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Tuberal hypothalamic expression of the glial intermediate filaments, glial fibrillary acidic protein and vimentin across the turkey hen (*Meleagris gallopavo*) reproductive cycle: Further evidence for a role of glial structural plasticity in seasonal reproduction

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ABSTRACT

Glia regulate the hypothalamic–pituitary–gonadal (HPG) axis in birds and mammals. This is accomplished mechanically by ensheathing gonadotrophin-releasing hormone I (GnRH) nerve terminals thereby blocking access to the pituitary blood supply, or chemically in a paracrine manner. Such regulation requires appropriate spatial associations between glia and nerve terminals. Female turkeys (*Meleagris gallopavo*) use day length as a primary breeding cue. Long days activate the HPG-axis until the hen enters a photorefractory state when previously stimulatory day lengths no longer support HPG-axis activity. Hens must then be exposed to short days before reactivation of the reproductive axis occurs. As adult hens have discrete inactive reproductive states in addition to a fertile state, they are useful for examining the glial contribution to reproductive function. We immunostained tuberal hypothalami from short and long-day photosensitive hens, plus long-day photorefractory hens to examine expression of two intermediate filaments that affect glial morphology: glial fibrillary acidic protein (GFAP) and vimentin. GFAP expression was drastically reduced in the central median eminence of long day photosensitive hens, especially within the internal zone. Vimentin expression was similar among groups. However, vimentin-immunoreactive fibers abutting the portal vasculature were significantly negatively correlated with GFAP expression in the median eminence, which is consistent with our hypothesis for a reciprocal relationship between GFAP and vimentin expression. It appears that up-regulation of GFAP expression in the central median eminence of turkey hens is associated with periods of reproductive quiescence and that photofractoriness is associated with the lack of a glial cytoskeletal response to long days.

1. Introduction

While glia were once believed to primarily serve an architectural purpose in the nervous system, research over the past few decades has shown that these cells regulate hypothalamic–pituitary–gonadal (HPG) axis activity. This view is evidenced by findings that the spatial relationship between glia and GnRH nerve terminals varies over vertebrate reproductive cycles. Electron microscopy revealed that in diestrus female rats, which have low levels of GnRH release, glial processes ensheath GnRH nerve terminals with much higher frequency than is observed in proestrus rats, when the GnRH surge occurs (Prevot et al., 1999). Tanycytes, glia whose processes extend from their point of origin in the ependymal layer of the third ventricle (VIII) to the external zone of the median eminence (ME-EZ), are a well-established source of this ensheathment (Prevot et al., 1999; Rodriguez et al., 2005). Wrapping of tancyctic endfeet around GnRH nerve terminals in the ME-EZ is believed to create a barrier to neuropeptide release into the hypophyseal-portal-vasculature which prevents GnRH from reaching the pituitary and stimulating release of the gondatrophins, thereby suppressing gonadal activity (Rodriguez et al.,

Abbreviations: LD, long day; ir, immunoreactive; IA, infundibular area; ME, median eminence; ME-IZ, internal zone of ME; ME-EZ, external zone of ME; PR, photorefractory; SD, short day; TNB-HS, Tris-NaCl-blocking reagent with horse serum.

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2005). Astrocytes, glia characterized by the stellate shape that they may exhibit (Montgomery, 1994), have also been reported to ensheath GnRH nerve terminals in non-human primates (Witkin et al., 1995), humans (Baroncini et al., 2007) and possibly in sheep (Lehman et al., 1988), but the exact function of astrocytic–GnRH terminal ensheathment is poorly understood (Dhandapani et al., 2003). It is known, however, that tanycytes (Nakao et al., 2006; Prevot et al., 2003; Yoshimura et al., 2003) and astrocytes (Kuo and Micevych, 2012; Ojeda et al., 2003) produce factors, such as transport proteins, neurosteroids and enzymes, that influence GnRH release.

Changes in the spatial relationship between GnRH terminals are also observed in seasonally breeding birds. In male Japanese quail (*Coturnix japonica*), inducement of GnRH release and subsequent gonadal activation by exposure to reproductively stimulatory long days corresponds with a significant decline in the number of putative GnRH nerve terminals encased by glia, as compared with reproductively quiescent short-day quail (Yamamura et al., 2004). Turkeys (*Meleagris gallopavo*) also use long photoperiods as a proximal cue for reproduction (Siopes, 1994). As with many other avian photoperiodic breeders, female turkeys (Siopes, 1994) develop a state of photorefractoriness in which previously stimulatory day lengths become insufficient to maintain reproductive activity and the HPG-axis shuts down in anticipation of unfavorable environmental conditions (Nicholls et al., 1988); exposure to short days for several weeks is necessary to reestablish photosensitivity (Siopes, 1989). In sharp contrast to reproductively inactive short-day but photosensitive hens, as well as photorefractory hens, the rostral and central ME of reproductively active long-day turkey hens express high levels of type 2-iodothyronine deiodinase, a glial derived enzyme that increases thyroid hormone activity (Steinman et al., 2008). Thyroid hormone in turn has been reported to induce tanycyte retraction from GnRH terminals in Japanese quail (Yamamura et al., 2006). Likewise long-day turkeys show high expression of residual proteins marking recent expression of c-fos, a transcription factor and indirect marker of cellular activation (Morgan and Curran, 1991), in the anterior portion of the ME, while short day and photorefractory hens show little evidence of c-fos immunoreactivity (Millam et al., 2003; Steinman et al., 2008). Many of the c-fos-immunoreactive (ir) nuclei in the long-day turkey appear inside stellate astrocytes (Steinman et al., 2008). These findings suggest that glia in the ME of photorefractory hens seem to themselves show a refractoriness to long days.

This apparent glial photorefractoriness coupled with the fact that reproductively mature turkeys have a seasonal cycle with one reproductively active state and at least two distinct inactive states makes turkeys a useful model of how glia regulate HPG-activity. Herein reproductive state refers to the combination of photoperiod and degree of photosensitivity. We sought to integrate this paradigm with an examination of the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, as they are integral components of glial cytoskeletons that can influence glial spatial associations with neurons (Eng and Lee, 1995). Intermediate filaments support cytoarchitecture in glia and motility of free-moving astrocytes by influencing the dimension and density of glial processes. GFAP which is often used as a marker of astrocytes (Chiu et al., 1981; Eng and Ghirnikar, 1994), but also occurs in tanycytes and other glia (Chojnacki et al., 2009), is significantly less expressed in the ME-EZ of rats in the ovulatory phase of their estrous cycle (Parkash and Kaur, 2005). Vimentin occurs in tanycytes (Pixley et al., 1984) and immature astrocytes (Dahl et al., 1981; Voigt, 1989) and has been shown to exhibit reduced expression under short photoperiods in reproductively suppressed hamsters (Bolborea et al., 2011; Kameda et al., 2003). Therefore, reduced GFAP expression and increased vimentin expression may be associated with periods of high GnRH release. Consequently, we used immunohistochemistry to test the hypothesis that GFAP and vimentin expression occur in a reciprocal fashion, as turkeys transition between states of reproductive activity and quiescence. Specifically, we predicted that reduced GFAP expression would be accompanied by elevated vimentin expression in the ME of photostimulated long day hens as compared to photosensitive short day and photorefractory turkeys. We also examined expression of these two intermediate filaments in the infundibular area (IA), another photoresponsive portion of the tuberal hypothalamus dorsal to the ME (Meddle and Follett, 1995; Millam et al., 2003; Steinman et al., 2008). Results indicated that GFAP expression was in fact reduced in the central portion of the ME of long day hens, but surprisingly, the effect was most pronounced in the inner portion (ME-IZ). There was no effect of reproductive state on vimentin expression in any of the areas analyzed.

2. Materials and methods

2.1. Experimental animals

The study used somatically mature turkey hens (Nicholas 06 02 strain, Nicholas Turkeys, Lewisburg, WV, USA) that had completed fewer than two reproductive cycles. One group of photosensitive hens was maintained under a short day photoschedule (8L:16D; SD group) for 10–14 weeks before euthanasia. A subset of these hens were photostimulated by switching them from short to long days (16L:8D) for 48 h prior to euthanasia (LD group). A final group of hens (PR) was exposed to 35–46 weeks of long days until they became photorefractory as evidenced by a cessation of egg production and palpation of rigid, tight pubic bones. They were then placed on short days for 2 weeks, which is insufficient to reestablish photosensitivity [24], and again exposed to long days for 48 h before euthanasia. Upon post-mortem inspection, PR hens were observed to have completely regressed ovaries. This protocol is the same as has been previously used to test photoinducibilty of turkey hen hypothalamic regions in our lab (Millam et al., 2003; Steinman et al., 2008). Hens were euthanized by an intravenous overdose of chloral hydrate (20%) in water, and then underwent perfusion fixation via the carotid artery by exsanguinating with sodium phosphate-buffered saline (PBS) and heparin and then fixing with 10 ml of cold 4% paraformaldehyde. Brains were removed and the hypothalamus was isolated by dissecting away the surrounding tissue, transferred to paraformaldehyde overnight, and cryoprotected in 20% sucrose in PBS for 24 h. Tissue was flash frozen on dry ice and stored at −80 °C until cryosectioning at 25 μm thickness, thaw-mounted (Superfrost; Fisher Scientific, Pittsburgh, PA, USA), and stored at 4 °C in 95–100% ethanol. All animals were treated according to protocols approved by the Institutional Animal Care and Use Committees of the University of California, Davis and North Carolina State University.

2.2. Immunohistochemistry

The rostral two-thirds of the ME (approximately 1.5 mm in distance, encompassing six sections chosen at every 250 μm) was single-labeled for either GFAP or vimentin. This portion of the ME was examined because previous turkey work indicated that it is highly responsive to photoperiod in photosensitive, but not PR birds (Millam et al., 2003; Steinman et al., 2008). Slides were stained in batches that included at least two different reproductive states. Tissue was rehydrated in 0.1 M PBS then incubated in 200 μg/ml trypsin in PBS for 20 min and washed in Tris-buffered saline (TBS, 1 m Tris, 1.5 m NaCl, pH 7.5). Slides were placed in 0.3% hydrogen peroxide for 30 min and washed in PBS. Tissue was blocked for 3 h at room temperature in Tris-NaCl-blocking reagent