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M. Proszkowiec-Weglarz, Joëlle Dupont, Nicole Rideau, C. Gespach, Jean Simon, et al.. Insulin immuno-neutralization decreases food intake in chickens without altering hypothalamic transcripts involved in food intake and metabolism. *Poultry Science*, 2017, 96 (12), pp.4409-4418. 10.3382/ps/pex247 . hal-02624622

**HAL Id: hal-02624622**

**<https://hal.inrae.fr/hal-02624622>**

Submitted on 26 May 2020

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# Insulin immuno-neutralization decreases food intake in chickens without altering hypothalamic transcripts involved in food intake and metabolism

M. Proszkowiec-Weglarz,<sup>\*,1</sup> J. Dupont,<sup>†</sup> N. Rideau,<sup>†</sup> C. Gespach,<sup>‡</sup> J. Simon,<sup>†</sup> and T. E. Porter<sup>\*,2</sup>

<sup>\*</sup>Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742; <sup>†</sup>Station de Recherches Avicoles (UR 83), INRA, 37380 Nouzilly, France; and <sup>‡</sup>INSERM U938, Molecular and Clinical Oncology, Hôpital Saint Antoine, Université Pierre et Marie Curie Paris 6, 75012 Paris, France

**ABSTRACT** In mammals, insulin regulates blood glucose levels and plays a key regulatory role in appetite via the hypothalamus. In contrast, chickens are characterized by atypical glucose homeostasis, with relatively high blood glucose levels, reduced glucose sensitivity of pancreatic beta cells, and large resistance to exogenous insulin. The aim of the present study was to investigate in chickens the effects of 5 h fasting and 5 h insulin immuno-neutralization on hypothalamic mRNA levels of 23 genes associated with food intake, energy balance, and glucose metabolism. We observed that insulin immune-neutralization by administration of anti-porcine insulin guinea pig serum (AI) significantly de-

creased food intake and increased plasma glucose levels in chickens, while 5 h fasting produced a limited and non-significant reduction in plasma glucose. In addition, 5 h fasting increased levels of *NPY*, *TAS1R1*, *DIO2*, *LEPR*, *GLUT1*, *GLUT3*, *GLUT8*, and *GCK* mRNA. In contrast, AI had no impact on the levels of any selected mRNA. Therefore, our results demonstrate that in chickens, food intake inhibition or satiety mechanisms induced by insulin immuno-neutralization do not rely on hypothalamic abundance of the 23 transcripts analyzed. The hypothalamic transcripts that were increased in the fasted group are likely components of a mechanism of adaptation to fasting in chickens.

**Key words:** insulin, food intake, metabolism, hypothalamus, gene expression

2017 Poultry Science 96:4409–4418  
<http://dx.doi.org/10.3382/ps/pe247>

## INTRODUCTION

The hypothalamus plays a pivotal role in feed intake and energy balance regulation in birds (Kuenzel, 1994; Kuenzel et al., 1999). With few exceptions, such as a lack of response to specific orexigenic peptides (Orexin A and B, galanin, motilin and melanin concentrating hormone) (Furuse, 2002), feed intake and energy homeostasis seem to be regulated in a similar way in chickens as in mammals (Furuse, 2002; Boswell, 2005; Kuenzel et al., 1999; Richards and Proszkowiec-Weglarz, 2007). Pro-opiomelanocortin (**POMC**), Agouty-related protein (**AGRP**), and neuropeptide Y (**NPY**) are synthesized in the arcuate nucleus of the hypothalamus, where they play opposing roles in feed intake regulation

in mammals (Ollmann et al., 1997; Kalra et al., 1999; Schwartz et al., 2000; Mizuno et al., 2003). Through its receptor, insulin contributes to nutrient sensing, regulation of energy expenditure, food intake control (Nandi et al., 2004), and regulation of liver glucose production (Inoue, 2016). Foxo1 and Gpr17 have been recently proposed as the targets of the insulin receptor (**IR**) cascade in AGRP neurons of the arcuate nucleus to regulate peripheral metabolism and energy balance and insulin sensitivity (Ren et al., 2012; Ren et al., 2015; Scherer et al., 2016). In birds the infundibular nucleus plays a similar role to that of the mammalian arcuate nucleus (Wang et al., 2001).

Chickens exhibit several peculiarities in plasma glucose level control, glucose transporters, insulin release, and IR signaling (Hazelwood, 1986; Rideau, 1998; Simon et al., 2000; Dupont et al., 2012). Despite these peculiarities, a cross-talk exists in birds between insulin and the hypothalamus. Hyperphagia develops and glucose-stimulated insulin levels are enhanced following lesions of the ventromedial hypothalamus (**VMH**) in geese and chickens (Jacoby et al., 1995). Intraventricular injection of insulin inhibits food intake in young chickens via the central melanocortin system (Shiraishi et al., 2008). Chicken brain IRs show specific glycosylation, enhanced affinity, and different tyrosine kinase activity in response to

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Received May 6, 2017.

Accepted August 10, 2017.

<sup>1</sup>Current address: Animal Biosciences and Biotechnology Laboratory, USDA-Agricultural Research Service, Beltsville, MD 20705

<sup>2</sup>Corresponding author: [teporter@umd.edu](mailto:teporter@umd.edu)

nutritional alterations vs. peripheral IRs (Simon and Leroith, 1986). Moreover, IRs have been localized by immunohistochemistry in neurons of several structures of hypothalamus (the paraventricular nucleus, ventromedial and lateral regions, and infundibular nucleus) and in the infundibulum, they co-localize with  $\alpha$ -melanocyte stimulating hormone and NPY neurons (Shiraishi et al., 2011). Central insulin injection modified hypothalamic levels of several mRNAs. In one study, *POMC*, cocaine- and amphetamine-regulated transcript (*CART*) and corticotropin-releasing hormone (*CRH*) mRNAs were up-regulated, whereas *NPY* or *AGRP* mRNAs were unaltered (Honda et al., 2007). In another study, *POMC* mRNA increased and *NPY* mRNA decreased (Shiraishi et al., 2008). In contrast, intraperitoneal insulin injection did not modify *NPY* mRNA levels in the hypothalamus of selected high or selected low weight chickens at 90 days of age (Zhang et al., 2015). The lack of insulin effect on hypothalamic *NPY* mRNA in these lines following intraperitoneal injection (vs. central injection in other studies) may rely on the time course for insulin access and/or age of the chickens. In another study however, intraperitoneal insulin injection increased hypothalamic tryptophan hydroxylase 2 mRNA in 5 day-old chickens of selected high or selected low weight chickens (Rice et al., 2014). Hypothalamic *NPY* mRNA content was higher in selected high weight chickens than in selected low weight chickens (Zhang et al., 2015), though at a younger age opposite results were observed (Rice et al., 2014). Changes in hypothalamic *NPY* mRNA have also been observed in response to changes in nutritional status. *NPY* mRNA levels increased following feed restriction (Boswell et al., 1999a,b) or prolonged fasting, except in the lateral area (Zhou et al., 2005) and in day-old chickens unfed for 48 h (Higgins et al., 2010). In the latter model, a large number of genes (among which a large number of receptors) were differentially expressed in the hypothalamus between the various nutritional states (fed, unfed for 48 h, or refed).

The goal of the present study was to gain insight into insulin control of gene expression in the chicken hypothalamus, using 23 selected mRNAs and two experimental models. In the first model, insulin deficiency was induced in fed broiler chickens (16 or 17 days of age) by immune-insulin privation for 5 h. Insulin deprived chickens exhibited a large decrease in food intake, large increases in plasma glucose, non-esterified fatty acids (NEFA), amino acids (alpha-NH<sub>2</sub>-non protein), and glucagon, and a decrease in plasma T<sub>3</sub>. In 3 peripheral tissues of the same chickens used in the present study, transcriptome studies identified a rather large number of insulin-dependent genes: 1,573 in liver and 1,225 in muscle, but much less in abdominal adipose tissue following immune insulin privation (Ji et al., 2012; Simon et al., 2012). A short fasting period (5 h) was included as a second model, as fasting represents another status of "insulin privation". Plasma insulin levels were not, however, measured in this experiment because of the

presence of anti-insulin antibody in the insulin-deprived group. As compared to fed control chickens, 5 h fasting slightly decreased plasma glucose (though not significantly) and amino acid levels, increased plasma NEFA and glucagon levels, and decreased plasma T<sub>3</sub>. Therefore, several changes at the plasma level are shared by these two experimental models. The 23 mRNAs assessed included most of those discussed earlier. Others were chosen mainly from our study on effects of prolonged fasting in day-old chickens (48 h without feed) in the hypothalamus (Higgins et al., 2010) or had been identified as differentially expressed in the hypothalamus during development of genetically selected fat or lean chickens (Byerly et al., 2010). Fat and lean chickens differ in their glucose-insulin relationship, with a slightly higher insulin sensitivity in fat chickens (Simon and Leclercq, 1985). Finally, other selected mRNAs were identified as insulin-dependent in liver or muscle, after insulin privation (Dupont et al., 2008; Simon et al., 2012). As a whole, selected mRNAs are involved in feed intake regulation or neuronal, endocrine, or transcriptional control or glucose metabolism and glucose sensing.

## MATERIALS AND METHODS

### *Animals and Experimental Protocol*

The animals and experimental protocol used were described previously (Dupont et al., 2008). Briefly, male broiler chickens (ISA 915, Institute de Selection Animale, Saint Brieuc, France) were fed a conventional balanced diet ad libitum (3,050 kCal or 12.8 MJ/kg metabolizable energy, 22% proteins (N6.25), based on corn, wheat, peas, soybean meal, corn gluten, and rapeseed oil) and exposed to a 14L:10D light regimen. At 16 to 17 d of age, chickens of similar body weight were divided into five experimental groups (n = 7 birds each) as follows: fed groups that received a single intra-venous (i.v.) injection (1.5 mL/kg) of either normal guinea pig serum (**NS**, PromoCell, Heidelberg, Germany) or anti-porcine insulin guinea pig serum (**AI**) (**C.1** and **AI.1** groups, respectively), fed groups that received three i.v. injections (1.5 mL/kg each) at time 0, 2, and 4 h of either NS or AI (**C.5** and **AI.5** groups, respectively), and a fasted group that was fasted for 5 h and received three i.v. injections of NS at the times described above (**F.5** group). The 5 h time point was based on availability of antiserum. Insufficient anti-serum was available to assess longer time points, while still administering the antiserum at 2 h intervals. Four replicates were performed at 16 d and three at 17 d of age to allow for collection of all samples within an hour on each day. Immune sera were prepared as described previously (Simon et al., 2000). Food intake was measured 1 h (C.1 and AI.1) or 5 h (C.5 and AI.5) after the first i.v. injection. At the same time, blood was sampled under EDTA, cooled on ice, and the plasma rapidly collected by centrifugation. Birds were killed by cervical

**Table 1.** Gene-specific primers used for the analysis of mRNA levels using quantitative real-time RT-PCR.<sup>a</sup>

Gene Name <sup>b</sup>	GenBank Accession No. <sup>c</sup>	Forward primers (5'-3')	Reverse primer (5'-3')	PCR size (bp)
AGRP	NM_0,010,31457	AAGTCTGGCCTGGGAAGAG	CCCCCTGCAGAAGATGAG	122
ACTB	X0082	CAGGATGCAGAAAGGATCACA	TAGAGCCTCCAATCCAGACAGAGTA	101
CHAT	NM_204,610	TGATGAGGGCAGAGTTGACA	TGCGTCCTTAAATCTCTGCAT	124
CRH	NM_0,011,23031	CACAGCAACAGGAAACTGATGGAAA	AAAGAGGTGACATCAGAGCAGCACTATG	101
DIO2	NM_204,114	ACTGTTTGGAGGGCGCTAAACC	AAACACTAGCCCTCCAGAATACCTT	110
DPYSL2	NM_204,494	CTGCCAAGACCCACAACATA	TTCCCTTGGCTGATAACCCAC	91
EGR1	NM_204,136	TCAGCACTTTCAGACATGACATCA	AGTACCAGTGGAAAGAGGTGAATGC	101
ENO2	NM_204,876	GCCTCCTGCTCAAAGTCAAC	CATTCTCCTGGGCCAACTTA	75
GCK	AF525739	ACGAAGCACCAGATGTACTCC	GGAGATGCACTCCGAGATGT	86
GLUT1	NM_205,209	GGGATCAATGCGGTTTTCTA	CGAAGAGCGAAAACAACAGTG	124
GLUT2	NM_207,178	TATCGTCAACAGGCATCCTCA	GAAGAAGTGCAGCAGAGCAG	118
GLUT3	NM_205,511	CTCAACCAGCTGGGCATAGT	AGTGGCCAAAGTGCTTCAGT	89
GLUT8	NM_204,375	TGCAGGCAAATTGAAGCTAA	TGCCTGTTACTTGCTGGAGA	124
GPI	NM_0,010,06128	CCATGCTGGGAGTGTGGTAT	GCTGGAAGTAGGCAGAAAAG	99
HK1	NM_204,101	CGTTACCTGGCATTTCGGACT	CTTGCAAGGGAAGGAGAATG	88
LEP	LN794246 <sup>d</sup>	GGCGATTCCAACGCCTACT	CCGCGATGGCTTGCA	102
LEPR	NM_204,323	TTCCAAACCCCAAGAATTGCT	CAAATGACATTGCTTCAGGGTG	102
MC4R	NM_0,010,31514	CGGGAGGCTGCTATGAACAA	AGCTGATGATGCCAGAGTCA	101
NPY	NM_205,473	GGGAAAGCACAGAAAACATTCC	AAATCCCATCACCACATCGAA	101
PGAM1	NM_0,010,31556	AGGCTCAGGTGAAGATCTGG	TGCTGATGGTGCTGAAGAAG	87
POMC	NM_0,010,31098	CGCTACGGCGGCTTCA	TCTTGTAGGCGCTTTTGACGAT	88
TAS1R1	XM_425,734	GCGTGGCAGAAAACACCAGAT	GCTTGACCAATATGCGCTTGT	64
TH	NM_204,805	CGGACTGCTGTCTATGAGCTA	CAGTTGCTCCCAGAGATGC	102
TRH	NM_0,010,30383	TGGATGACATCCTGCAGAGATC	GGAAAGCCATTGTGGCAGA	116

<sup>a</sup>All primers used for expression analysis were designed using the Primer3 program (<http://fakker.wi.mit.edu/primer3/input.htm>; Rozen and Skaletsky, 2000).

<sup>b</sup>Abbreviation of the gene names are defined in Table 2 and text.

<sup>c</sup>Reference chicken gene sequences that contain the corresponding PCR products list.

<sup>d</sup>From Seroussi et al. (2016).

**Table 2.** Effect of insulin immuno-neutralization and fasting on mRNA expression levels in the hypothalamus of chickens.

Gene Name	Treatment				
	C-1	AI-1	C-5	AI-5	F-5
POMC	100.00 ± 12.28	97.57 ± 16.44	86.76 ± 17.87	92.48 ± 22.36	114.46 ± 30.34
MC4R	100.00 ± 7.27	90.11 ± 7.74	93.76 ± 4.60	85.92 ± 7.79	107.97 ± 10.46
EGR1	100.00 ± 5.98 <sup>a</sup>	97.68 ± 8.78 <sup>a</sup>	92.64 ± 4.92 <sup>a,b</sup>	76.07 ± 5.13 <sup>b</sup>	102.96 ± 9.26 <sup>a</sup>
CRH	100.00 ± 15.19 <sup>a,b</sup>	82.32 ± 7.95 <sup>a,b</sup>	84.01 ± 12.21 <sup>a,b</sup>	74.38 ± 9.60 <sup>b</sup>	116.17 ± 18.16 <sup>a</sup>
TRH	100.00 ± 6.80	95.50 ± 8.08	94.39 ± 4.77	86.01 ± 7.76	108.61 ± 10.47
ENO2	100.00 ± 4.97	99.28 ± 1.88	98.05 ± 1.82	97.07 ± 7.60	108.34 ± 7.25
PGAM1	100.00 ± 11.84	83.22 ± 3.40	80.06 ± 6.88	85.88 ± 10.78	92.19 ± 14.14
GPI	100.00 ± 4.55	98.51 ± 3.44	99.29 ± 1.48	94.82 ± 8.30	109.99 ± 7.47
DPYSL2	100.00 ± 5.60	98.65 ± 5.27	97.12 ± 2.94	96.52 ± 7.45	104.13 ± 9.36
HK1	100.00 ± 4.92	97.63 ± 2.69	102.17 ± 4.67	96.79 ± 6.78	102.50 ± 6.02

Reverse transcription quantitative PCR (RT-qPCR) was used to quantify mRNA levels for pro-opiomelanocortin (*POMC*), melanocortin 4 receptor (*MC4R*), early response gene (*EGR1*), corticotropin-releasing hormone (*CRH*), thyrotropin-releasing hormone (*TRH*), neural enolase 2 (*ENO2*), phosphoglycerate mutase 1 (*PGAM1*), glucose phosphate isomerase (*GPI*), dihydropyrimidinase like protein (*PRYSL2*), and hexokinase 1 (*HK1*) in hypothalami collected from ad libitum fed chickens 1 h after one normal serum (NS) injection (C-1) or one anti-insulin serum (AI) injection (AI-1) and after 5 h during which three injection of NS (C-5) or AI (AI-5) were administered. Birds fasted for 5 h (F-5) were also analyzed for comparison. Each value represents the mean ± SE of seven birds (n = 7).

<sup>a,b</sup>Different letters denote statistically significant differences for mean comparisons ( $P < 0.05$ ).

dislocation after blood sample collection. Hypothalamic tissue samples were collected manually using a dissecting microscope as described previously (Byerly et al., 2009; Higgins et al., 2010) based on published information (Kuenzel and Masson, 1988) and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Initial incisions were made just anterior to the oculomotor nerve (*nervus oculomotorius*) and posterior to the *tuberculum olfactorium*. Next, lateral cuts were made 2 mm from the midline to yield a rectangular piece of tissue. This was placed on its side,

and a final cut was made at a depth immediately below the subseptal organ (*organum subseptale*) and parallel to the basal surface of the hypothalamus. Plasma glucose levels were measured by the glucose oxidase method (Glucose Beckman Analyzer 2, Beckman, Palo Alto, CA). All procedures were approved by the Agricultural Agency and the Scientific Research Agency of INRA and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

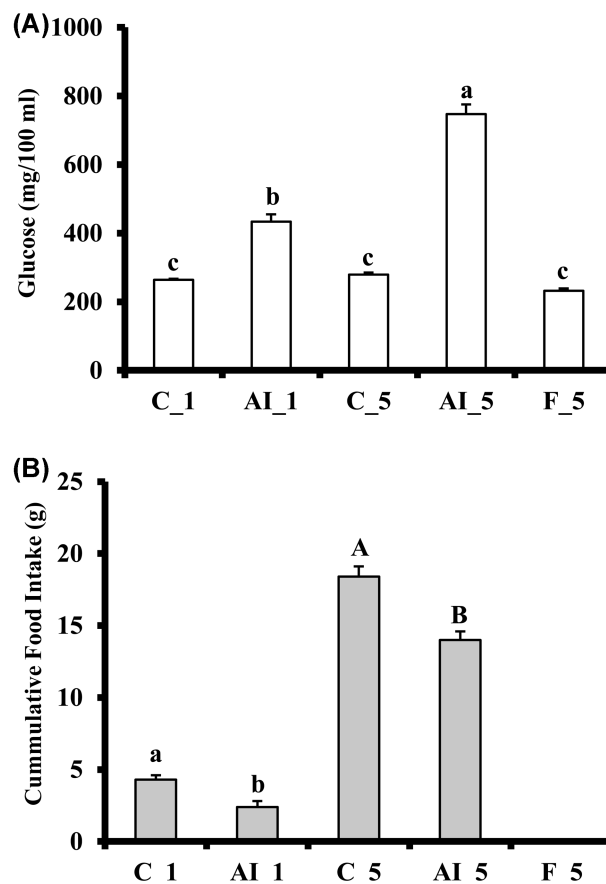


## RNA Isolation and Reverse Transcription-Quantitative PCR

Total RNA was extracted using RNeasy Midi kits (Qiagen, Valencia, CA), according to the manufacturer's protocol and quantified using the RiboGreen Quantitation kit (Invitrogen, Carlsbad, CA). Two-step reverse transcription-quantitative PCR (RT-qPCR) was performed to analyze hypothalamic mRNA levels. The RT reactions (20  $\mu$ L) consisted of 1  $\mu$ g of RNA, 50 units Superscript III reverse transcriptase (Invitrogen), 40 units of an RNase inhibitor (Invitrogen), 0.5 mM dNTPs, and 2.5  $\mu$ M anchored oligo dT primers. A pool of all RNA (1  $\mu$ g) from all treatment groups was used as a negative control for genomic DNA contamination and was processed as the other samples, but with omission of Superscript III enzyme. The RT reactions were diluted to 200  $\mu$ L before being subjected to PCR. PCR was performed in 15  $\mu$ L reactions containing 1  $\mu$ L of diluted RT reaction, 400 nM of each gene-specific primer, 7.5  $\mu$ L of 2  $\times$  PCR buffer (100 mM KCl, 20 mM Tris-HCl, pH 9, 0.2% Triton X-100), 3.8 mM MgCl<sub>2</sub>, 0.12 U/ $\mu$ L Taq polymerase, 400 nM dNTPs, 40 nM fluorescein (Invitrogen), and SYBR Green I Nucleic Acid Gel Stain (Invitrogen) diluted 1:10,000, and the MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Thermal cycling parameters were: 1 cycle at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Dissociation curve analysis and gel electrophoresis were employed to ensure that a single PCR amplicon of appropriate size was amplified in each reaction. PCR products were sequenced to confirm their identities. Primer sequences, designed using Primer3 software (Rozen and Skaletsky, 2000), are listed in Table 1. The data obtained were normalized to the housekeeping gene  $\beta$ -actin (ACTB), and the data were transformed using the equation  $2^{-Ct}$ , where Ct represents the fractional cycle number when the amount of amplified product reached a fixed threshold for fluorescence. The data are presented as fold changes relative to the C.1 group for statistical analysis.

## Statistical Analysis

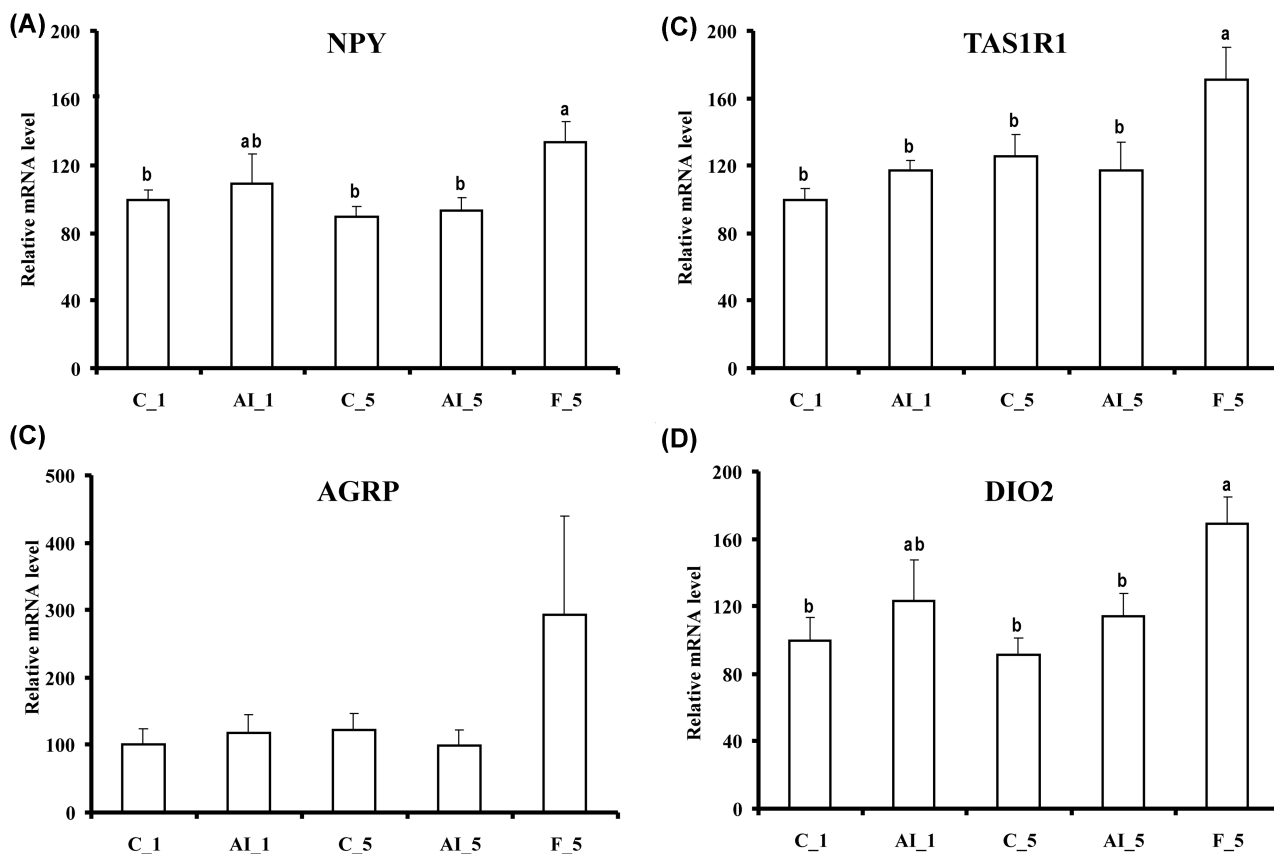
All data were analyzed by one-way ANOVA using the general linear models (GLM) procedure of the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA). The treatment means for food intake and plasma glucose level were compared by Fisher's test or Student *t* test, while the Duncan's multiple range test option of the GLM procedure for SAS was used to determine significance of mean differences for gene expression analysis. Significance was set at  $P < 0.05$ .



**Figure 1.** Effects of insulin immune-neutralization and fasting on (A) cumulative feed intake and (B) plasma glucose levels. At 16 to 17 d of age, chickens at similar body weight were divided into five experimental groups ( $n = 7$  birds each) as follows: fed groups that received a single i.v. injection (1.5 mL/kg) of either normal guinea pig serum (NS, PromoCell, Heidelberg, Germany) or anti-porcine insulin guinea pig serum (AI) (C.1 and AI.1 groups, respectively), fed groups that received three i.v. injections (1.5 mL/kg each) at time 0, 2, and 4 h of either NS or AIS (C.5 and AI.5 groups, respectively), and a fasted group that was fasted for 5 h and received three i.v. injections of NS at the times described above (F.5 group). Each value represents the mean  $\pm$  SE of seven birds ( $n = 7$ ). Due to the large differences in feed intake between 1 h (C.1 and AI.1) and 5 h (C.5 and AI.5) after the first i.v. injection, results for feed intake at 1 h and 5 h were analyzed separately in order to test for differences due specifically to AI. Different letters denote statistically significant differences for mean comparisons ( $P < 0.05$ ).

## RESULTS

Insulin immune-neutralization for 1 (AI.1) or 5 h (AI.5) significantly ( $P < 0.008$  and  $P < 0.0001$ , respectively) decreased feed intake in comparison to corresponding control groups (Figure 1). Plasma glucose levels were significantly increased after insulin immune-neutralization at both time points, while chicks fasted for 5 h had similar ( $P > 0.05$ ) plasma glucose levels as chicks fed *ad libitum* (Figure 1). These findings indicate that AI depressed insulin activity which led to increased glucose levels and decreased feed intake. Despite the large and multiple effects observed following insulin immuno-neutralization at the level of plasma parameters, liver or muscle IR signaling, and



**Figure 2.** Effects of insulin immune-neutralization and fasting on hypothalamic levels of mRNA for (A) neuropeptide Y (NPY), (B) agouty gene related protein (AGRP), (C) sweet taste receptor 1 (TAS1R1), and (D) iodothyronine deiodinase 2 (DIO2). See legend to Figure 1 for further details.

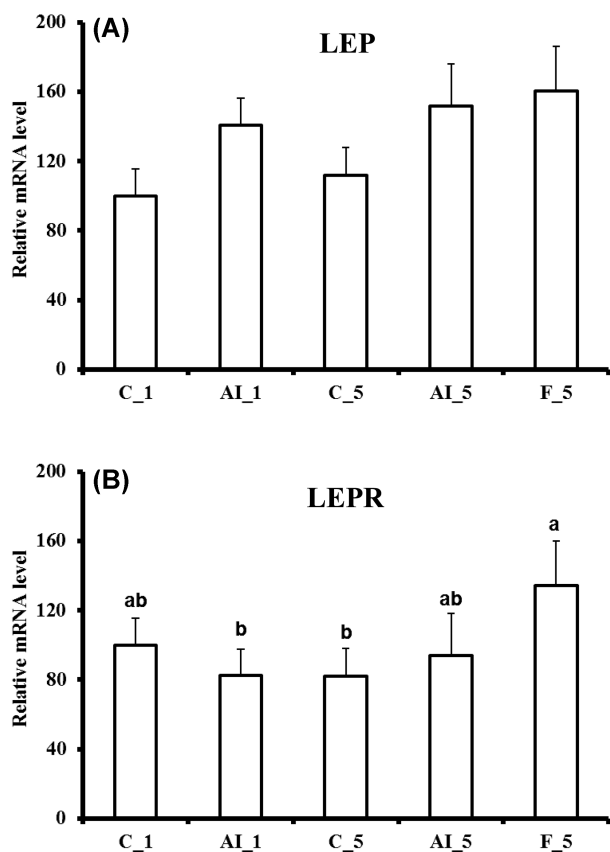
liver or muscle transcriptome (Dupont et al., 2008; Simon et al., 2012), levels of none of the selected mRNAs were modified in the hypothalamus at either 1 h or 5 h insulin privation, as compared to the respective fed controls (Table 2). *EGR1* mRNA, which decreased both in liver and leg muscle after insulin-neutralization (Dupont et al., 2008; Simon et al., 2012), was lower in the hypothalamus in the 5 h insulin neutralization group compared to the 5 h fasted group but not to the 5 h fed group. A similar situation was observed for *CRH* mRNA (Table 2). Origin of the opposite changes in *EGR1* and *CRH* in response to insulin-neutralization and fasting are unknown.

In contrast, 5 h fasting altered the levels of several mRNAs. *NPY*, sweet taste receptor 1 (*TAS1R1*), and iodothyronine deiodinase 2 (*DIO2*) mRNA levels increased as compared to both fed controls or 5 h insulin deprived fed chickens (Figure 2), and *LEPR* mRNA levels were increased by 5 h fasting as compared to fed controls (Figure 3). *LEP* messenger levels were unchanged (Figure 3). Fasting also increased mRNAs for several glucose transporters (*GLUT1*, *GLUT3*, and *GLUT8*) and *GCK*, also as compared to both fed controls or 5 h insulin deprived chickens (Figure 4). Choline O-acetyltransferase (*CHAT*) and tyrosine hydroxylase (*TH*) mRNA levels were unchanged (Figure 5), suggesting a lack of activation of cholinergic and dopaminergic systems in the hypothalamus, at the mRNA level,

in any of the present conditions. Finally, levels of eight other mRNAs were unaffected by any of the treatments (see Table 2).

## DISCUSSION

In the present study, 5 h insulin immune neutralization in fed chickens had no effect on hypothalamic levels of the mRNAs investigated (only *EGR1* tended to decrease). This was highly surprising, considering the inhibition of food intake, changes in levels of numerous mRNAs in peripheral tissues, known effects of central insulin injection on the chicken hypothalamus, and the multiple and major effects of insulin in the mammalian hypothalamus. Though, to our knowledge, insulin has not been measured in chicken cerebrospinal fluid (csf), it would be very unlikely if both csf insulin and glucose levels had not been altered during the 5 h insulin neutralization, as the half-life of plasma insulin is about 5 min in chickens and about 7 min in turkeys (Langslow, 1976; McMurtry et al., 1987). Uptake of plasma glucose and insulin through blood-brain barrier and relationships between plasma glucose and insulin levels and csf glucose and insulin levels have been investigated in mammals. Changes in csf are tempered in comparison with peripheral variations of glucose or insulin, but do exist, showing some delay and variations of lower magnitude. The adjustment of csf insulin is disturbed in



**Figure 3.** Effects of insulin immune-neutralization and fasting on hypothalamic levels of mRNA for (A) leptin (LEP) and (B) leptin receptor (LEPR). See legend to Figure 1 for further details.

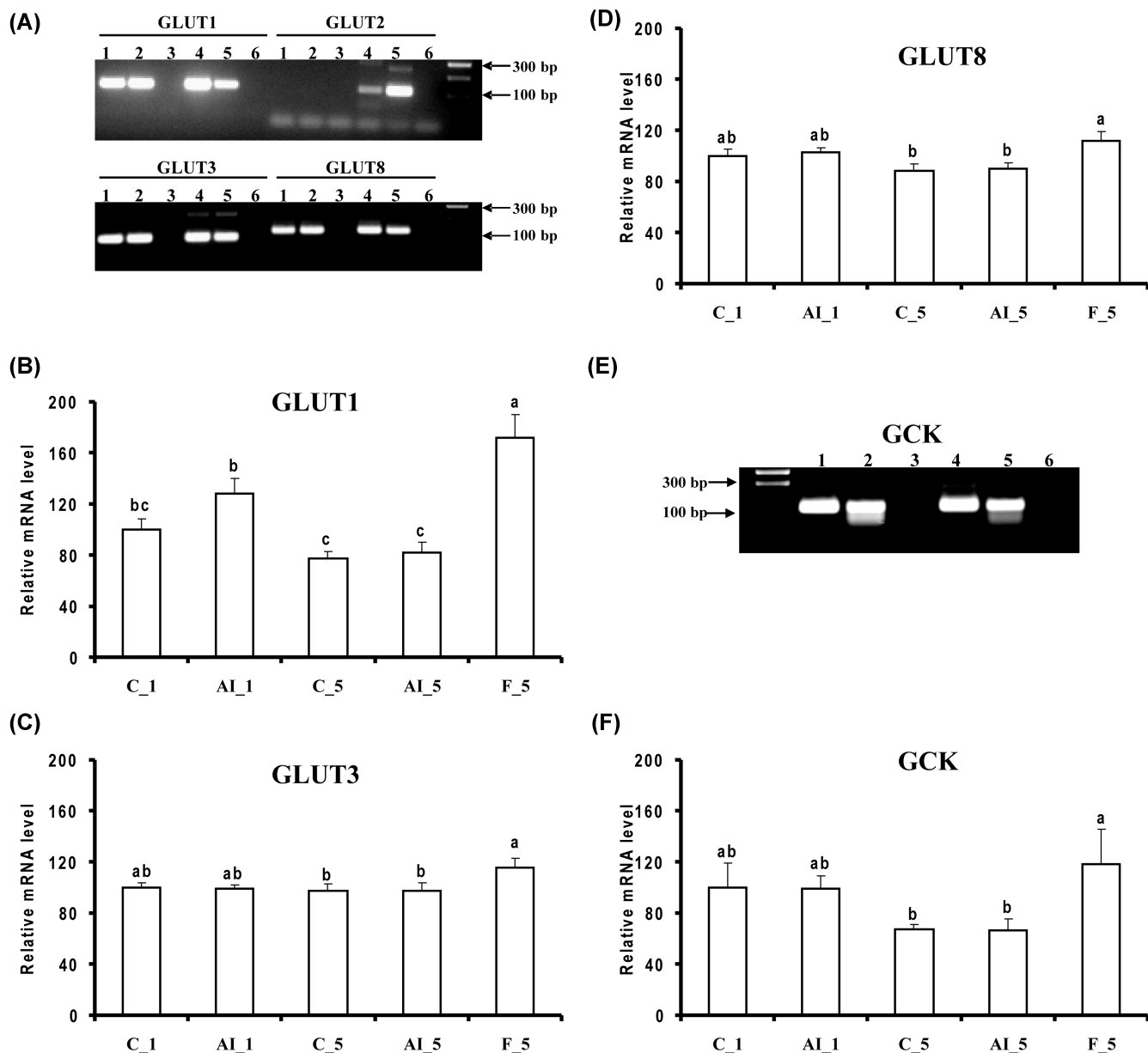
some pathologies (Begg, 2015). In contrast, changes in csf glucose level have been observed in chickens in various conditions: a decrease in response to peripheral hypoglycemia (induced by intravenous tolbutamide or insulin injection) with a delay of about 20 to 40 minutes (Anderson and Hazelwood, 1969; Simon and Leclercq, 1985) or in response to overnight fasting of 16 h (Simon and Leclercq, 1985). Since no changes were observed for any of hypothalamic mRNAs presently investigated despite the rather extreme experimental conditions, the inhibition of food intake following insulin neutralization must rely on mechanisms other than control at the transcription level in the hypothalamus within the period of time investigated. Effects on neurotransmitter release within the hypothalamus are a likely possibility. A change in glucose sensing or metabolism and/or a decrease in insulin signaling is another possibility. Up to now, major steps of the insulin-IR cascade have not been characterized in the chicken brain. Only IR tyrosine kinase activity has been measured and shown to be refractory to extreme nutritional changes (Simon and Leroith, 1986). In peripheral tissues, the early steps of insulin receptor signaling appear to be responsive to insulin in the liver but refractory in muscle and adipose tissue (Dupont et al., 2004; Dupont et al., 2012). So far, only the phosphorylation of Src homology 2 domain containing transforming protein 1 adaptor protein

(Shc), an IR substrate, appeared insulin sensitive in muscle (Dupont et al., 2004).

Plasma glucose levels were not significantly different between 5 h fasting and fed control chickens. A catabolic state had likely already developed during the 5 h fast, as suggested by the significant increases in plasma NEFA and glucagon levels and significant decrease in amino acid index reported previously (Dupont et al., 2008). The 5 h fast certainly represents a transition step during which a hunger state develops and orexigenic mechanisms are stimulated. Though some orexigenic gene mRNA levels did not change in the 5 h fasting group (e.g., *POMC*, *MC4R*), expression of 8 genes was altered out of the 23 measured. These genes are involved in the control of food intake, endocrine control of metabolism, or glucose metabolism.

Hypothalamic *NPY* and *DIO2* mRNAs were increased by 5 h fasting. Similar increases were previously observed for *NPY* and *DIO2* mRNA following fasting in growing chickens or in 48 h non-fed, day-old chickens (Boswell et al., 1999a,b; Zhou et al., 2005; Higgins et al., 2010). In liver, *DIO2* mRNA was, in contrast, down-regulated by both 5 h fasting and 5 h insulin deprivation (Dupont et al., 2008). Expression of two other genes of the endocrine system was changed in the hypothalamus following fasting. *CRH* (as compared to insulin-immuno-neutralization) and *LEPR* (as compared to fed controls) mRNA levels were increased following fasting. *CRH* has been previously shown to decrease feed intake in chickens (Denbow et al., 1999), and differential *CRH* signaling may influence appetite regulatory mechanisms in chickens with different body weight and feed consumption (Cline et al., 2009). Moreover, *CRH* acts as a mediator for a number of anorexic peptides in chickens (Honda et al., 2007; Tachibana et al., 2004; Tachibana et al., 2006). An increase in *CRH* mRNA was previously observed when insulin was administered in chickens (Honda et al., 2007). Alternatively, the increase in *CRH* mRNA levels following fasting might be a result of fasting-induced stress. The increase in *LEPR* mRNA is consistent with our previous results (Higgins et al., 2010). The chicken *LEP* gene, which has been identified only very recently, is expressed in the hypothalamus (Seroussi et al., 2016). In the present study, hypothalamic *LEP* mRNA levels were unchanged by fasting.

*TAS1R1*, which has been proposed in mice as a hypothalamic sensor of glucose (Ren et al., 2009), is expressed at a higher level in chickens selected for increased body fat than in their counterparts selected for leanness (Byerly et al., 2010). In the present study, hypothalamic *TAS1R1* showed no changes when glucose levels largely increased following insulin neutralization but did increase following the 5 h fasting period, most likely in response to transient alterations in plasma glucose levels. Since glucose is the energetic fuel in brain, the increase in hypothalamic *TAS1R1* mRNA may signal a quick adaptation to transient decreases in glucose levels during fasting. A similar adaptation is also



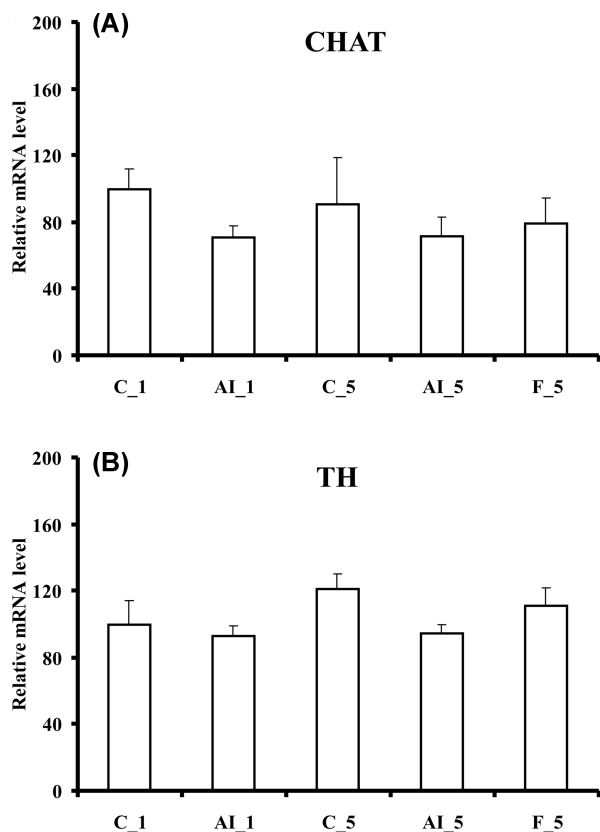
**Figure 4.** Effects of insulin immune-neutralization and fasting on hypothalamic levels of mRNA for genes involved in glucose transport and metabolism. (A) PCR products for glucose transporters 1, 2, 3, and 8 (GLUT1, GLUT 2, GLUT3, and GLUT8, respectively). Hypothalamic levels of mRNA for (B) GLUT1, (C) GLUT2, (D) GLUT3, and (F) GCK were determined by reverse transcription-quantitative PCR. (E) PCR products for glucokinase (GCK). Numbers 1 to 6 in panels A and E refer to different tissues. Samples are identified as follows: 1, C-5; 2, AI-5; 3, no RT sample; 4, brain sample from 3 wk old chicken; 5 liver sample from embryonic day 18 and 6, blank. See legend to Figure 1 for further details.

suggested by the changes observed at the level of mRNAs coding for *GLUT1*, *GLUT3*, *GLUT8*, and *GCK*.

In mammals, a large family of glucose transporters facilitates cell glucose uptake. Among these, GLUT4, which largely accounts for insulin sensitive glucose uptake in muscle and adipocytes, is also present in the hypothalamus, where it appears to play a critical role in glucose uptake (Ren et al., 2015). The *GLUT4* gene is not present in the yet incompletely sequenced chicken genome. Recently, GLUT12 appeared to act as an insulin-sensitive glucose transporter in chicken muscles and may play a similar role in this species as GLUT4 in mammals (Coudert et al., 2015). To our knowledge,

the presence of GLUT12 in chicken hypothalamus has not been shown. Herein, other GLUT mRNAs were investigated. GLUT1 is expressed ubiquitously and facilitates basal glucose uptake. GLUT2 has a low affinity to glucose, and its expression is limited to the basolateral membrane of hepatocytes, kidney, small intestine, and pancreatic beta-cells. In mammals GLUT2 is also expressed in the hypothalamus, contributing to the control of food intake (Stolarczyk et al., 2010). GLUT3 is a high affinity transporter responsible for glucose uptake in tissues and cells where extracellular substrate concentration is low (e.g., neuronal cells) (Kono et al., 2005), while GLUT8 has been shown to be insulin





**Figure 5.** Effects of insulin immune-neutralization and fasting on hypothalamic levels of mRNA for (A) choline O-acetyltransferase (*CHAT*) and (B) tyrosine hydroxylase (*TH*). See legend to Figure 1 for further details.

responsive in the blastocyst of mammals (Carayannopoulos et al., 2000). The presence of *GLUT1*, *GLUT3*, and *GLUT8* mRNAs was confirmed by PCR in brain and liver tissues of chickens. In contrast, mRNA expression of *GLUT2* was not detectable in the chicken hypothalamus in the present study (data not shown). Similar expression patterns were observed by Kono et al. (2005) in chicken tissues. The 5 h fasting treatment increased hypothalamic mRNA levels for *GLUT1*, *GLUT3*, and *GLUT8* (Figure 4), with *GLUT1* demonstrating the largest effect.

Mammalian hexokinase IV (glucokinase, **GCK**) plays a crucial role in glucose homeostasis (Postic et al., 2001; Matschinsky, 2002) by phosphorylating hexose into hexose-6-phosphate in the first step of glycolysis. GCK is predominantly located in pancreatic beta-cells and in the liver, but also is present in glucose-sensitive tissues including the hypothalamus (Magnuson, 1992; Schuit et al., 2001; Penicaud et al., 2002). Within the hypothalamus of mammals, GCK has been shown to be expressed in the arcuate nucleus, lateral nucleus, dorsomedial nucleus, ventromedial nucleus, and paraventricular nucleus (Lynch et al., 2000; Maekawa et al., 2000; Li et al., 2003). Even though the chicken *GCK* gene has not been identified in the chicken genome, a 1,326 bp liver cDNA fragment that shows high homology with human *GCK* cDNA has been sequenced (Berradi et al., 2005). Chicken *GCK* has been shown to be insulin de-

pendent in fed chickens (Dupont et al., 2008), *GCK* mRNA increased in liver in response to a meal (Rideau et al., 2008) and protein levels decreased during fasting (Dupont et al., 2008; Rideau et al., 2008). Furthermore, the use of a specific activator of mammalian GCK supported the existence a functional and potent GCK in chicken. This glucokinase activator induced hypoglycemia and inhibited food intake without recruiting insulin, suggesting that liver glucokinase activation by itself accounted for the hypoglycemia (Rideau et al., 2010). We detected *GCK* mRNA in the chicken hypothalamus (Figure 4E). To our knowledge, this is the first report showing the presence of the mRNA for this enzyme in the avian hypothalamus. Previously, Berradi et al. (2005) were not able to detect chicken GCK in chicken brain at the protein level. It is, however, possible that the protein level of GCK in whole brain is too low to be detected. In mammals, GCK is recognized as a specific marker for glucose sensing neurons (Yang et al., 1999; Dunn-Meynell et al., 2002; Kang et al., 2004, 2006).

In conclusion, 5 h insulin immune-neutralization in fed chickens was without any effect on the various mRNAs presently investigated in the hypothalamus, despite an early decrease in food intake. A change in glucose and/or insulin signaling are likely to be involved in this “satiety” mechanism. The fact that all the mRNAs studied remained at a “normal” fed level is largely unexpected. By contrast, 5 h of fasting altered mRNA levels for 8 genes involved in food intake, glucose transport, and glucose metabolism. This short period of fasting was clearly perceived as a signal of the development of a glucose shortage event. Therefore, these “fast responder” genes are likely components of a mechanism of emergency adaptation to food deprivation in the chicken.

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