Journal of Chemical Neuroanatomy 41 (2011) 137–147

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Neural distribution of the nuclear progesterone receptor in the túngara frog, *Physalaemus pustulosus*☆

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ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 24 December 2010
Accepted 3 January 2011
Available online 20 January 2011

Keywords:
Progesterone receptor
Amphibian
Neuroanatomy
Cytoarchitecture
Mate choice
Tyrosine hydroxylase

ABSTRACT

The gonadal steroid hormone progesterone plays an important role across all vertebrates in mediating female reproductive physiology and behavior. Many effects of progesterone are mediated by a nuclear progesterone receptor (PR), which is crucial for integration of external signals and internal physiological cues in the brain to produce an appropriate behavioral output. The túngara frog, *Physalaemus pustulosus*, is an excellent model system for the study of mechanisms by which sensory signals, such as auditory communication, are processed within neural circuits where mate choice decisions are made. To establish a framework for studying the neural basis of mate choice and social behavior in this species, we first describe the cytoarchitecture of the brain using Nissl-stained sections. Then, in order to better understand where progesterone acts to regulate social decisions, we determined the distribution of PR protein throughout the brain of *P. pustulosus* by immunohistochemistry. We found PR immunoreactivity in key brain regions known to modulate the processing of auditory cues and social behavior in other vertebrates. Due to its widespread distribution, PR likely also plays important roles in non-limbic brain regions that mediate non-social information processing. Further, we have colocalized PR with tyrosine hydroxylase, providing a functional context for the role of progesterone in mediating motivation and motor behavior. Our results significantly extend our understanding of hormonal modulation in the anuran brain and support the important role of the nuclear progesterone receptor in modulating female mate choice and receptivity in amphibians and across vertebrates.

1. Introduction

Individuals integrate external cues through sensory systems, and these environmental signals can have both immediate and long-term effects on brain processes and behavior. One key channel for affecting such long-term changes is the modulation of gene expression (Morgan and Curran, 1989, 1991; Clayton, 2000; Hofmann, 2003, 2010; Aubin-Horth and Renn, 2009). Social decision-making requires an integration of external and internal cues in the brain where information is processed and behavioral decisions are implemented by dedicated brain circuits. Sex steroid hormones can alter neural circuit function and properties (Ball and Balthazart, 2004; Beach, 1948; Lehrman, 1965). Since (classical) steroid hormone receptors act as transcription regulators, these

☆ Grant Sponsor: NSF grant IOS 0843712, the Alfred P. Sloan Foundation, and a Dwight W. and Blanche Faye Reeder Centennial Fellowship in Systematic and Evolutionary Biology to HAH.

Abbreviations: A, anterior thalamic nucleus; AA, anterior amygdaloid area; Acc, nucleus accumbens; Ad, anterodorsal tegmental nucleus; AH, anterior hypothalamus; aob, accessory olfactory bulb; Av, anteroventral tegmental nucleus; BST, bed nucleus of the stria terminalis; C, central thalamic nucleus; Cb, cerebellum; CeA, central amygdala; DB, diagonal band of Broca; DH, dorsal hypothalamic nucleus; Dp, dorsal pallium; DP, dorsal pallidum; e, postolfactory eminence; Ep, posterior entopeduncular nucleus; Gc, griseum centrale rhombencephali; gl, glomerular layer of the olfactory bulb; gr, granule cell layer of the olfactory bulb; Hv, ventral habenula; La, lateral thalamic nucleus, anterior division; LA, lateral amygdale; LH, lateral hypothalamic nucleus; Lp, lateral pallium; Lpd, lateral thalamic nucleus, posterodorsale; Lpv, lateral thalamic nucleus, posteroventrale; Ls, lateral septum; M, dorsal midline; MeA, medial amygdale; Mgd, magnocellular preoptic nucleus, dorsal part; Mgv, magnocellular preoptic nucleus, ventral part; ml, mitral cell layer of the olfactory bulb; Mp, medial pallium; Ms, medial septum; ON, optic nerve; Npv, nucleus of the periventricular organ; P, posterior thalamic nucleus; Pd, nucleus posterodorsalis tegmenti; POa, anterior preoptic area; Pv, nucleus posteroventralis tegmenti; Rm, nucleus reticularis medius; Rs, nucleus reticularis superior; SC, suprachiasmatic nucleus; Str, Striatum; Tect, optic tectum; Tel, telencephalon; Tor-L, torus semicircularis, laminar nucleus; Tor-P, torus semicircularis, principal nucleus; Tor-V, torus semicircularis, ventral area; TP, posterior tuberculum; Vd, descending trigeminal tract; VH, ventral hypothalamic nucleus; VLd, ventrolateral thalamic nucleus, dorsal part; VLv, ventrolateral thalamic nucleus, ventral part; Vm, nucleus motorius nervi trigemini; VM, ventromedial thalamic nucleus; VP, ventral pallidum.

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doi:10.1016/j.jchemneu.2011.01.002

pathways are good candidates for integrating external signals into gene expression changes. An animal's hormonal state can mediate the integration of external cues and the way auditory signals are perceived. For example, in females of the plainfin midshipman fish, *Porichthys notatus*, hormonal state affects auditory sensitivity to male vocalizations (Sisneros et al., 2004). Gonadal steroid hormones can also have rapid non-genomic effects on behavior (Remage-Healey and Bass, 2006; Mani et al., 2009).

The effects of progesterone can be mediated by genomic and non-genomic mechanisms. Effects on gene transcription are transduced by the nuclear progesterone receptor (PR), and thus the characteristics of PR action arise from its specificity to its ligand and the DNA response element as well as its spatial and temporal pattern of expression. Importantly, besides a recently characterized conventional G protein-coupled progesterone receptor (Thomas, 2008; Mani et al., 2009), PR itself can also mediate non-genomic effects of progesterone, when it participates in a phosphorylation signal-transduction cascade (Zhu et al., 2008). In the context of behavior, the best characterized non-genomic interaction is between dopamine receptors and PR in facilitating female receptivity in rats (Mani et al., 2000; Frye, 2001). More generally, progesterone has been found to regulate diverse social behavior patterns in many vertebrates species, such as male and female sexual behavior, parental behavior, addiction, and aggression (Schneider et al., 2003; Crews, 2005; Wagner, 2006; Frye, 2007; Kabelik et al., 2008).

In amphibians, progesterone appears to influence female mate choice and receptivity, which is the best-studied social decision-making behavior in this vertebrate group. In female anurans, including túngara frogs, *Physalaemus pustulosus*, plasma progesterone levels are much higher during amplexus (Harvey et al., 1997; Itoh and Ishii, 1990), when females display the maximum frequency of reproductive behavior (Lynch and Wilczynski, 2005). Both estradiol and progesterone are required for receptive behavior in the clawed frog, *Xenopus laevis*, although receptivity did not increase with either hormone alone (Kelley, 1982). In the American toad, co-injection of progesterone and prostaglandin increases female receptivity to male mating calls as measured by the intensity and duration of phonotaxis, although treatment of prostaglandins alone will not elicit this behavior (Schmidt, 1985). Although recent work in female túngara frogs has shown that progesterone is not necessary for phonotaxis movement (Chakraborty and Burmeister, 2009), its role in receptivity or the mate choice process itself remains to be investigated. Since progesterone likely plays an important role in amphibian reproduction, it is surprising that the neural distribution of the progesterone receptor in this group is unknown. This information would give us a better understanding of which brain regions may be sites of modulation of social behavior by progesterone, especially in the light of recent insights into the neural circuitry underlying mate choice and female receptivity in the túngara frog (Hoke et al., 2004, 2005, 2007, 2008; Burmeister et al., 2008).

The túngara frog is an excellent model system to study the mechanisms by which sensory cues are transduced into molecular events within the neural circuits that govern behavioral decisions, such as mate choice. As in most anurans, túngara males produce species-specific advertisement calls that females use for species recognition and assessment of male quality (Ryan, 1985). Females will respond to both natural and synthetic calls in phonotaxis experiments, exhibiting a robust and repeatable approach towards broadcast calls, a behavior that is an unequivocal indication of mating call preference (e.g. Ryan and Rand, 1995; Phelps et al., 2006). Importantly, the anuran auditory system is biased towards detection and perception of conspecific mating calls (Wilczynski and Capranica, 1984), and details of these processes in túngara frogs have been revealed through studies of electrophysiology (Ryan et al., 1990; Wilczynski et al., 2001) and analysis of immediate early gene expression (Hoke et al., 2004, 2005, 2007, 2008; Burmeister et al., 2008).

Based on insights in mammals, birds and teleosts, there are two neural networks that seem to regulate social behavior and/or encode the salience of (social) stimuli. First, many studies indicate that the mesolimbic reward system (including the mid-brain dopaminergic system) is the neural network where evaluations of stimulus salience take place (Deco and Rolls, 2005; Wickens et al., 2007). Second, the neural substrates underlying social behavior, including female sexual behavior, have been proposed by Newman (1999) to form a "social behavior network", mostly based on work in mammals. The core nodes of this network are involved in multiple forms of social behavior, are reciprocally connected, and – by definition – contain sex steroid hormone receptors. This framework has since been expanded to reptiles, birds, and teleosts (Newman, 1999; Crews, 2003; Goodson, 2005; O'Connell and Hofmann, 2011), yet has not been specifically applied to amphibians, although the involvement of several hypothalamic nodes of Newman's network has been discussed by Hoke et al. (2005). While the brain regions involved in the dopaminergic reward system and Newman's social behavior network are well studied in mammals, and increasingly in other amniotes, determining the homologs of these brain areas in the amphibian brain has been a challenge, especially for forebrain regions in the basal nuclei (Bruce and Braford, 2009; Marín et al., 1998). However, a consensus is emerging from neurochemical, hodological, and developmental studies that provide support for putative homologies for most of the relevant areas in the amphibian brain (Endepols et al., 2000; Marín et al., 1998; Smeets et al., 2000; Bruce and Braford, 2009; O'Connell and Hofmann, in press). These two neural networks can be used as a useful framework for understanding the neural underpinnings of female mate-choice and social decision-making in amphibians and in other vertebrates.

The main aim of this study is to test the hypothesis that PR is expressed in fore- and midbrain regions important for the regulation of social behavior and evaluation of stimulus salience. Towards this aim, we determined the distribution of PR in the female túngara frog brain, as progesterone plays an important role in female receptivity and mate choice in many vertebrate species. We also describe the basic architecture of the túngara frog brain, as no cytoarchitectonic description exists despite the importance of this model system for the study of female mate choice and sexual selection (Ryan, 2010). Together, a better understanding of the basic morphology of the túngara frog brain and the distribution of PR will facilitate functional studies directly related to the neural basis of mate choice and auditory communication. Finally, we also colocalize PR with tyrosine hydroxylase, in order to lay a foundation for functional studies into the interaction of PR and dopaminergic systems in the anuran brain.

2. Materials and methods

2.1. Animals

The animals chosen for this study were females housed in a breeding colony. The frogs were descendants of animals collected in Panama and maintained in 19-l aquaria or larger landscaping ponds that were converted to terraria. Frogs were maintained at 25 °C on a diet of crickets and wingless fruit flies, a 12:12 light cycle, and misted several days a week to maintain moisture and humidity levels similar to their native habitat.

We adopted the neuroanatomical nomenclature of Marín et al. (1998) for basal nuclei, Northcutt and Kicliter (1980) for the telencephalon, Neary and Northcutt (1983) for the diencephalon, Wilczynski (1988) for the divisions of the torus (as originally described by Potter, 1965), and Gonzalez and Smeets (1994) for the hindbrain. All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.2. Cresyl violet staining for cytoarchitecture

Túngara females ($n = 5$) were sacrificed and the brain and skull were rapidly dissected and incubated in 4% formaldehyde in 1× phosphate-buffered saline (PBS;