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Developmental expression of Kv1 voltage-gated potassium channels in the avian hypothalamus

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Abstract

Specialized hypothalamic neurons integrate the homeostatic balance between food intake and energy expenditure, processes that may become dysregulated during the development of diabetes, obesity, and other metabolic disorders. Shaker family voltage-gated potassium channels (Kv1) contribute to the maintenance of resting membrane potential, action potential characteristics, and neurotransmitter release in many populations of neurons, although hypothalamic Kv1 channel expression has been largely unexplored. Whole-cell patch clamp recordings from avian hypothalamic brain slices demonstrate a developmental shift in the electrophysiological properties of avian arcuate nucleus neurons, identifying an increase in outward ionic current that corresponds with action potential maturation. Additionally, RT-PCR experiments identified the early expression of Kv1.2, Kv1.3, and Kv1.5 mRNA in the embryonic avian hypothalamus, suggesting that these channels may underlie the electrophysiological changes observed in these neurons. Real-time quantitative PCR analysis on intact microdissections of embryonic hypothalamic tissue revealed a concomitant increase in Kv1.2 and Kv1.5 gene expression at key electrophysiological time points during development. This study is the first to demonstrate hypothalamic mRNA expression of Kv1 channels in developing avian embryos and may suggest a role for voltage-gated ion channel regulation in the physiological patterning of embryonic hypothalamic circuits governing energy homeostasis.

Keywords

Hypothalamus; Potassium channel; Kv1; Embryonic chicken; Energy homeostasis; Development

1. Introduction

Shaker family voltage-gated potassium channels (Kv1) are major determinants of membrane excitability in many types of neurons [1, 2], contributing to the maintenance of resting membrane potential [3], action potential repolarization [4], interspike interval [5], and

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neurotransmitter release [6]. Variable expression of Kv1 channels in the plasma membrane of neurons can alter neuronal excitability [7, 8, 9, 10], thus altering the activity of certain brain regions. Aberrant neuronal excitability during the developmental programming of neuronal circuits may alter the long-term physiological behavior of an organism [11]. Thus, it is important to understand whether Kv1 channels contribute to the electrophysiological events that occur during gestational development.

The hypothalamus is a brain region that mediates the regulation of critical metabolic processes throughout the body [12, 13]. For example, prenatal temperatures have been shown to influence postnatal hypothalamic thermosensitivity in birds [14], underscoring the importance of the gestational environment on the long term physiology of the organism. Despite recent interest in the influence of prenatal factors on hypothalamic development and consequent adult metabolism, little remains known about the electrophysiological development of hypothalamic neurons [15, 16, 17]. Specialized neurons in the arcuate nucleus (ARC) of the hypothalamus can integrate the homeostatic balance between food intake and energy expenditure via peripheral signaling, mechanisms that are well conserved between mammals and birds [18, 19]. Therefore, understanding the developmental expression and function of Kv1 channels in the arcuate nucleus of the avian hypothalamus may provide insight into the early patterning of electrophysiological activity in these neurons.

The Kv1 family of voltage-gated potassium channels is composed of eight distinct genes that code for separate alpha subunit proteins, Kv1.1 through Kv1.8 [20]. Homomultimeric or heteromultimeric assembly of these subunits through a T1 tetramerization domain leads to the formation of functional Kv1 ion channels [4, 21, 22]. Four specific intronless alpha subunits, Kv1.2, Kv1.3, Kv1.5 and Kv1.8, have been cloned in the chicken genome and demonstrate conservation of important mammalian structural regions including the T1 tetramerization domain, the ion transport domain, and the PDZ-binding domain [23]. However, whether Kv1 channel regulation plays a role in the electrophysiological development of embryonic hypothalamic circuits remains unclear. In this study, we test the hypothesis that Kv1 channels are expressed in the avian hypothalamus during critical time points during gestation, where they may contribute to the early patterning of homeostatic circuits.

Several reports indicate that Kv1 family channels are present in avian tissues [23, 24, 25, 26, 27]. For example, Kv1.2, Kv1.3 and Kv1.5 mRNA has been detected in several regions of the embryonic avian cochlea [23]. Additional studies demonstrate Kv1.1 and Kv1.2 immunoreactivity in neuronal cell bodies of the chick tangential nucleus [27]. Furthermore, α -dendrotoxin sensitive delayed rectifier currents have been shown to affect the electrophysiological function of nucleus magnocellularis neurons in the avian brainstem [25, 26]. Here, we report the presence of Kv1 channel mRNA in the avian embryonic hypothalamus and provide evidence for a developmental increase in gene expression that may underlie the electrophysiological changes observed in these cells.

2. Materials and methods

2.1. Tissue harvest

Fertile white leghorn chicken eggs were obtained from Sunrise Farms, Catskill, NY 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) or embryonic day 12 (E12). For electrophysiological recordings, embryos were sacrificed by decapitation and whole brains were embedded in 1% agarose for microtome sectioning. For RT-PCR and quantitative real-time PCR, hypothalamic tissue was microdissected on ice and stored in RNAlater at 4°C until further RNA isolation.

2.2. Hypothalamic slice cultures

250µm coronal hypothalamic brain slices were generated using a Leica VT1200S vibrating microtome and maintained in oxygenated (95% O₂ / 5% CO₂) artificial CSF (aCSF) composed of the following (in mM): 125 NaCl, 4 KCl, 1 MgCl₂, 1 KH₂PO₄, 24 NaHCO₃, 2 CaCl₂, 11 glucose. Slices were allowed to recover for 1 hr at 37°C before initial electrophysiological recordings. All brain slices were visualized using differential interference contrast (DIC) imaging on a Nikon upright fixed stage microscope. To minimize circadian artifacts within hypothalamic tissues, electrophysiological recordings for all developmental time points were consistently performed during the same time of day.

2.3. Electrophysiology

Hypothalamic brain slices were affixed to the recording chamber and the arcuate nucleus was visually identified using an avian neuroanatomy atlas (Fig. 1) [28]. Standard whole-cell patch clamp recordings were performed at 37°C using an Axopatch 200A amplifier and pCLAMP 9 software. Slices were superfused with aCSF (2 mL/min) and the internal pipette solution was composed of the following (in mM): 140 KCl, 1 CaCl₂, 1 MgCl₂, 10 EDTA, 10 HEPES, pH 7.3. For voltage-clamp experiments, neurons were held at -60 mV, followed by a 20 ms hyperpolarization to -90 mV and stepped in 10 mV increments between -70 mV to 50 mV. Leak currents (P/8) were subtracted from all traces. For current-clamp experiments, resting membrane potential was recorded for 100 ms, after which a 50 pA hyperpolarizing pulse of current was injected, followed by four depolarizing current pulses delivered in 50 pA increments. Action potential overshoot was measured at the 150 pA current pulse. All electrodes were pulled from borosilicate glass and fire polished to resistances ranging from 5 – 10 MΩ. All data represent individual neurons from separate slice recordings.

2.4. Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Using an avian neuroanatomy atlas [28], whole hypothalamic tissue was dissected from E8 and E12 embryos. The pituitary gland and infundibulum were carefully removed to expose the intact hypothalamus at the ventral surface of the brain. Hypothalamic tissue surrounding the third ventricle was extracted using the optic chiasm and mammillary bodies as anterior and posterior borders, respectively. Hypothalamic tissue was stored in RNAlater at 4°C until further use and RNA was isolated using RNeasy mini kits from Qiagen according to the manufacturer's protocol. Equal amounts of RNA were reversed transcribed and equal

amounts of cDNA were amplified using the OneTaq RT-PCR kit from New England Biolabs. Previously published primer sets were used to amplify gene fragments specific to Kv1 channels and -actin primers were used as a positive control for a reference gene [23]. Because Kv1 family channel genes are intronless, all cDNA synthesis reactions were run side by side with a no reverse transcriptase (RT) condition as a negative control. Resulting PCR products were separated via electrophoresis using a 2% agarose gel to resolve bands at predicted fragment sizes. Gels were incubated in ethidium bromide and bands were visualized using UV transillumination on the GelDoc XR from Bio Rad.

2.5. Real-Time Quantitative PCR (qPCR)

Hypothalamic cDNA samples for real-time quantitative PCR (qPCR) were prepared as described above. Primer sets designed and used for qPCR experiments are outlined in Table 1. All qPCR primers were validated by the University of Vermont DNA analysis facility and qPCR reactions were performed using the Applied Biosystems 7500 Fast Real-Time PCR System. All qPCR reactions included (–) RT controls. Data were normalized to the β -actin reference gene and analyzed using the standard curve method. Amplified qPCR products were sequenced to confirm the identity of the cDNA.

2.6. Statistical Analysis

Data are presented as the mean $\pm$ standard error (SEM). Unpaired Student's *t*-tests assuming unequal variances were used to determine statistical differences. Unless otherwise noted, differences were considered significant if $p < 0.05$.

3. Results

3.1. Electrophysiological recordings from avian arcuate nucleus neurons during various developmental time points.

Using the whole-cell patch clamp technique, we found an increase in the steady-state outward current recorded from avian hypothalamic neurons in the ARC of E12 embryos (Fig. 2B; $n=8$) as compared to E8 embryos (Fig. 2A; $n=6$). Current traces for each developmental time point represent the average of unpaired measurements recorded from individual slices. To determine whether this observed increase in outward current was due to a developmental change in membrane surface area over time, we also measured the membrane capacitance of each neuron. While the data presented in Fig. 2C indicate a significant increase in membrane capacitance from E8 ($13.4 \text{ pF} \pm 1.03$) to E12 ($18.8 \text{ pF} \pm 1.79$; $P < 0.01$), capacitance compensated current-voltage relationships shown in Fig. 2D indicate a significant increase in outward current from E8 to E12 over a broad range of voltage steps ($P < 0.05$). These data indicate that the increase in cell size of hypothalamic ARC neurons is also accompanied by a concomitant increase in the outward current conductance measured from these cells.

3.2. Kv1 channel mRNA expression in avian hypothalamic neurons during various developmental time points

To begin to assess the underlying component of the observed developmental changes in electrophysiological characteristics, we tested the hypothesis that Kv1 channels are present

in hypothalamic neurons during early development in the avian embryo. Functional Kv1 voltagegated potassium channels can form heteromultimers with certain alpha subunits from the same family [22]. For example, Kv1.2 and Kv1.5 have been shown to form heteromultimeric complexes in vascular smooth muscle cells [29, 30], while Kv1.3 and Kv1.5 association has been reported in macrophages [31]. We utilized previously published primer sets [23] to determine the presence of Kv1.2, Kv1.3, Kv1.5, and Kv1.8 channels in the genomic DNA of E12 chickens (Fig. 3A). Predicted fragment sizes were amplified for four Kv1 channels: Kv1.2, 448bp; Kv1.3, 474bp; Kv1.5, 347bp; and Kv1.8, 364bp. To determine whether these Kv1 channels are expressed in the avian hypothalamus, microdissections were performed to isolate hypothalamic tissue from E8 and E12 embryos. Each reaction included a no reverse transcriptase (-RT) negative control due to the intronless nature of Kv1 family channel genes. Analysis of mRNA expression using RT-PCR demonstrates the presence of Kv1.2, Kv1.3, and Kv1.5 mRNA in the hypothalamus of E8 embryos (Fig. 3B). Similarly, Kv1.2, Kv1.3, and Kv1.5 mRNA was also present in hypothalamic microdissections isolated from E12 embryos (Fig 3C). While the presence of Kv1.8 was confirmed in the chicken genome (Fig. 3A), Kv1.8 mRNA remained undetectable in the hypothalamic extracts at both E8 and E12 developmental time points (Fig. 3B and C).

3.3. Developmental increase in avian hypothalamic Kv1 channel mRNA

Several ion channel candidates could potentially mediate the changes in outward ionic current observed in Fig. 2. Therefore, we performed quantitative real-time PCR (qPCR) analysis on hypothalamic microdissections to test the hypothesis that Kv1 channel mRNA expression is upregulated in the avian hypothalamus from E8 to E12. Independent tissue isolates from separate hypothalamic microdissections were used to generate multiple cDNA preparations for each embryonic day. The newly designed primer sets outlined in Table 1 were tested using RT-PCR (Fig. 4A). The qPCR analysis shown in Fig. 4B revealed a significant increase in both hypothalamic Kv1.2 and Kv1.5 mRNA expression between E8 and E12 ($n=3$; $P < 0.05$). Although no significant difference was detected in Kv1.3 mRNA expression between E8 and E12, these data are the first to indicate the presence of this channel in the developing chicken hypothalamus. Because Kv1.8 mRNA was undetectable at E8 and E12 using RT-PCR (Fig. 3B and C), this channel was not included in the qPCR analysis.

3.4. Action potential characteristics from avian arcuate nucleus neurons during various developmental time points.

To determine if the developmental increase in outward ionic current observed in Fig 2 was linked to alterations in membrane voltage, we also measured action potential characteristics from avian hypothalamic brain slices at E8 and E12 time points. Current-clamp recordings from E8 avian arcuate neurons demonstrate a small amplitude response at the onset of current injection which was considered to be an immature action potential (Fig. 5A). Consistent with the increase in outward ionic current shown in Fig 2A, B, and D, E12 arcuate neurons also displayed a robust phasic action potential upon current injection (Fig. 5B). Although no difference in neuronal resting membrane potential was detected between E8 ($-56.3 \text{ mV} \pm 14.68$; $n=3$) and E12 ($-60.9 \text{ mV} \pm 5.40$; $n=7$) time points (Fig. 5C), there was a significant increase in the action potential overshoot from E8 ($6.7 \text{ mV} \pm 2.00$) to E12

($16.7 \text{ mV} \pm 4.50$; $P < 0.05$) (Fig. 5D). The threshold to action potential firing in all cells was approximately -35 mV .

4. Discussion

Voltage-gated potassium channels have been well studied in adult neurons, however their role in regulating neuronal physiology during development remains poorly understood. In the developing amphibian nervous system, delayed rectifier K^+ current (I_{Kv}) has been shown to facilitate the transition from early action potentials with long durations to mature action potentials exhibiting brief spikes [32, 33, 34]. Delayed rectifier currents have also been isolated in mammalian embryonic neurons [35] and the specific Shaker channel, $Kv1.1$, is differentially expressed during development in murine neurons [36]. Here, we present the first report of electrophysiological recordings from hypothalamic neurons of the embryonic chicken. We identify an important developmental shift in the outward ionic current between E8 and E12 time points (Fig. 2D) which corresponds with an electrophysiological maturation of action potentials (Fig. 5B) in chicken hypothalamic neurons.

This study is also the first to identify Shaker family $Kv1$ channel mRNA in the embryonic avian hypothalamus (Fig. 3B, C), suggesting that these channels may play a role in the patterning of neuronal circuits governing homeostatic processes including food intake and energy expenditure. Previous studies have identified $Kv1$ channels in neurons of the rat hypothalamic paraventricular nucleus, speculating that channel regulation may modulate neuronal activity in the adult mammalian hypothalamus [37]. Because alterations in neuronal excitability during early development can profoundly alter the wiring of hypothalamic circuits [38], we aimed to characterize the expression of various $Kv1$ channels in the embryonic chicken. While $Kv1$ channel genes were previously cloned and identified in cochlear tissues of the embryonic chicken [23], our study is the first to demonstrate hypothalamic mRNA expression of $Kv1.2$, $Kv1.3$, and $Kv1.5$ channels in developing avian embryos and may suggest a role for voltage-gated ion channel regulation in the physiological patterning of embryonic hypothalamic circuits. Future electrophysiology experiments using 4-aminopyridine, a non-selective inhibitor of voltage-gated potassium channels [39], should aid in the confirmation of functional potassium channel subtypes. Furthermore, pharmacological application of venom-derived toxins [40, 41, 42] should confirm the link between specific $Kv1$ alpha subunit expression and the observed shift in the electrophysiological characteristics of developing hypothalamic neurons.

Evidence has shown that certain Shaker channels can form heteromultimeric complexes in an array of cell types including vascular myocytes [29], macrophages [31], and glial cells [43]. In particular, $Kv1.2$ and $Kv1.5$ have been shown to form heteromultimeric complexes that appear to have a key role in maintaining membrane potential in smooth muscle cells [29]. Similarly, heteromultimeric association between $Kv1.3$ and $Kv1.5$ has been reported to alter the localization, targeting, and subcellular distribution of these channels [44]. Our study demonstrates that hypothalamic neurons also express several $Kv1$ alpha subunits, which may form heteromultimeric complexes that influence hypothalamic neuronal function. Here we report a developmental increase in the expression of $Kv1.2$ and $Kv1.5$ channels in the avian hypothalamus that is correlated with an increase in outward current and action potential

maturation in these cells. Therefore, this study may also provide insight into the role of voltagegated potassium channels in the physiological development of embryonic hypothalamic circuits.

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Abbreviations

Kv1	Shaker family voltage-gated potassium channel
E8	embryonic day 8
E12	embryonic day 12
aCSF	artificial cerebrospinal fluid
Opt	optic tract
3v	third ventricle
ARC	arcuate nucleus
VMH	ventromedial hypothalamus
ME	median eminence
Elec	electrode

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Highlights

- Avian hypothalamic neurons demonstrate a developmental increase in outward current
- E12 hypothalamic neurons exhibit robust phasic action potentials compared to E8
- Kv1.2, Kv1.3, and Kv1.5 mRNA is present in embryonic avian hypothalamic neurons
- Increase in Kv1.2 and Kv1.5 likely contributes to electrophysiological changes

Fig. 1. Embryonic avian hypothalamic slice cultures for electrophysiological recordings. Representative photomicrograph of an E12 chicken coronal brain slice affixed to the electrophysiological recording chamber (left). Corresponding regions of interest as adapted from an avian neuroanatomy atlas of a 2-week old chicken brain (right) [34]. Opt, optic tract; 3v, third ventricle; ARC, arcuate nucleus; VMH, ventromedial hypothalamus; ME, median eminence; Elec, electrode. (Scale bar = 200 μ m)

Fig. 2. Developmental changes in outward current recorded from avian hypothalamic neurons. Average voltage-clamp recordings from neurons of E8 (A; $n = 6$) and E12 (B; $n = 8$) hypothalamic brain slices. Membrane capacitance was estimated by calculating the integral of the initial capacitance transient in response to a 10 mV hyperpolarizing pulse (C). Capacitance compensated steady state current-voltage relationships (D) demonstrate a significant developmental increase in outward currents recorded from hypothalamic neurons (* $p < 0.05$).

Fig. 3. Kv1 channel mRNA expression in the embryonic avian hypothalamus.

Genomic DNA analysis (A) reveals predicted fragment sizes for the following Kv1 channels: Kv1.2 (448 bp); Kv1.3 (474 bp); Kv1.5 (347 bp). RT-PCR products for Kv1 channels expressed at E8 (B) and E12 (C) were separated via electrophoresis. All cDNA synthesis reactions were run side by side with a no reverse transcriptase (-RT) condition as a negative control. β -actin primers were used as a positive control for a reference gene.

Fig. 4. Developmental increase in Kv1 channel gene expression in the avian hypothalamus.

A) Real-time quantitative PCR primers were designed to amplify predicted fragment sizes for Kv1.2 (98bp), Kv1.3 (100bp), Kv1.5 (102bp), and β -actin (100bp) expressed in hypothalamic tissue. B) Hypothalamic Kv1.2 and Kv1.5 gene expression increases from E8 to E12 ($n = 3$; $p < 0.05$); no significant change in gene expression was detected for Kv1.3 ($n = 3$). Real-time quantitative PCR data was calculated using the standard curve method and all Kv1 channel expression data was normalized to the β -actin reference gene for each condition.

Fig. 5. Developmental changes in action potential characteristics in avian hypothalamic neurons. Representative current-clamp recordings from arcuate neurons of E8 (A) and E12 (B) hypothalamic brain slices. Current was administered for either 400 ms (A) or 200 ms (B). While no difference in resting membrane potential (C) was detected between E8 ($n = 3$) and E12 ($n = 7$) time points, a significant increase in the action potential overshoot (D) was observed (* $p < 0.05$).

Table 1.

Forward and reverse qPCR primer sequences

mRNA	Primer sequence	Annealing temp	Amplicon size
Kv1.2	5' – GGA AGA AGC CAT GGA GAT GT – 3' 5' – GCA ACC AGA CCT GTC TCT GA – 3'	59 °C	98 bp
Kv1.3	5' – CAG GGA TGA CCA CGA CTA TG – 3' 5' – AAC AGC AGA GGA CGA GGA AT – 3'	59 °C	100 bp
Kv1.5	5'- CAG GTC TGG CTC ATC TTT GA -3' 5'- GCA GAA GGT GAT GAT GGA GA -3'	59 °C	102 bp
β-actin	5'- GCG CAA GTA TTC TGT CTG GA -3' 5'- GGG GCC AGA CTC ATC ATA CT -3'	59 °C	100 bp