalated products of two or more of these genes.

The present data show that the ratio of ERα and ERβ gene transcripts in caudal ARH NPY/AgRP neurones is increased during both acute and chronic hypoglycaemia, albeit to a significantly lesser extent under the latter conditions, whereas the baseline ratio is decreased after precedent dosing with NPH. These results are intriguing in light of the evidence indicating that ERα and ERβ exert dissimilar effects on NPY and AgRP mRNA levels *in vitro*, and that modifications in variant ratios are correlated with different neurotransmitter mRNA expression patterns under those conditions (21). Collectively, the present study establishes the need to gather information on distinctive versus mutual regulatory effects of ERα- versus ERβ on NPY and AgRP gene/protein expression *in vivo*, and to gain insight on the role of these receptor variants in normo- and hypoglycaemic patterns of neurotransmitter release. There is also a need to evaluate the respective roles of hyperinsulinemia and hypoglycaemia *per se* in ER transcriptional acclimation to ongoing NPH administration. Preliminary data from our laboratory implicate oestrogen in whole-ARH InsRb and GR gene responses to NPH-induced hypoglycaemia; additional studies are needed to evaluate the impact of ERα and ERβ on NPY/AgRP neuronal expression of InsRb and GR under normo- and hypoglycaemic conditions.

The current experimental design included animals injected with vehicle on the final day of the study (e.g. groups VVVV and IIIV), as controls for potential effects of the physical act of subcutaneous drug injection. Data from groups IIIV versus IIII were thus compared for the purpose of assessing neurotransmitter and receptor gene transcription rates in NPY neurones harvested from non-insulin- versus insulin-treated rats after precedent 3-day NPH dosing. Although it is presumed that mRNA measures from animals in group IIIV represent basal transcription rates of the various genes evaluated, a potential caveat is that, for one of more of these genes, baseline transcript levels may vary to some undetermined extent over the 2-h period between the time of injection and sacrifice.

In consideration of likely region-based functional heterogeneity of ARH NPY/AgRP neurones, as indicated by differences in reactivity of rostral versus caudal subpopulations to lactation (9, 38) and differentiation of GABA- and non-GABA-sensitive cells according to dorsal versus ventral location (39), the present analysis was restricted to cell samples harvested from the ventromedial portion of the caudal ARH. The results obtained in the present study do not preclude the possibility that NPY/AgRP cells located in other areas of the ARH may not express one or more of the hormone receptor genes evaluated here, and may be characterised by dissimilar patterns of neurotransmitter, receptor and GCK gene expression during acute and/or chronic NPH-induced hypoglycaemia.

The oestradiol replacement protocol employed in the present study was designed to replicate plasma oestradiol levels that characterise the low end of normal physiological range in intact cycling female rats (31). However, a caveat to this treatment strategy is that it yields static circulating hormone levels. Thus, in the absence of oestrous cycle-like fluctuations in plasma oestradiol concentrations, we cannot discount the possibility that insulin-induced hypoglycaemia may elicit differential NPY neurone transcriptional responses in the presence of unvarying versus dynamic patterns of circulating oestradiol.

In summary, the present data derived from single-cell multi-transcriptional profiling of immunocytochemically-characterised caudal ARH NPY neurones provide novel evidence for differential NPY and AgRP transcriptional responses to acute and chronic intermediate insulin-induced hypoglycaemia and, furthermore, demonstrate that these mRNA levels diverge (e.g. are up- or down-regulated, respectively) during serial NPH dosing. The present evidence indicating that ARH NPY/AgRP neurones co-express InsRb, GR, ERα and ERβ mRNA suggests that these cells may function as a common target for insulin, glucocorticoid and oestrogen signalling *in vivo*. The current observations of relative reductions in InsRb, GR and ERβ transcripts during recurring insulin-induced hypoglycaemia imply that habituation of NPY/AgRP neurones to this metabolic stress may include decreased cellular sensitivity to insulin, glucocorticoids and ERβ-mediated oestrogenic signalling during repeated exposure to this metabolic stress. Future studies are needed to explore the role of adaptive adjustments in glucose/hormone receptivity in the modification of NPY and AgRP gene expression.

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References

1 Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord* 2001; **25** (Suppl. 5): S63–S67.
2 Morton GJ, Schwartz MW. The NPY/AgRP neuron and energy homeostasis. *Int J Obes Relat Metab Disord* 2001; **25** (Suppl. 5): S56–S62.
3 Beck B. Neuropeptides and obesity. *Nutrition* 2000; **16**: 916–923.
4 van den Top M, Spanswick D. Integration of metabolic stimuli in the hypothalamic arcuate nucleus. *Prog Brain Res* 2006; **153**: 141–154.

5 Guijarro A, Laviano A, Mequid MM. Hypothalamic integration of immune function and metabolism. *Prog Brain Res* 2006; **153**: 367–405.
6 Parikh R, Marks JL. Metabolic and orexigenic effects of intraventricular neuropeptide Y are attenuated by food deprivation. *J Neuroendocrinol* 1997; **9**: 789–795.
7 Kalra SP. Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocr Rev* 1993; **14**: 507–538.
8 Hahn TM, Breninger JF, Baskin DG, Schwartz MW. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci* 1998; **1**: 271–272.
9 Chen P, Li C, Haskell-Luevanon C, Cone RD, Smith MS. Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. *Endocrinology* 1999; **140**: 2645–2650.
10 Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997; **278**: 135–137.
11 Baskin DG, Figlewicz Latteman D, Seeley RJ, Woods SC, Porte D, Schwartz MW. Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res* 1999; **848**: 114–123.
12 Sipols AJ, Baskin DG, Schwartz MW. Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. *Diabetes* 1995; **44**: 147–151.
13 Savontaus E, Conwell IM, Wardlaw SL. Effects of adrenalectomy on AGRP, POMC, NPY and CART gene expression in the basal hypothalamus of fed and fasted rats. *Brain Res* 2002; **958**: 130–138.
14 Shimizu H, Arima H, Watanabe M, Goto M, Banno R, Sato I, Ozaki N, Nagasaki H, Oiso Y. Glucocorticoids increase neuropeptide Y and agouti-related peptide gene expression via adenosine monophosphate-activated protein kinase signaling in the arcuate nucleus of rats. *Endocrinology* 2008; **149**: 4544–4553.
15 Levine JE, Bauer-Dantoin AC, Besecke LM, Conaghan LA, Legan SJ, Meredith JM, Strobl FJ, Urban JH, Vogelsong KM, Wolfe AM. Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. *Recent Prog Horm Res* 1991; **47**: 97–151.
16 McEwen BS. The molecular and neuroanatomical basis for estrogen effects in the central nervous system. *J Clin Endocrinol Metab* 1999; **84**: 1790–1797.
17 Shughrue PJ, Scrimo PJ, Merchentaler I. Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. *Endocrinology* 1998; **139**: 5267–5270.
18 Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson J. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 1997; **138**: 863–870.
19 Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J. A cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996; **93**: 5925–5930.
20 Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997; **277**: 1508–1510.
21 Titolo D, Cai F, Belsham D. Coordinate regulation of neuropeptide Y and agouti-related peptide gene expression by estrogen depends on the ratio of estrogen receptor (ER) alpha to ERbeta in clonal hypothalamic neurons. *Mol Endocrinol* 2006; **20**: 2080–2092.
22 Cryer PE. Hypoglycemia: still the limiting factor in the glycemic management of diabetes. *Endocr Pract* 2008; **14**: 750–756.
23 Nedungadi TP, Briski KP. Effects of estradiol on acute and chronic insulin-induced hypoglycemia-associated patterns of arcuate neuropeptide Y, proopiomelanocortin, and cocaine- and amphetamine related-transcript gene expression in the ovariectomized female rat. *Neuroendocrinology* 2007; **86**: 270–276.
24 Briski KP, Parihar M. Effects of orchidectomy on adaptation of arcuate neuropeptide Y, proopiomelanocortin, and cocaine- and amphetamine related-transcript gene profiles to recurring insulin-induced hypoglycemia in the male rat. *Neuropeptides* 2008; **42**: 585–591.
25 Matschinsky FM, Magnuson MK, Zelent D, Jettson TL, Doliba N, Han Y, Taub R, Grimsby K. The network of glucokinase-expressing cells in glucose homeostasis and the potential of glucokinase activators for diabetes therapy. *Diabetes* 2006; **55**: 1–12.
26 Lynch TM, Tomkins LS, Brooks HL, Dunn-Meynell AA, Levin BE. Localization of glucokinase gene expression in rat brain. *Diabetes* 2000; **49**: 693–700.
27 Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, Levin BE. Glucokinase is the likely mediator of glucosensing in both glucose excited and glucose inhibited central neurons. *Diabetes* 2002; **51**: 2056–2065.
28 Nedungadi TP, Genabai NK, Parihar M, Briski KP. Acclimation of hypothalamic arcuate glucokinase gene and protein expression to recurring insulin-induced hypoglycemia: impact of gender. *Neuroendocrinology* 2009; **89**: 377–386.
29 Genabai NK, Briski KP. Adaptation of arcuate insulin receptor, estrogen receptor-alpha, estrogen receptor-beta, and type II glucocorticoid receptor gene profiles to intermediate insulin-induced hypoglycemia in estrogen-treated ovariectomized female rats. *J Mol Neurosci* 2009; PMID: 19953342.
30 Briski KP, Marshall ES, Sylvester PW. Effects of estradiol on glucoprivic transactivation of catecholaminergic neurons in the female rat caudal brainstem. *Neuroendocrinology* 2001; **73**: 369–377.
31 Goodman RL. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. *Endocrinology* 1987; **102**: 142–150.
32 Paranjape SA, Briski KP. Recurrent insulin-induced hypoglycemia causes site-specific patterns of habituation or amplification of CNS neuronal genomic activation. *Neuroscience* 2005; **130**: 957–970.
33 Nedungadi TP, Goleman WL, Paranjape SA, Kale AY, Briski KP. Effects of estradiol on glycemic and CNS neuronal activational responses to recurrent insulin-induced hypoglycemia in the ovariectomized female rat. *Neuroendocrinology* 2007; **84**: 235–243.
34 Tschenett A, Singewald N, Carli M, Balducci C, Salchner P, Vezzani A, Herzog H, Sperk G. Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. *Eur J Neurosci* 2003; **18**: 143–148.
35 Painsipp E, Wultsch T, Edelsbrunner ME, Tasan RO, Singewald N, Herzog H, Holzer P. Reduced anxiety-like and depression-related behavior in neuropeptide Y Y4 receptor knockout mice. *Genes Brain Behav* 2008; **7**: 532–542.
36 Karlsson RM, Choe JS, Cameron HA, Thorsell A, Crawley JN, Holmes A, Heilig M. The neuropeptide Y Y1 receptor subtype is necessary for the anxiolytic-like effects of neuropeptide Y, but not the antidepressant-like effects of fluoxetine, in mice. *Psychopharmacology* 2008; **195**: 547–557.
37 Akabayashi A, Zaia CT, Silva I, Chae HJ, Leibowitz SF. Neuropeptide Y in the arcuate nucleus is modulated by alterations in glucose metabolism. *Brain Res* 1993; **621**: 343–348.
38 Li C, Chen P, Smith MS. The acute suckling stimulus induces expression of neuropeptide Y (NPY) in cells in the dorsomedial hypothalamus and increases NPY expression in the arcuate nucleus. *Endocrinology* 1998; **139**: 1645–1642.
39 Horvath TL, Bechmann I, Naftolin F, Kalra SP, Leranth C. Heterogeneity in the neuropeptide Y-containing neurons of the rat arcuate nucleus: GABAergic and non-GABAergic subpopulations. *Brain Res* 1997; **756**: 283–286.