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Insulin Receptor Localization in the Embryonic Avian Hypothalamus

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Abstract

The hypothalamus is a brain region critical for the homeostatic regulation of appetite and energy expenditure. Hypothalamic neuronal activity that is altered during development can produce permanent physiological changes later in life. For example, circulating hormones such as insulin have been shown to influence hypothalamic neuronal projections, leading to altered metabolism in adult rodents. While insulin signaling in the post-hatch chicken has been shown to mirror that of mammals, the developmental role of insulin in the avian embryonic hypothalamus remains largely unexplored. Here we present the earliest known evidence for insulin receptor (InsR) expression in embryonic avian hypothalamic nuclei governing energy homeostasis. RT-PCR analysis reveals InsR mRNA in E8, E10, and E12 neurons while western blot data demonstrate protein expression in E12 avian whole brain and hypothalamic lysates. Immunohistochemical analysis of avian hypothalamic brain slices demonstrates a shift in InsR localization from paraventricular expression in E8 to a more defined concentration of InsR in developmental regions resembling the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) in E12 time points. In addition, InsR expression appears in a heterogeneous pattern, suggesting receptor localization to subpopulations of avian hypothalamic neurons as early as E8. With increasing evidence suggesting energy homeostasis pathways may be altered via the gestational environment, it is important to understand how insulin signaling may affect embryogenesis. Our research provides evidence for the earliest known embryonic expression of InsR protein in the avian hypothalamus and may suggest a developmental role for insulin signaling in the early patterning of metabolic pathways in the central nervous system.

Keywords

Insulin Receptor; Hypothalamic Development; Avian Embryo; Energy Homeostasis; Metabolism

1. Introduction

The hypothalamus is a brain region that mediates a variety of autonomic functions. In particular, it is responsible for the integration of appetite signals and energy sensing information from the periphery in order to maintain homeostasis[1–5]. Several hypothalamic nuclei bordering the third ventricle work together to orchestrate alterations in energy

homeostasis. The location of these metabolic nuclei in relation to the third ventricle allows circulating molecules to cross the blood brain barrier, bind to surface receptors expressed on neuronal membranes, and influence the function of specific cells [2]. Located adjacent to the median eminence, the arcuate nucleus (ARC) receives signals from circulating factors in the periphery and relays this information to surrounding hypothalamic areas. First order neurons in the ARC exist as subpopulations of cells classified by orexigenic and anorexigenic function. The activation of orexigenic cells stimulates food seeking behavior in post-natal organisms and relies on agouti-related peptide (AgRP), γ -aminobutyric acid (GABA), and Neuropeptide-Y (NPY) to influence second order neurons in the lateral hypothalamic area (LH), paraventricular nucleus (PVN), and dorsomedial nucleus of the hypothalamus (DMN) [3,4]. Anorexigenic cells function to suppress appetite and increase energy expenditure via proopiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) release onto neurons in the LH and PVN[5]. Insulin, a major hormone involved in communicating metabolic signals from the periphery, can cross into the interstitial space of the hypothalamus via transcytosis and bind to its cognate insulin receptor (InsR), rendering the brain a novel insulin-sensing organ[2,6,7]. In adult mammals, both orexigenic and anorexigenic neurons express InsRs, though little is known about the expression of these receptors during development[8]. Our work aims to determine whether InsRs are expressed in hypothalamic nuclei governing energy homeostasis during critical time points in embryonic development.

Contrary to other regions of the brain, hypothalamic development is not entirely pre-natal; neurons in metabolic regions continue developing after birth[9]. In rodents, ARC neuron connections to other hypothalamic nuclei are not complete until approximately two weeks after birth [10] and post-hatch development also occurs in avian hypothalamic metabolic regions[11]. However, hypothalamic neurons are also sensitive to changes in the surrounding environment during development, which could produce permanent alterations in neuronal circuitry. For example, epigenetics and maternal high-fat diet have been shown to alter hypothalamic regulation in both thermosensitive and metabolic regions, highlighting the importance of the external environment on hypothalamic development[11,12]. Metabolic hormones, such as insulin, are key determinants in the formation of neonatal hypothalamic feeding pathways later in development[12], although it is unclear if these ligand-receptor interactions are also significant in the early patterning of these circuits. Therefore, targeting the emergence of InsRs during embryogenesis is critical to understanding the developmental patterning of metabolic circuits.

Research has shown that the insulineric systems driving energy homeostasis are conserved between post-natal birds and mammals[13–15] and that the avian species demonstrates similar causes of obesity and metabolic disorders as other mammals[16]. In addition, the tyrosine kinase activity of the avian InsR has been shown to parallel that of the mammalian InsR[17], underscoring further similarities between species. The avian model provides the opportunity to study the development of metabolic nuclei in an environment that is independent of maternal influence and allows for manipulation of the organism at critical developmental time points. For example, altering the developmental environment of the chicken embryo with high glucose exposure leads to permanent changes in InsR mRNA expression [18] and decreased neuronal glucose sensitivity in post-natal chicks[19]. Here,

we examine embryonic hypothalamic InsR expression and localization in metabolic nuclei in order to provide evidence for insulin sensitivity during the very early stages of avian hypothalamic development.

In addition to drawing further comparisons of metabolic pathways between species, this research establishes a developmental model system which can be used to study neuronal circuit formation independent of maternal influence.

The current study is the first to demonstrate early expression of InsR in the embryonic chicken and provides significant evidence localizing this receptor to early hypothalamic nuclei resembling the PVN, ventromedial hypothalamus (VMH), and ARC. We demonstrate heterogeneous InsR expression in the avian hypothalamus as early as embryonic day 8 (E8), suggesting that insulin signaling may affect the developmental patterning of neuronal circuits in a subpopulation of hypothalamic neurons. In addition, this embryonic InsR expression data establishes insulin signaling as a potential determinant of early neuronal development in energy homeostasis pathways.

2. Materials and methods

2.1 Tissue harvest

Fertile white leghorn chicken eggs were obtained from Charles River, Wilmington, MA and stored at 6 °C. Eggs were transferred and maintained in a forced draft incubator at 37.5°C with 55% relative humidity until embryonic day 8 (E8), E10, and E12 and tissues were processed for experimentation as described. Time points were selected based on critical windows of hypothalamic development and the differentiation of the neuroendocrine axis [20]

2.2 Neuronal Dissociation

Forebrain tissue was harvested from E12 avian embryos and placed in 2mL ice cold Dulbecco's PBS (DPBS) supplemented with 0.1% glucose and 1.0% penicillin/streptomycin (Thermo Fisher Scientific). Forebrain neurons were mechanically dispersed by trituration with a sterile flame polished Pasteur pipette and allowed to settle on ice for 5 min. The supernatant was transferred to a new collection tube and maintained on ice. Nondispersed tissues were resuspended in 2mL supplemented DPBS, triturated, and allowed to settle on ice for another 5 min. Supernatants were combined and centrifuged for 5 min at 1000rcf. The resulting cell pellet was gently resuspended in NeuroBasal Plus medium (Thermo Fisher Scientific) supplemented with B-27, GlutaMAX, and 1.0% penicillin/streptomycin (Thermo Fisher Scientific) and dissociated neurons were plated on 35mm poly-D-lysine coated culture dishes. Cells were maintained at 37°C with 5% CO₂ and humidification. After 24 hours, nonneuronal cells were growth arrested with 5uM cytosine arabinoside (Sigma Aldrich) and maintained in culture medium.

2.3 Immunofluorescence

Neuronal cultures were fixed in 4% paraformaldehyde in PBS for 20 min at RT, permeabilized in 0.3% Triton-X-100, and subsequently blocked for 1 hr in 5% normal goat

serum. Anti InsR- β monoclonal antibody (BD Biosciences; 610109) was applied (0.5 μ g/mL) overnight at 4°C in dilution buffer (1% BSA and 0.3% Triton X-100 in PBS). Slides were rinsed in PBS and incubated in Alexaflour goat anti-mouse 555 (Invitrogen, A32727) fluorescent secondary antibody (4 μ g/mL) for 1 hr at room temperature. Slides were rinsed in PBS and mounted using Prolong Gold antifade reagent (Thermo Fisher Scientific) to preserve fluorescence. Fluorescence microscopy was performed using a Nikon Diaphot inverted microscope and all images were background subtracted using a secondary only control.

2.4 Western Blotting

E12 forebrain and E12 hypothalamic tissues were harvested and Dounce homogenized in RIPA buffer (Thermo Fisher Scientific) supplemented with a Halt protease inhibitor cocktail (Thermo Fisher Scientific) and phenylmethanesulfonyl fluoride (Sigma Aldrich). Samples were sonicated briefly and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was then diluted in an equal volume of Laemmli's sample buffer (BioRad), boiled for 5 min, and electrophoresed on 12% acrylamide gels. Proteins were transferred to a nitrocellulose membrane and blocked in TBS containing 0.05% Tween-20 (TBST) and 5% milk. Anti-InsR β monoclonal antibody (BD Biosciences; 610109) was applied overnight in TBST at 4°C followed by three 5 min washes in TBST. Membranes were then incubated in IRDye 800 (LI-COR Biosciences) in TBST for 1 hour at RT, rinsed in TBST, and imaged using the Odyssey Clx Infrared Imaging System (LI-COR Biosciences).

2.5 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Hypothalamic tissue was dissected from E8, E10, and E12 embryos as previously described [21] and stored in RNAlater at 4°C until further use. Hypothalamic neuronal cultures were harvested at 7 DIV and RNA was isolated using RNeasy mini kits (Qiagen). Equal amounts of RNA were reversed transcribed and equal amounts of cDNA were amplified using the One Taq RT-PCR kit (New England Biolabs). Previously published primer sets were used to amplify gene fragments specific to the insulin receptor (InsR) tyrosine kinase domain located on the β subunit [22]. As a negative control, all cDNA synthesis reactions were run side by side with a no reverse transcriptase (-RT) condition. Resulting PCR products were separated via electrophoresis using a 2% agarose gel to resolve the InsR band at the predicted fragment size of 58 base pairs. After a 10 min incubation in ethidium bromide, gels were washed three times in dH₂O, and bands were visualized using UV transillumination on the GelDoc XR (BioRad).

2.6. Immunohistochemistry

Whole brains from each developmental time point were dissected under sterile conditions, fixed in 4% paraformaldehyde overnight at 4°C, and subsequently cryoprotected in 30% sucrose in PBS overnight at 4°C. Whole brains were then frozen in Tissue-Tek O.C.T. compound (Fisher HealthCare) and sliced to a thickness of 30 μ m using a Leica CM1520 cryostat at -30°C. Sections were transferred to subbed slides (Southern Biotech) and blocked for 1 hour in blocking buffer consisting of 5% normal goat serum containing 0.3% Triton-X-100 for permeabilization. Anti InsR β monoclonal antibody (BD Biosciences; 610109) was applied (0.5 μ g/mL) for 18–24 hours at 4°C in dilution buffer (1% BSA and

0.3% Triton X-100 in PBS). Slides were rinsed in PBS and treated with Alexafluor goat anti-mouse 555 (Invitrogen, A32727) fluorescent secondary antibody (4 μ g/mL) for 90 min at room temperature. Slides were rinsed in PBS and mounted using Prolong Gold antifade reagent containing DAPI to preserve fluorescence. All images were compared with mouse IgG1 isotype control for confirmed InsR staining. Confocal microscopy was performed on a Zeiss 510 META laser scanning confocal microscope supported by NIH Award Number 1S10RR019246 from the National Center for Research Resources at the University of Vermont Microscopy center.

3. Results

3.1. Insulin receptor mRNA and protein are expressed during avian embryonic brain development

It is well established that insulin signaling occurs in the central nervous system of adult mammalian species [3,23,24], however embryonic avian insulin signaling remains largely unexplored. Therefore, to determine the presence of the insulin receptor in the avian model, we first characterized the expression of InsR protein in both dissociated (Fig. 1A and B) and intact (Fig. 1C) whole brain tissue derived from E12 embryos. Immunofluorescence staining confirmed the presence of InsR in the soma and processes of avian forebrain neurons (Fig. 1A and B; n = 5). Consistent with other reports of brain insulin receptor expression [25], the monoclonal anti-InsR- β antibody detected a protein band at approximately 120kDa (Fig. 1C), slightly higher than the predicted weight of 95kDa for peripheral InsR (n = 4). Differences in the molecular weight banding between brain and peripheral insulin receptors may stem from changes in posttranslational modification, including the glycosylation pattern of receptor subunits [25,26].

Since pancreatic insulin levels have been reported to increase 10-fold from E5 to E12 [27] and plasma insulin levels have been detected as early as E10 [28], this may suggest a developmental role for insulin signaling in the embryonic chicken. To determine whether InsR expression is localized to the hypothalamus during early developmental time points, we performed RT-PCR analysis on intact hypothalamic tissue (Fig. 1D) and dissociated neurons derived from the E12 hypothalamus (Fig. 1E). Using previously published primer sets [22], InsR mRNA was detected in the avian hypothalamus at E8, E10, and E12 at the predicted fragment size of 58bp (Fig. 1D and E; n = 3). Similar to whole brain tissue, western blot analysis of hypothalamic lysates with a monoclonal InsR- β antibody revealed a prominent band at 120kDa (Fig. 1F; n = 3).

3.2. Insulin receptor protein localizes to hypothalamic regions governing energy homeostasis in the avian embryo

In adult mammals, brain InsR expression is prominent in the arcuate nucleus (ARC) of the hypothalamus, a region most notably associated with the regulation of food intake and energy expenditure [2,3,5]. In 5-day old post hatch chickens, InsR expression is also found in the ARC, where the receptor co-localizes with both orexigenic NPY neurons and anorectic POMC neurons [13]. In the post hatch chicken, various metabolic nuclei are present throughout the hypothalamus, where they vary in shape and size depending on the

anatomical plane of section [29]; however, little is known about the architecture of these nuclei during embryogenesis. In order to determine whether InsR expression is also found within metabolic nuclei of the embryonic chicken hypothalamus, we used immunohistochemical staining in coronal sections of brain tissue. Our data reveals hypothalamic InsR protein expression as early as E8 (Fig. 2B and C), where it localizes to an area resembling the paraventricular nucleus (PVN) surrounding the third ventricle (n = 6). While the immunofluorescence staining does not specify nuclei borders, it is clear from the DAPI overlay that some neurons express InsR while others lack InsR staining (Fig. 3). These data indicate heterogeneous InsR expression and may suggest the receptor is specific to neuronal subpopulations within the hypothalamus as early as E8.

Anatomically, there are marked differences in hypothalamic structure between E8 and E12 developmental time points. In addition to distinct differences in InsR staining patterns, DAPI nuclear staining in Fig. 2A (E8) and Fig. 2D (E12) demonstrates the overall architecture of the E12 hypothalamus has grown wider and elongated. Insulin receptor staining exists in both anterior and posterior coronal sections of E12 hypothalamus. In anterior sections, staining is present in the most lateral region of the hypothalamus, suggesting the emergence of LH nuclei staining (data not shown). Posterior sections show prominent InsR staining between the third ventricle and the most lateral hypothalamic borders (Fig. 4B and E; n = 8). Further anatomical characterization of this embryonic brain region demonstrates a marked resemblance to the VMH when compared to the stereotaxic chick atlas. In addition to the distinct VMH-like pocket in E12 tissue, posterior hypothalamic sections also exhibit staining near the median eminence (Fig. 2E and F; n = 8). This is the first demonstration of a localized cluster of InsR positive cells in an embryonic area resembling the ARC and suggests the formation of first order insulin sensitive hypothalamic neurons at a critical developmental time point.

4. Discussion

Differences in energy homeostasis among individuals are complex processes that may be initiated at critical points during gestation [30]. This can be confirmed through several rodent perinatal undernutrition studies examining nutritional alterations during the weaning period[5]. With influences largely stemming from the gestational environment, metabolic impairments later in life have been shown to correlate with specific problems that arise during development of the organism[31,32]. Studies linking maternal nutrition and the developing organism's predisposition to disease are increasing in number[30,33]. This highlights the necessity for further discussion on the underlying mechanisms causing obesity. While obesity has historically been thought of as a "willpower" disorder, evidence shows that complexities arising from genetic predisposition, neuronal development in the central nervous system, and fetal nutritional exposure all play a role in contributing to obesity later in life[12,34]. Therefore, it is important to understand when key molecular interactions involved in appetite signaling arise and how alterations may produce changes in neuronal development.

Several studies indicate that the role of insulin signaling in the regulation of energy balance is conserved between birds and mammals. For example, the central infusion of insulin in

both chicks and baboons has been shown to suppress feeding behavior[35,36]. Furthermore, neuropeptide expression in hypothalamic neurons demonstrates a high degree of evolutionary conservation between birds and mammals[37]. Investigations of how gestational environments influence the development of metabolism are critical to understanding the progression of disease later in life[38]. While mammalian systems offer a challenging approach to diagnose risk factors due to the dependence of the embryo on the mother, the chicken embryo provides a controlled setting for experimental manipulation and is an excellent developmental model for exploring metabolic programming independent from maternal influence. The avian embryo offers the flexibility to exogenously manipulate specific factors during development and establish differences in receptor expression in metabolic nuclei. Previous research has implemented embryonic glucose manipulation to examine insulin receptor expression in postnatal chickens[18]. To begin to understand the developmental alterations of these metabolic circuits during embryogenesis, our study pinpoints InsR expression at critical developmental time points, thus establishing early receptor localization and the conservation of metabolic pathways between species.

Changes in membrane excitability due to insulin receptor signaling have also been found to alter neuronal circuits. In mammalian olfactory bulb neurons, alterations in membrane excitability can have permanent effects on neuronal function through the suppression of peak current amplitude in voltage-gated potassium channels (Kv) [25]. In particular, the Kv1.3 potassium channel is known to play a critical role in determining neuronal excitability when directly phosphorylated by the kinase activity of the insulin receptor[39]. Previous evidence from our lab demonstrates mRNA expression of the insulin-sensitive Kv1.3 channel in the avian hypothalamus[21]. When paired with data from our current study, Kv1.3 mRNA is expressed at the same embryonic time points as InsR (E8, E10, E12), suggesting a potential protein interaction in both mammals and chickens and further illustrating the importance of examining insulin dependent processes in various species.

Since insulin is predominantly released by pancreatic beta cells, it is also important to consider the timing of both pancreatic and hypothalamic development. Evidence suggests that proteins critical to hypothalamic nuclei formation are present at the same time the pancreas begins to produce insulin [40–43]. In the avian embryo, pancreatic insulin levels have been reported as early as embryonic day 5 (E5) [27] and circulating levels of plasma insulin have been detected at E10 [28], suggesting an early role for insulin signaling during development. Insulin has inhibitory effects on rostral neurons (LH and PVN nuclei) differentiating from embryonic stem cells [44]. Our current study demonstrates InsR expression in areas of the chicken embryo similar in structure to the PVN at E8 (Fig. 3B and C), and the LH, VMH, and ARC as early as E12 (Fig. 3E and F). These findings are congruent with both pancreatic and plasma insulin expression and suggest that the insulin hormone may play a key role in avian hypothalamic circuit formation at early stages of embryonic development.

The findings presented in this study reveal the earliest known InsR expression in the developing avian hypothalamus and suggest that insulin signaling may play a functional role in brain development. Although specific hypothalamic nuclei are challenging to analyze this early in avian brain development, immunohistochemical analysis demonstrates distinctive

boundaries within brain tissue that align with previously mapped regions of the 2-week old chick[29]. In addition, the heterogeneous InsR expression pattern suggests that the receptor localizes to specific subpopulations of neurons at early time points in hypothalamic development. There is a clear maturation of InsR positive neurons between E8 and E12 in areas resembling the VMH and ARC, which may be linked to the concurrent expression of plasma insulin at this stage in development[28]. While the presence and location of hypothalamic InsR provides insight into the mechanism of insulin sensitive neural development, the function and timing of insulin action is critical to understanding the hormones effects on the development of metabolic neuronal circuitry. Further experiments are needed to characterize the exact nature of neuronal subpopulations as well as the functional response to exogenously applied insulin hormone. Overall, the data presented in this study provide evidence for the role of insulin signaling at early periods during hypothalamic development, underscoring the importance of cellular environments in the development and maintenance of metabolic control centers in the brain.

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Abbreviations

DAPI	4',6-diamidino-2-phenylindole
InsR	Insulin Receptor
E8	embryonic day 8
E10	embryonic day 10
E12	embryonic day 12
3v	third ventricle
ARC	arcuate nucleus
VMH	ventromedial hypothalamus
LH	lateral hypothalamus
PVN	paraventricular nucleus
POMC	proopiomelanocortin
CART	cocaine and amphetamine related transcript
AgRP	agouti-related peptide
NPY	neuropeptide Y

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Fig. 1. Insulin receptor expression in the embryonic avian brain.

A) Immunofluorescence staining of InsR protein in dissociated neuronal cultures (7 DIV) derived from embryonic day 12 (E12) avian whole brain tissue. Scale bar = 100 μ m; n=5. B) High power magnification of InsR labeled neuron from the dotted region indicated in A. Scale bar = 50 μ m. C) Western blot analysis of InsR protein detected in E12 whole brain tissue. M = marker; n = 4. D) RT-PCR analysis of InsR expressed at E8, E10, and E12 in the intact avian hypothalamus. A no reverse transcriptase (-RT) condition was performed in parallel as a negative control for genomic DNA contamination. M = marker; n = 3. E) RT-PCR analysis of InsR expressed in dissociated E12 avian hypothalamic cultures. M = marker; n = 3. F) Western blot analysis of InsR protein detected in E12 hypothalamic lysates. M = marker; n = 3.

Fig. 2. Changes in insulin receptor localization during hypothalamic development.

Representative low power composite (5×5 array) fluorescence micrographs of E8 (top) and E12 (bottom) coronal hypothalamic sections labeled with DAPI nuclear staining (A, D) and anti-InsR antibody (B, E) and subsequently merged (C, F). Dotted lines highlight increased InsR intensity in areas that resemble the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) of E12 as compared to adjacent tissue. 3v = third ventricle; scale bar = $200\mu\text{m}$; $n = 6$ (E8); $n = 8$ (E12).

Fig. 3. Heterogeneous insulin receptor expression in the E8 hypothalamus.

Representative high power fluorescence micrograph of an E8 coronal hypothalamic section depicting the developing paraventricular (PVN) region labeled with A) DAPI nuclear staining and B) anti-InsR antibody and subsequently merged (C). Scale bar = 20µm; n = 6.

Fig. 4. Insulin receptor expression in the E12 ventromedial nucleus of the hypothalamus. Fluorescence micrographs of E12 coronal hypothalamic sections labeled with DAPI nuclear staining (A, D) and anti-InsR antibody (B, E) and subsequently merged (C, F). Dotted line depicts a clear border between InsR staining in the developing ventromedial hypothalamus (VMH) compared to adjacent tissue. 3v = third ventricle; top scale bar = 100μm; bottom scale bar = 20 μm; n = 8.