available at www.sciencedirect.com

SCIENCE DIRECT®

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Short Communication

Sex differences in neuronal morphology in the killifish hypothalamus

Lisa E. Lauer[a], *Margaret M. McCarthy*[b], *Jessica Mong*[c], *Andrew S. Kane*[a,d,*]

[a]*Aquatic Pathobiology Center, Virginia-Maryland Regional College of Veterinary Medicine, College Park, MD 20742, USA*
[b]*Departments of Physiology and Psychiatry, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[c]*Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[d]*Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

ARTICLE INFO

Article history:
Accepted 2 November 2005
Available online 23 January 2006

Keywords:
Sexual dimorphism
Killifish
Hypothalamus
Teleost
Dendrite
Spine
Fundulus heteroclitus

ABSTRACT

This study examined the neuroarchitecture of the male and female killifish (*Fundulus heteroclitus*) hypothalamus to evaluate whether sexual dimorphism of this brain region exists in fishes as it does in mammals and other vertebrates. The rostral medulla, a brain region distinct from the hypothalamic–pituitary–gonadal axis, was also examined to determine if any observed differences were region-specific. With the use of Golgi–Cox impregnation, five dendritic characteristics were measured from neurons of both the hypothalamus and medulla including: spine density, number of branch points, dendrite length, surface area and volume. Dendritic spines are associated with excitatory synapses, and changes in density are associated with a variety of normal and pathological changes. Consistent with mammalian studies, we found that adult female killifish have 25% greater dendritic spine densities in the hypothalamus than male killifish (densities of 0.34 ± 0.06 μm^{-1} and 0.25 ± 0.08 μm^{-1}, respectively). By contrast, no statistically significant difference between males and females was detected in spine densities in the rostral medulla. This finding supports the conclusion that hypothalamic sexual dimorphism is conserved in killifish.

Sexual dimorphism of the CNS has been described in all vertebrate classes, with the mammalian brain the most extensively studied. During development, steroid hormones from the differentiated gonads organize the brain into a masculine or feminine form. This process of aromatase-induced conversion of testosterone to estradiol is well-documented in mammals, as are the resulting morphological sex differences. Sexual differentiation is manifested partly through morphological sex differences in the ultrastructure of organelles, dendritic organization and the volume of distinct cell groups within the brain (Arnold and Gorski, 1984; De Vries, 2004; MacLusky and Naftolin, 1981; Simerly, 2002). A variety of neuroanatomical, morphometric sex differences have been described and include differences in the size of entire brain regions, volume of distinct sub-nuclei or projections, sex differences in soma size, dendritic length, branch number, spine synapses and total dendrite surface area. Morphometric sex differences of the largest magnitude generally occur in the hypothalamic nuclei of mammals (Amateau and McCarthy, 2004; Ayoub et al., 1983; Cherry et al., 1992; Commins and Yahr, 1984; Dohler et al., 1982; Gorski et al., 1980; Handa et al., 1985; Hines et al., 1985; Matsumoto

* *Corresponding author*. Aquatic Pathobiology Center, 8075 Greenmead Drive, College Park, MD 20742, USA.
E-mail address: akane@umaryland.edu (A.S. Kane).

0006-8993/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2005.11.030

Fig. 1 – Sexual dimorphic characteristics of the adult male (A) and female (B) *F. heteroclitus*. Male killifish are typically olive green and pale yellow in color with vertical silver bars and iridescent spots scattered along the body and fins. In spawning condition, the yellow coloring on the ventral half of the male changes from a pale to a brilliant yellow. Female killifish are typically brownish green in color, and their dorsal region is darker than their ventral region. The female exhibits black vertical bars, and no markings are found on the fins. The black bars fade when the female is in spawning condition.

and Arai, 1980, 1981, 1983, 1986; Mong et al., 1999; Tobet et al., 1986) and birds (Balthazart and Adkins–Regan, 2002) or in the song control nuclei of birds (Schlinger and Brenowitz, 2002; Wade and Arnold, 2004). Dendritic spines are the primary site of excitatory synaptic input and are a readily quantifiable marker of synapses. The density and/or number of spines on dendrites of hypothalamic neurons are markedly dimorphic, being higher in the preoptic area and ventromedial nucleus of males compared to females (Amateau and McCarthy, 2004; Pozzo-Miller and Aoki, 1991) but lower in the arcuate nucleus of males compared to females (Matsumoto and Arai, 1981, 1986; Mong et al., 1999). Thus, there is considerable regional heterogeneity in the directionality of dendritic spine density sex differences within the mammalian hypothalamus, and, in each instance, the role of estradiol is critical. CNS sexual dimorphisms of avian species are found in portions of the forebrain, collectively known as the vocal control region, and involve those nuclei related to song control. These nuclei are significantly larger in the male than in the female, with an increase in number and size of neurons (Arnold and Gorski, 1984). Differences between the two genders in the volume of preoptic nuclei within the CNS of amphibians and reptiles have also been reported. For example, in toads (*Bufo japonicus*), the regions of the brain involved in mate calling, the anterior portion of the preoptic nucleus and the amygdala pars medialis, are significantly larger in males than in females (Takami and Urano, 1984). Relatively little is known about the process of gender variation in the brains of fishes or the resulting morphological differences. To date, Bass (1992, 1996) provides the only evidence of gender difference in fish neuronal architecture. Similar to findings in birds and amphibians, Bass observed that the neuronal structures involved with reproductive vocalizations differ between male and female plainfin midshipman (*Porichthys notatus*). This species has two male morphs with divergent vocalizing patterns, which also differ in their neuronal architecture. The long hums of the Type I morph are notably distinguishable from the short grunts of both the Type II morph and the female. The dendrites and soma of the sonic motoneurons that innervate the sonic muscles are one to three times larger in Type I males than they are in both Type II males and females (Bass, 1992, 1996).

As part of the hypothalamic–gonadal–pituitary axis, the hypothalamus contributes greatly to reproductive function and behavior in vertebrates. The hypothalamus has been found to be the most sexually dimorphic region of the brain in higher vertebrate classes, however, no publications to date have focused on gender differences in hypothalamic neuronal structure in fishes. Thus, the objective of this study was to

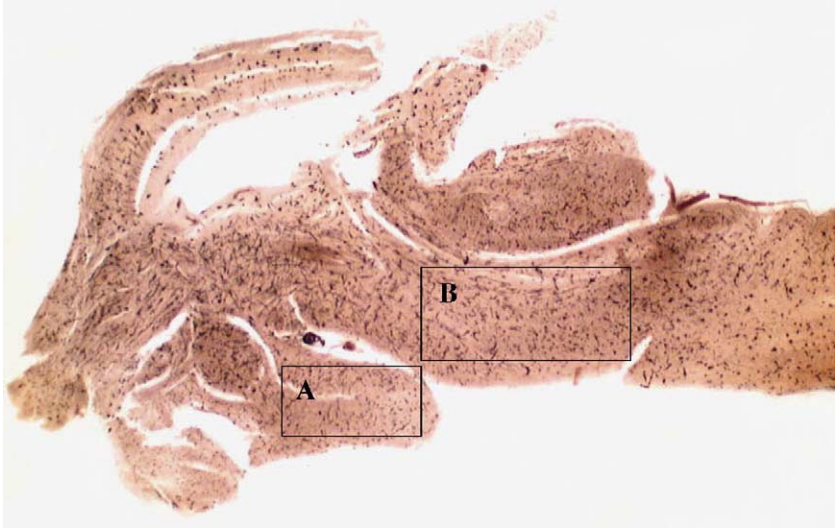

Fig. 2 – Sagittal section of a Golgi–Cox impregnated killifish brain. Anatomical regions analyzed for neuronal sexual dimorphisms included the hypothalamus (region labeled A) and the rostral medulla (region labeled B). Scale bar = 1 mm.

determine the existence of neuronal sexual dimorphisms in the hypothalamic region of the killifish (*Fundulus heteroclitus*, also known as mummichog). Dendrites from the rostral medulla were also analyzed to examine regional differences in neuronal architecture, apart from the HPG. The killifish is a good teleost model since it is sexually dimorphic in body size and coloration (Fig. 1), ubiquitous throughout the Chesapeake Bay and estuarine waters of North America and has notable ecological importance as a prey species.

Killifish were collected using a beach seine from a reference site located in a tidal portion of the Chesapeake Bay. Fish were transported to the Aquatic Pathobiology Center and were acclimated to laboratory conditions for at least 2 months prior to the experiment. Fish were maintained at 20–22 °C in 160-l aquaria at a salinity of 3‰ with a 16 h light:8 h dark cycle. Mature killifish (6–8 g) of both genders were randomly chosen from the acclimated population, anesthetized with buffered tricaine-methanesulfate (MS-222) and humanely sacrificed by decapitation prior to the removal of intact brains for study. These procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee and involved minimal stress to the animals.

Visualization of neurons was accomplished by the immersion of intact brains in Golgi–Cox solution for heavy metal impregnation (Glaser and Van der Loos, 1981). After 4 days of impregnation, brains were processed as previously described by Gibb and Kolb (1998) with some modifications. Briefly, the brains were transferred to a 30% sucrose solution for 3 days. Sagittal sections were cut to a thickness of 100 μm using a vibratome. These sections were placed into 20% sucrose in dH_2O and were subsequently mounted onto gelatin-coated slides. The slides were developed for 30 min with NH_4OH diluted 1:1 with dH_2O to precipitate the metals (Fig. 3).

Dendrites from two regions of the killifish brain, the hypothalamus and rostral medulla (Fig. 2), were analyzed for spine density, branching, length, volume and surface area. There was a significant difference ($P < 0.008$) in the hypothalamic spine density between the two gender groups, with 25% greater density observed in females than was observed in males (Table 1). No significant gender difference was seen in total dendritic length, number of dendrite branching points, total surface area or total volume.

In the rostral medulla, there was no significant difference between the gender groups regarding dendrite branching or volume. Interestingly, the spine density in males was 21% greater than the density observed in females (Table 1). In addition, the dendrite length in the female rostral medulla was 22% longer than what was observed in males ($P < 0.004$). Similarly, the surface area of dendrites in females was 25% greater than what was observed in males ($P < 0.01$).

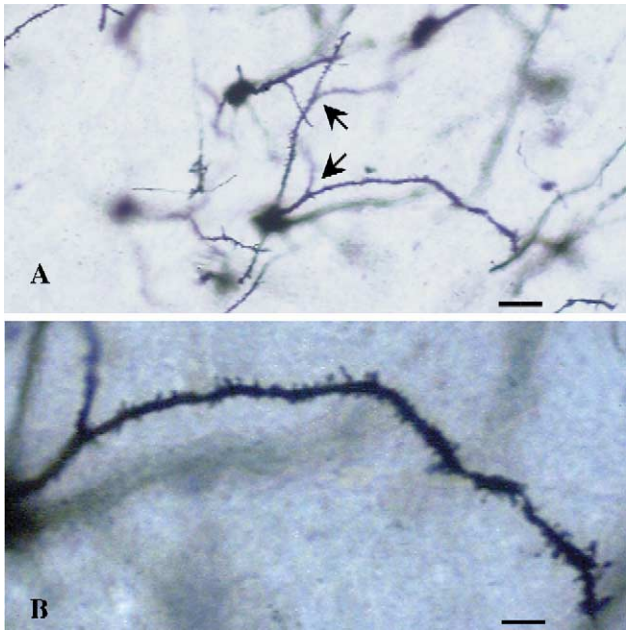

Fig. 3 – (A) Photomicrograph of hypothalamic dendrite impregnated with Golgi–Cox solution. Branches (arrows) were designated as any protrusion from the dendrite that was >5 μm. Branches demonstrated here are leaving the plane of focus. Scale bar = 40 μm. (B) Impregnated dendrite with spines, defined as any protrusions 5 μm or less. Scale bar = 10 μm.

Analyses of dendrite characteristics in the killifish brain demonstrated that sex differences in hypothalamic neuronal morphology exist in teleosts. It was determined that the sex difference found in the hypothalamus was region-specific when contrasted with results from the rostral medulla. Hypothalamic spine density was greater in the female brain than in the male, a gender difference that is consistent with the arcuate nucleus in mammals (Matsumoto and Arai, 1981, 1986; Mong et al., 1999, 2001). Morphometric sex differences have also been reported for the fish brain, but in many instances, interpretation is complicated by multiple reproductive morphs of one sex or the ability to change sexes over the lifespan (Goodson and Bass, 2000; Grober and Bass, 2002). Nonetheless, a variety of sex differences in fish brains have been reported,

Table 1 – Morphometry results from male and female killifish dendrite observations. Data are means ± SEs

	Spine density (μm^{-1})	Branching (#/dendrite)	Length (μm)	Volume (μm^3)	Surface area (μm^2)
Hypothalamus					
Male	0.25 ± 0.08	1.4 ± 0.6	201 ± 56	357 ± 133	887 ± 270
Female	0.37 ± 0.04	1.7 ± 0.8	227 ± 47	411 ± 103	1021 ± 211
Medulla					
Male	0.18 ± 0.04	0.7 ± 0.3	146 ± 9	270.5 ± 126	270 ± 52
Female	0.15 ± 0.03	0.8 ± 0.2	188 ± 8	366.9 ± 81	362 ± 34

including differences in the LHRH neurons of goldfish (Parhar et al., 2001) and in the baseline and hormonal regulation of the rate-limiting enzyme in GABA synthesis, glutamic acid decarboxylase (Bosma et al., 2001; Trudeau et al., 2000). Other differences between the male and female fish brain include the distribution of galanin neurons (Jadhao et al., 2001), preprotachykinin expression (Peyon et al., 2000) and distribution, amount, and activity of the gonadal steroid receptors and the estradiol synthetic enzyme, aromatase (Forlano et al., 2001; Joakim Larsson et al., 2002; Kim et al., 2002; Melo and Ramsdell, 2001). There have also been reports of sex differences in the position of the preoptic area and other brain regions of the red salmon (Jadhao and Meyer, 2000), but to the best of our knowledge, there have been no systematic investigations of morphometric sex differences at the cellular level in fish.

Sex differences in dendritic spine density in particular brain regions have been correlated with adult sex-typic behavior (Amateau and McCarthy, 2004) or the control of gonadotropin secretion from the anterior pituitary (Garcia-Segura et al., 1994). Hormonal modulation of dendritic spines in the hippocampus has been correlated with changes in learning (Wooley, 1999) and stress responses (Shors et al., 2001). While we have provided confirmation that sexual differences in dendritic spine density are conserved in vertebrates, the functional significance of this sexually dimorphic brain characteristic in killifish requires further study.

REFERENCES

Amateau, S.K., McCarthy, M.M., 2004. Induction of PGE(2) by estradiol mediates developmental masculinization of sex behavior. Nat. Neurosci. 7 (6), 643–650.

Arnold, A.P., Gorski, R.A., 1984. Gonadal steroid induction of structural sex differences in the central nervous system. Annu. Rev. Neurosci. 7, 413–442.

Ayoub, D.M., Greenough, W.T., Juraska, J.M., 1983. Sex differences in dendritic structure in the preoptic area of the juvenile macaque monkey brain. Science 219, 197–198.

Balthazart, J., Adkins-Regan, E., 2002. Sexual differentiation of brain and behavior in birds. In: Pfaff, D.W. (Ed.), Hormones Brain and Behavior, vol. IV. Elsevier, Amsterdam, pp. 223–301.

Bass, A., 1992. Dimorphic male brains and alternative reproductive tactics in a vocalizing fish. Trends Neurosci. 15, 139–145.

Bass, A.H., 1996. Shaping brain sexuality. Amer. Sci. 84, 352–363.

Bosma, P.T., Blazquez, M., Fraser, E.J., Schulz, R.W., Docherty, K., Trudeau, V.L., 2001. Sex steroid regulation of glutamate decarboxylase mRNA expression in goldfish brain is sexually dimorphic. J. Neurochem. 76, 945–956.

Cherry, J.A., Tobet, S.A., DeVoogd, T.J., Baum, M.J., 1992. Effects of sex and androgen treatment on dendritic dimensions of neurons in the sexually dimorphic preoptic/anterior hypothalamic area of male and female ferrets. J. Comp. Neurol. 323, 577–585.

Commins, D., Yahr, P., 1984. Adult testosterone levels influence the morphology of a sexually dimorphic area in the Mongolian gerbil brain. J. Comp. Neurol. 224, 132–140.

De Vries, G.J., 2004. Minireview: sex differences in adult and developing brains: compensation, compensation, compensation. Endocrinology 145, 1063–1068.

Dohler, K.D., Coquelin, A., Davis, F., Hines, M., Shryne, J.E., Gorski, R.A., 1982. Differentiation of the sexually dimorphic nucleus in the preoptic area of the rat brain is determined by the perinatal hormone environment. Neurosci. Lett. 33, 295–298.

Forlano, P.M., Deitcher, D.L., Myers, D.A., Bass, A.H., 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J. Neurosci. 21, 8943–8955.

Garcia-Segura, L.M., Chowen, J.A., Duenas, M., Torres-Aleman, I., Naftolin, F., 1994. Gonadal steroids as promoters of neuro-glial plasticity. Psychoneuroendocrinology 19, 445–453.

Gibb, R., Kolb, B., 1998. A method for vibratome sectioning of Golgi–Cox stained whole rat brain. J. Neurosci. Methods 79, 1–4.

Glaser, E.M., Van der Loos, L.H., 1981. Analysis of thick brain sections by obverse-reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. J. Neurosci. Methods 4, 117–125.

Goodson, J.L., Bass, A.H., 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403, 769–772.

Gorski, R.A., Harlan, R.E., Jacobson, C.D., Shryne, J.E., Southam, A.M., 1980. Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. J. Comp. Neurol. 193, 529–539.

Grober, M.S., Bass, A.H., 2002. Life history, neuroendocrinology and behavior in fish. In: Pfaff, D.W. (Ed.), Hormones Brain and Behavior, vol. II. Elsevier, Amsterdam, pp. 331–347.

Handa, R.J., Corbier, P., Shryne, J.E., Schoonmaker, J.N., Gorski, R.A., 1985. Differential effects of the perinatal steroid environment on three sexually dimorphic parameters of the rat brain. Biol. Reprod. 32, 855–864.

Hines, M., Davis, F., Coquelin, A., Goy, R.W., Gorski, R.A., 1985. Sexually dimorphic regions in the medial preoptic area and the bed nucleus of the stria terminalis of the guinea pig brain: a description and an investigation of their relationship to gonadal steroids in adulthood. J. Neurosci. 5, 40–47.

Jadhao, A.G., Meyer, D.L., 2000. Sexually dimorphic distribution of galanin in the preoptic area of red salmon, *Oncorhynchus nerka*. Cell Tissue Res. 302, 199–203.

Jadhao, A.G., D'Aniello, B., Malz, C.R., Pinelli, C., Meyer, D.L., 2001. Intrasexual and intersexual dimorphisms of the red salmon prosencephalon. Cell Tissue Res. 304, 121–140.

Joakim Larsson, D.G., Sperry, T.S., Thomas, P., 2002. Regulation of androgen receptors in Atlantic croaker brains by testosterone and estradiol. Gen. Comp. Endocrinol. 128, 224–230.

Kim, S.J., Ogasawara, K., Park, J.G., Takemura, A., Nakamura, M., 2002. Sequence and expression of androgen receptor and estrogen receptor gene in the sex types of protogynous wrasse, *Halichoeres trimaculatus*. Gen. Comp. Endocrinol. 127, 165–173.

MacLusky, N.J., Naftolin, F., 1981. Sexual differentiation of the central nervous system. Science 211, 1294–1302.

Matsumoto, A., Arai, Y., 1980. Sexual dimorphism in 'wiring pattern' in the hypothalamic arcuate nucleus and its modification by neonatal hormonal environment. Brain Res. 190, 238–242.

Matsumoto, A., Arai, Y., 1981. Effect of androgen on sexual differentiation of synaptic organization in the hypothalamic arcuate nucleus: on ontogenetic study. Neuroendocrinology 33, 166–169.

Matsumoto, A., Arai, Y., 1983. Sex difference in volume of the ventromedial nucleus of the hypothalamus in the rat. Endocrinol. Jpn. 30, 277–280.

Matsumoto, A., Arai, Y., 1986. Development of sexual dimorphism in synaptic organization in the ventromedial nucleus of the hypothalamus in rats. Neurosci. Lett. 68, 165–168.

Melo, A.C., Ramsdell, J.S., 2001. Sexual dimorphism of brain aromatase activity in medaka: induction of a female phenotype by estradiol. Environ. Health Perspect. 109, 257–264.

Mong, J.A., Glaser, E., McCarthy, M.M., 1999. Gonadal steroids promote glial differentiation and alter neuronal morphology in

the developing hypothalamus in a regionally specific manner. J. Neurosci. 19, 1464–1472.
Mong, J.A., Roberts, R.C., Kelly, J.J., McCarthy, M.M., 2001. Gonadal steroids reduce the density of axospinous synapses in the developing rat arcuate nucleus: an electron microscopy analysis. J. Comp. Neurol. 432, 259–267.
Parhar, I.S., Tosaki, H., Sakuma, Y., Kobayashi, M., 2001. Sex differences in the brain of goldfish: gonadotropin-releasing hormone and vasotocinergic neurons. Neuroscience 104, 1099–1110.
Peyon, P., Saied, H., Lin, X., Peter, R.E., 2000. Preprotachykinin gene expression in goldfish brain: sexual, seasonal, and postprandial variations. Peptides 21, 225–231.
Pozzo-Miller, L.D., Aoki, A., 1991. Stereological analysis of the hypothalamic ventromedial nucleus: II. Hormone induced changes in the synaptogenic pattern. Dev. Brain Res. 61, 189–196.
Schlinger, B.A., Brenowitz, E.A., 2002. Neural and hormonal control of birdsong. In: Pfaff, D.W. (Ed.), Hormones Brain and Behavior, vol. II. Elsevier, Amsterdam, pp. 799–839.
Shors, T.J., Chua, C., Falduto, J., 2001. Sex differences and opposite effects of stress on dendritic spine density in the male versus the female hippocampus. J. Neurosci. 21, 6292–6297.
Simerly, R.B., 2002. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu. Rev. Neurosci. 25, 507–536.
Takami, S., Urano, A., 1984. The volume of the toad medial amygdala–anterior preoptic complex is sexually dimorphic and seasonally variable. Neurosci. Lett. 44, 253–258.
Tobet, S.A., Zahniser, D.J., Baum, M.J., 1986. Sexual dimorphisms in the preoptic/anterior hypothalamic area of ferrets: effects of adult exposure to sex steroids. Brain Res. 364, 249–257.
Trudeau, V.L., Bosma, P.T., Collins, M., Priede, I.G., Docherty, K., 2000. Sexually dimorphic expression of glutamate decarboxylase mRNA in the hypothalamus of the deep sea armed grenadier, Coryphaenoides (Nematonurus) armatus. Brain Behav. Evol. 56, 269–275.
Wade, J., Arnold, A.P., 2004. Sexual differentiation of the zebra finch song system. Ann. N. Y. Acad. Sci. 1016, 540–559.
Wooley, C.S., 1999. Effects of estrogen in the CNS. Cur. Opin. Neurobiol. 9, 349–354.