Large-scale identification of proteins involved in the development of a sexually dimorphic behavior

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Abstract

Sexually dimorphic behaviors develop under the influence of sex steroids, which induce reversible changes in the underlying neural network of the brain. However, little is known about the proteins that mediate these activational effects of sex steroids. Here, we used a proteomics approach for large-scale identification of proteins involved in the development of a sexually dimorphic behavior — the electric organ discharge of brown ghost knifefish, Apteronotus leptorhynchus. In this weakly electric fish, the discharge frequency is controlled by the medullary pacemaker nucleus, and is higher in males than in females. After lowering the discharge frequency by chronic administration of β-estradiol, two-dimensional difference gel electrophoresis revealed 62 proteins spots in tissue samples from the pacemaker nucleus that exhibited significant changes in abundance of more than 1.5-fold. The 20 identified protein spots indicated, among others, a potential involvement of astrocytes in the establishment of the behavioral dimorphism. Indeed, immunohistochemical analysis demonstrated higher expression of the astrocytic marker protein GFAP and increased gap-junction coupling between astrocytes in females, compared to males. We hypothesize that changes in the size of the glial syncytium, glial coupling and/or number of glia-specific potassium channels lead to alterations in the firing frequency of the pacemaker nucleus via a mechanism mediating uptake of extracellular potassium ions from the extracellular space.

Keywords: sexual dimorphism; weakly electric fish; proteomics; neural oscillator; astrocytes
Sex differences in behavior are widespread among animals and occur most commonly during social interactions, particularly in the context of courtship and parental care. It is well established that sex steroids play an overarching role in the control of this sexual dimorphism of behavior by influencing the organization of brain structures during development, and by inducing reversible changes in neural circuits of the adult brain (Baum 2003; Morris et al. 2004). However, whereas sex differences in brain structures that control specific sexually dimorphic behaviors have been well characterized (Kelley 1988; Cooke et al. 1998), little is known about the multitude of genes and proteins that regulate these behaviors and mediate the activational effects of sex steroids (Xu et al. 2012). To address this issue, we employed an unbiased, large-scale approach to identify such proteins, using the electric organ discharge (EOD) of the weakly electric brown ghost knifefish (*Apteronotus leptorhynchus*) as a behavioral model that exhibits a reversible sexual dimorphism.

In this species, the EOD is produced continuously by synchronous depolarization of modified axonal terminals of spinal electromotor neurons that constitute the electric organ (de Oliveira-Castro 1955; Bennett 1971; Waxman et al. 1972). The frequency of the electric discharge is determined by the firing frequency of pacemaker and relay neurons in the pacemaker nucleus (Pn) in the medulla oblongata (Dye and Meyer 1986). Within the species-specific range of 650-1000 Hz in adult fish, males discharge at higher frequencies than females (Meyer et al. 1987). The EOD frequency, as well as the firing frequency of the Pn, is sensitive to the action of steroids in adult fish of both sexes, with estrogens inducing decreases in frequency (Meyer et al. 1987; Schaefer and Zakon 1996).
Taking advantage of this steroid-controlled behavioral and neural plasticity, we examined global changes in protein abundance in the Pn after lowering the EOD frequency through implantation of crystalline $\beta$-estradiol. Using two-dimensional difference gel electrophoresis (2-D DIGE), 62 proteins spots that exhibited significant changes in abundance of more than 1.5-fold were detected. The proteins associated with 20 of these spots were identified via peptide mass fingerprinting (PMF) and/or tandem mass spectrometry (MS/MS). The abundance of one of these proteins, glial fibrillary acidic protein (GFAP), increased almost twofold after $\beta$-estradiol implantation. Further analysis through immunohistochemistry and confocal laser scanning microscopy provided evidence that the spatial association of GFAP-expressing astrocytes with pacemaker and relay cells is significantly higher in females, compared to males. We hypothesize that such changes in the size and the properties of this glial syncytium lead to alterations in the firing frequency of the pacemaker nucleus via a mechanism involving potassium buffering.

**MATERIALS AND METHODS**

*Experimental animals.* Brown ghost knifefish (*Apteronotus leptorhynchus*; Gymnotiformes, Teleostei) were supplied by tropical fish importers and maintained in the laboratory as described previously (Gama Salgado and Zupanc 2011). A total of 139 fish (74 males, 64 females, and 1 fish that could not be sexed) were used. Their total length ranged from 80-189 mm, and their weight from 0.9-14.7 g. The gonadosomatic index ranged from 0.0006-0.0042 in males, and from 0.0021-0.0599 in females. Animal procedures were carried out in accordance with the relevant law, the *Deutsches Tierschutzgesetz*, of 1998.
EOD recordings. Differential recording of the fish’s EOD and determination of the frequency of the signal were performed as described previously (Gama Salgado and Zupanc 2011). The EOD frequency was adjusted to an ambient temperature of 26°C using a Q10 of 1.56 (Zupanc et al. 2003).

Treatment with β-estradiol. Under general anesthesia with 2% ethyl carbamate (urethane; Sigma) dissolved in aquarium water, fish were implanted with fine bore nylon tubing (flexible grade; length = 6 mm; outer diameter = 0.94 mm; inner diameter = 0.75 mm; Smith Medical International), packed with β-estradiol powder (Sigma-Aldrich). Implants were placed in the peritoneal cavity, and the wound was closed with Histoacryl Blue Topical Skin Adhesive (B. Braun Melsungen). Control fish received empty implants.

Isolation of Pn tissue. Fish were killed by immersion into an overdose of a 1.5 % solution of ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma-Aldrich) dissolved in aquarium water, and the heads were cooled with ice. The Pn was removed and immediately frozen in isopentane at -45°C, pooled with pacemaker nuclei of other fish, and stored at -80°C until further use.

2-D DIGE. Protein extraction, protein labeling with CyDye DIGE Fluors, and separation of protein extract by 2-D DIGE were performed using the Ettan DIGE system (Amersham Biosciences/GE Healthcare), as described previously (Ilieș et al. 2012). Briefly, pooled tissue samples from β-estradiol-treated and control fish, respectively, were homogenized through sonication in lysis buffer (Amersham Biosciences). After centrifugation, supernatant containing 50 μg protein from each sample were labeled with 400 pmol of Cy3 and Cy5, respectively. For
the internal standard, 25 μg protein from each sample were combined and then labeled with 400 pmol Cy2. Finally, the two samples and the standard were pooled and used for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Experiments were run in triplicate, separately for cytosolic and membrane fractions.

**Analysis of protein spots.** The labeled gels were digitized using an Ettan DIGE Imager (GE Healthcare) at a resolution of 100 μm per pixel. In-gel multi-dye co-detection of protein spots and quantification of protein abundance, as well as between-gels matching of spots and calculation of average fold changes were performed using DeCyder 2D software (Amersham Biosciences), separately for the cytosolic and membrane fractions. The matching of all protein spots exhibiting changes in abundance of more than 1.5 fold was verified manually. Statistical analysis was limited to protein spots found in all three replicates of either fraction.

**Protein identification.** Protein spots showing significant increases or decreases of more than 1.5 fold in either the cytosolic or the membrane fraction were selected for identification. Preparative gels were run as described above, using 500 μg of total protein. After Coomassie staining, spot maps were matched against reference spot maps determined from the analytical gels. Matched spots of interest were excised, de-stained, and subjected to in-gel digestion with modified trypsin (Roche Diagnostics) overnight at 37°C. The extracted peptides were desalted using a C18 Zip-Tip (Millipore), and identified by peptide mass fingerprinting (PMF) using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems), as described previously (Ilieş et al. 2012).

**Immunohistochemistry.** Fish were deeply anesthetized in a 2% solution of MS-222 in
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130 aquarium water and intracardially perfused with 2% freshly depolymerized paraformaldehyde
131 (Fisher) in 0.1 M phosphate buffer, pH 7.4. The brain was cryosectioned coronally at a thickness
132 of 16 μm. GFAP, Hu C/D, and connexin-43 antigenic sites were labeled using rabbit anti-GFAP
133 (Sigma-Aldrich) or chicken anti-GFAP (Abcam), mouse anti-Hu C/D (clone 16A11; Invitrogen),
134 and rabbit anti-connexin-43 (Cell Signaling) primary antibodies, followed by goat anti-rabbit
135 IgG conjugated to Alexa 488 or Alexa 546, goat anti-chicken IgG conjugated to Alexa 488, and
136 goat anti-mouse conjugated to Alexa 635 secondary antibodies (all from Invitrogen). The
137 sections were counterstained with DAPI.
138
139 Microscopy and image analysis. Confocal microscopy was performed using Zeiss LSM
140 700 and Zeiss LSM 710 laser scanning microscopes equipped with 25x and 63x objectives.
141 Optical sections were taken at a resolution of 0.2-0.5 μm per pixel, using Zen (Carl Zeiss)
142 software. Images were reconstructed in ImageJ (National Institutes of Health) using the Stitching
143 plugin (Preibisch et al. 2009). Background subtraction through tophat filtering, definition of
144 regions of interest based on Hu C/D labeling, and subsequent quantification of GFAP, Hu C/D,
145 and connexin-43 immunolabeling were performed in Matlab (Mathworks) using built-in
146 functions from the Image Analysis Toolbox.
147
148 RESULTS
149
150 Sexual dimorphism in EOD frequency. To confirm that brown ghost knifefish males and
151 females occupy different frequency domains, the EOD of 83 individuals was recorded, and they
152 were sexed through gonadal inspection. Analysis of the EOD frequencies adjusted to an ambient
temperature of 26°C demonstrated a distinct sexual dimorphism in the frequency of the electric
discharges (Fig. 1A). Whereas the mean EOD frequency of males was 879 Hz (median: 892 Hz;  
range: 704-987 Hz; n = 35 fish), the mean frequency of females was 737 Hz (median: 737 Hz;  
range: 671-809 Hz; n = 48 fish). This difference was highly significant (p < 0.0001; independent-
samples t-test).

**Effect of β-estradiol on EOD frequency.** Next, we experimentally manipulated the EOD
frequency of both males and females by intraperitoneal implantation of tubings filled with β-
estradiol, and we compared the changes in EOD frequency of these fish with the changes in  
frequency of control fish that had received empty implants. Administration of β-estradiol  
resulted in a gradual decrease of the EOD frequency in each of the fish examined (Fig. 1B).  
Eight days after the implantation, the frequency of β-estradiol-treated fish was on average 94 ±  
23 Hz lower than the pre-implantation baseline (p < 0.001; Z-test, Bonferroni correction for  
multiple comparisons; n = 14 fish). In all treated fish, both male and female, the observed  
frequency decrease was proportional to the baseline EOD frequency, with higher-frequency  
EODs showing larger reductions (Pearson’s ρ = 0.69, p < 0.01). By contrast, the EOD frequency  
of control fish did not exhibit any marked decrease beyond the initial effect of the implantation  
(Fig. 1B). Similar changes in the EOD frequency of control fish were reported previously (e.g.,  
Meyer et al. 1987), and are likely due to the traumatic effects of the implant surgery. Eight days  
after implantation, the frequency of control fish was on average 23 ± 16 Hz lower than the  
baseline (n = 17 fish), a significantly smaller effect than that observed in β-estradiol-treated fish  
(p < 0.001, independent-samples t-test, Bonferroni correction for multiple comparisons).
Protein expression profiles in the Pn: differences between β-estradiol-treated fish and controls. Eight days after the implantation of β-estradiol-filled or empty tubings, tissue was collected from whole pacemaker nuclei and processed for 2-D DIGE. An average number of 3248 ± 199 and 3006 ± 160 protein spots were detected in the gels run on the cytosolic and membrane fractions, respectively. A total of 1145 and 819 spots were matched across the three 2-D gels of the cytosolic and membrane fractions, respectively. In the cytosolic fraction, out of the 1145 protein spots, the standardized protein abundances of 14 spots (1.2% of total) were significantly increased by a factor of at least 1.5 in the Pn of β-estradiol-treated fish relative to the Pn of control fish, whereas the abundances of 10 spots (0.9%) were significantly reduced by a factor of at least 1.5 (p < 0.05, independent-samples t-test). In the membrane fraction, out of the 819 protein spots, the standardized protein abundances of 38 spots (4.6%) were significantly reduced by a factor of at least 1.5 (p < 0.05) in the Pn of β-estradiol-treated fish relative to the Pn of control fish, while none of the protein spots showing significantly increased abundances reached the 1.5-fold threshold (Fig. 2A). The protein spots displaying these differences were located throughout the 2-D gels, thus covering both small and large molecular weight domains, as well as the entire pI range analyzed.

Identification of differentially expressed proteins. Out of the 62 protein spots that exhibited significant changes in abundance of more than 1.5-fold in either the cytosolic or the membrane fraction, the proteins associated with 20 spots could be identified via PMF and/or MS/MS (Table 1; Fig. 2B, C). The following proteins showed an increase in abundance: fatty acid binding protein 11a (1 spot); GFAP (1 spot); NADH dehydrogenase (ubiquinone) Fe-S
protein 1 (1 spot); pyruvate kinase isozymes M1/M2 (1 spot). The following identified proteins showed a decrease in abundance: actin-related protein 2-A (1 spot); bactin1 protein (= beta-actin-1) (2 spots); fascin (1 spot); glutamine synthetase (1 spot); internexin neuronal intermediate filament protein, alpha (1 spot); internexin neuronal intermediate filament protein, alpha b (= gefiltin) (1 spot); IFT81 (= intraflagellar transport protein 81 homolog) (1 spot); isovaleryl-CoA dehydrogenase, mitochondrial (1 spot); 3-oxoacid CoA transferase 1a (1 spot); plastin 2 (1 spot); protein phosphatase 2 (formerly 2A) regulatory subunit A (PR 65) alpha isoform (1 spot); stathmin 1b (1 spot); succinate-CoA ligase, ADP-forming, beta subunit (1 spot); vesicle-fusing ATPase (2 spots).

Gross morphology of the Pn: absence of sex differences. Among the proteins that exhibited significant alterations in abundance after β-estradiol implantation, several are known to be involved in structural plasticity. Therefore, we examined whether any sexual dimorphism exists in the gross morphology of the Pn and its two major types of neurons, pacemaker and relay cells. Alternate 30-μm transverse brain sections from 3 males and 3 females were immunostained against the neuronal marker Hu C/D and analyzed (n = 14-18 sections per fish). No significant differences could be detected between males and females in the volume of the Pn, the numbers of relay and pacemaker neurons, and the average profile areas of these two neuronal cell types (Table 2; p > 0.50, independent-samples t-test).

Differences in GFAP expression between males and females. As shown by 2-D DIGE, the expression of GFAP in the Pn increased almost twofold after β-estradiol implantation. Assuming that similar changes occur during normal sexual maturation and persist as a sexually
dimorphic trait in adults, we hypothesized that the expression of GFAP in the Pn is higher in females than in males. In agreement with this hypothesis, confocal microscopy revealed numerous intensely labeled fibers in females (Fig. 3A), but fewer fibers and a lower labeling intensity in males (Fig. 3B). The total GFAP labeling (defined as the relative area immunostained, multiplied by the average labeling intensity after background correction) in the whole Pn was almost twice as high in females, compared to males ($p < 0.05$, independent-samples $t$-test; $n = 3$ males and 3 females; Fig. 3C left).

Next, we examined possible differences in the association between GFAP-labeled fibers and neurons in the Pn, identified by immunostaining against Hu C/D. The total GFAP labeling associated with the area covered by pacemaker and relay cells was significantly higher in females than in males ($p < 0.001$; Fig. 3C right). Similarly, the total GFAP labeling associated with the area covered by small interneurons (Turner and Moroz 1995) was higher in females than in males, but this difference was not significant ($p > 0.10$; Fig 3C middle).

**Differences between males and females in connexin-43 expression associated with GFAP-labeled astrocytes.** To further characterize the glial meshwork in which the pacemaker and relay cells are embedded, sections through the Pn were immunostained against connexin-43, a member of the connexin family of transmembrane gap junction proteins. Glia, and in particular astrocytes, have been found to express high levels of connexins, which play a critical role in the formation of a glial syncytium (Giaume and Liu 2012). Indeed, an abundance of punctate, plaque-like connexin-43 immunofluorescence was found in close proximity to GFAP-labeled fibers (Figs. 4A-A”). The relative area covered by connexin-43 immunoreactivity in a 20-µm-
thick annular region around the Hu C/D-immunolabeled pacemaker cells was almost 3-fold larger in females than in males ($p < 0.01$, independent-samples t-test; $n = 4$ males and 4 females; Figs. 4B, B’, C). The connexin-43-labeled area around relay cells was also increased in females, relative to males, but this difference was not significant ($p > 0.10$).

DISCUSSION

Proteomics approach to study the development of a sexually dimorphic behavior. A better understanding of how sexually dimorphic behaviors develop demands an integrative strategy, combining behavioral studies with investigations at the cellular and molecular levels (Zupanc 2010). Whereas significant progress has been made over the past few decades at the behavioral, endocrinological and, partly, cellular levels (Cooke et. al. 1998), the molecular basis of sexually dimorphic behaviors remains largely enigmatic. Here, we have — to our knowledge for the first time — employed proteomics to carry out an unbiased large-scale identification of proteins potentially involved in the development of the neural correlates underlying sexual dimorphism in a specific behavioral pattern. The present study serves as proof-of-principle that proteomic analysis represents a powerful method for future investigations that aim to explore the neural basis of behavioral plasticity.

Potential candidates involved in the development of sex differences in EOD frequency, as revealed through proteomic analysis. Proteomic analysis, combined with PMF and/or MS/MS, enabled us to identify the proteins associated with 20 spots whose abundance was significantly altered by a factor of at least 1.5 after implantation of β-estradiol. Several of these proteins,
including protein phosphatase 2, plastin-2, stathmin 1b, internexin alpha and alpha b, fascin, and beta-actin-1, indicate potential changes in the morphology of cells. If such structural changes indeed occur, they should be accompanied by an increase in energy demand and higher metabolic activity after β-estradiol administration — proposed changes that appear to be reflected by the elevated levels of NADH dehydrogenase FeS protein 1, pyruvate kinase isoenzymes M1/M2, and fatty acid binding protein 11a. We examined whether the structural changes indicated by proteomics analysis result in sexual dimorphism in the size of the Pn, or in the number and gross morphology of the pacemaker and relay cells, but we failed to detect any significant differences between males and females. We, therefore, hypothesize that, if structural changes indeed occur, they involve either a reorganization of neurons at the axonal and dendritic levels, or structural changes of non-neuronal cells. The latter hypothesis is consistent with the observed increase in GFAP abundance after β-estradiol treatment, possibly reflecting the generation of new glial cells, or the outgrowth of the existing glia. The proposed functional significance of such a remodeling of glia for the firing pattern of the neuronal network of the Pn is discussed in the next section.

The decrease in the abundance of glutamine synthetase after β-estradiol administration, as also revealed through proteomic analysis, could indicate a decrease in synaptically released glutamate — an interpretation that is consistent with the observed reduced abundance of vesicle-fusing ATPase. These changes are likely related to a second sexual dimorphism in the EOD of *A. leptorhynchus*. When subjected to sensory stimulation with the EOD of a neighboring fish, whose discharge frequency is similar to their own, males respond with the production of
transient amplitude and frequency modulations, called chirps. By contrast, females do not exhibit such a behavior (Zupanc and Maler 1993; Dulka and Maler 1994; Dunlap et al. 1998). Chirps are controlled by a sub-population of neurons in the central posterior/prepacemaker nucleus, the CP/PPn-C (Heiligenberg et al. 1981; Kawasaki and Heiligenberg 1988; Kawasaki et al. 1988; Zupanc and Maler 1992; Zupanc and Maler 1997; Metzner 1999; Zupanc 2002). The cells comprising this neuronal cluster project to relay cells in the pacemaker nucleus where they make glutamatergic synaptic contact involving non-NMDA glutamate receptors (Dye et al. 1989). This synaptic input from the CP/PPn-C results in rapid depolarization of the relay cells, which in turn leads to an acceleration of the firing frequency of both the pacemaker cells and the relay cells (Dye 1988), likely due to the extensive gap-junction coupling between these neurons (Bennett et al. 1967; Tokunaga et al. 1980; Elekes and Szabo 1985; Moortgat et al. 2000). Although never examined, it is plausible that the glutamatergic input from the CP/PPn-C to the relay cells of the Pn is much weaker in females than in males. If this is indeed the case, such a difference would explain the decrease revealed by proteomic analysis in abundance of glutamine synthetase and vesicle-fusing ATPase after β-estradiol administration.

Proposed role of GFAP-expressing astrocytes in the development of the sexual dimorphism in EOD frequency. By combining chronic administration of β-estradiol with proteomic analysis, we showed that 8 days after the start of the treatment the abundance of GFAP was significantly increased in the Pn. Complementary immunohistochemical experiments demonstrated a corresponding sexual dimorphism in GFAP expression in the Pn. The surface area of pacemaker and relay cells covered by GFAP-immunopositive astrocytes was markedly
higher in females than in males. Notably, this effect did not extend to the third neuronal type in the Pn, the small interneurons.

Confocal images showed that both pacemaker and relay cells are embedded in a dense meshwork of GFAP-expressing astrocytes, which form a gap-junction-coupled syncytium, as suggested by abundant expression of connexin-43 along GFAP-labeled processes. Notably, this coupling, particularly between those astrocytes that are associated with pacemaker cells, is markedly more pronounced in females than in males, as suggested by the quantitative analysis of connexin-43 labeling. The immunohistochemical demonstration of a dense astrocytic meshwork in the Pn is in agreement with prior ultrastructural studies that noted an intimate association of neurons and astrocytes in the Pn, particularly in the region of axon initial segments (Tokunaga et al. 1980; Elekes and Szabo 1985).

Physiological and immunohistochemical studies have demonstrated that three types of ions play important roles in the regulation of the electrical activity of the Pn, including modulation of the firing frequencies — Na\(^+\), K\(^+\), and Ca\(^{2+}\) (Dye 1991; Smith and Zakon 2000). Glial processes surrounding the pacemaker and relay cells express voltage-gated potassium channels (Smith et al. 2006). Based on these physiological and morphological observations, we propose that the astrocytic syncytium plays a role in the regulation of the firing frequency of the Pn, and that this control is mediated via uptake of K\(^+\) ions from the extracellular space through transporters or K\(^+\)-sensitive channels.

Potassium buffering by glia is a well-documented phenomenon (Kofaji and Newman 2004), but its possible involvement in the regulation of the firing frequency in neuronal
oscillatory networks has, to the best of our knowledge, never been examined. However, computer simulations of neuron-glia interactions mediated by ion flux have indicated that the firing pattern of tonically stimulated neurons can be altered by modifying the properties of adjacent astrocytes, including the density of specific types of membrane channels (Somjen et al. 2008). In the Pn, such a glia-mediated mechanism could accommodate the decrease in the oscillation frequency observed after chronic administration of β-estradiol, possibly by increasing the efficiency of removal of extracellular K⁺ ions through changes in one or several properties of the glial syncytium: (i) increase in the size of the glial syncytium; (ii) enhancement of the glial coupling through gap junctions; (iii) increase in the number of glia-specific potassium channels and/or Na⁺/K⁺ ATPases on the glial membrane.

The present study provides evidence for alterations in the first two properties, as a sexual dimorphism has been found in both the size of the glial syncytium associated with the pacemaker and relay cells, and the expression of the gap junction protein connexin-43 in glial cells. Assembly of these molecules into gap junctions, and the maintenance of their stability, are regulated by phosphorylation (Márques-Rosado et al. 2012). Phosphorylation might also play a prominent part in the modulation of the properties of membrane ion channels expressed in the astrocytic syncytium of the Pn, including potassium channels and/or Na⁺/K⁺ ATPases. Abundant evidence in other systems has demonstrated that a variety of ion channels, and perhaps all, are substrates for protein kinases and phosphoprotein phosphatases, and that this phosphorylation has profound effects on channel activity (Levitan 1994; Chen and Roche 2007). In the Pn, calcium-dependent phosphorylation plays a critical role in the regulation of N-methyl-D-
aspartate (NMDA) receptor-dependent plasticity (George et al. 2011). NMDA receptors expressed on relay cells are activated by glutamatergic input from the sublemniscal prepacemaker nucleus in the mesencephalon, mediating transient upward shifts by a few hertz of the pacemaker frequency.

The complexity of the discussed phenomenon clearly indicates that future investigations in this area will require a multidisciplinary approach, combining molecular, cellular, physiological, behavioral, and computational modeling experiments. By identifying the essential players in this process, and by dissecting the underlying mechanisms, the study of the Pn will provide an excellent opportunity to achieve a better understanding of the possible role of glia in the regulation of the activity of oscillatory neural networks, with implications that could reach significantly beyond the attempt to gain a better understanding of the structure and function of the Pn.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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FIGURE LEGENDS

Fig. 1. A: Box-and-whisker plot of EOD frequencies of male ($n = 35$) and female ($n = 48$) brown ghost knifefish. The discharge frequency was adjusted to an ambient temperature of 26°C. Whiskers mark the minimum and maximum values in each group, and boxes indicate the interquartile ranges. Middle lines denote medians. The difference in the EOD frequency between males and females is highly significant ($***; p < 0.0001$). The inset shows the trace of an EOD signal. B: Effect of β-estradiol on the EOD frequency of Apteronotus leptorhynchus. The EOD frequency was determined on 7 consecutive days before the operation (days -6 through 0) and on 8 days following the surgery (days 1 through 8). Fish received implants with (circles; $n = 14$) or without (triangles; $n = 17$) β-estradiol immediately following the measurement of the EOD frequency on day 0 (indicated by arrow). For each fish, the changes in EOD frequency were quantified relative to its average frequency over the 7-day pre-implantation period. The dashed line indicates no change from this baseline. Starting with day 4 after the implantation, the EOD frequencies of the β-estradiol-treated fish were significantly different from the discharge frequencies of the controls ($***; p < 0.001$, Bonferroni corrected) as well as from the pre-implantation frequencies ($p < 0.001$).

Fig. 2. Analysis of the protein spots that displayed significant increases or decreases in abundance in the Pn of β-estradiol-treated fish, compared to controls. The scatter plots show fold change versus statistical significance ($p$-value, independent-samples $t$-test) based on protein
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maps from the cytosolic and membrane fractions (A). Horizontal dashed lines indicate the +1.5- and -1.5-fold thresholds. The vertical dashed line marks the 0.05 significance level. Relative to controls, in the Pn of the β-estradiol-treated fish the abundances of 14 protein spots (red) were increased significantly (p < 0.05) more than 1.5-fold in the cytosolic fraction, while none of the protein spots of the membrane fraction met these two criteria. The abundances of 10 and 38 protein spots (green) were decreased significantly (p < 0.05) more than 1.5 fold in the cytosolic and membrane fraction, respectively. The middle panels (B) show magnified images of identified protein spots that exhibited significant increases (first row) or decreases (rows 2-5) in abundance in the Pn of β-estradiol-treated fish (Cy5 conjugation, red), compared to controls (Cy3 conjugation, green). Protein spot labels correspond to those shown in Table 1. Spot outlines are illustrated as detected by the DeCyder software. The bar chart (C) shows the average standardized abundances of the identified protein spots in tissue samples from the whole Pn of β-estradiol-treated fish (red), compared to samples from the Pn of control fish (green) (n = 3 gels, p < 0.05 in all cases). Error bars denote standard deviations. Spot labels (horizontal axis) correspond to those shown above in B and in Table 1. The dashed line separates protein spots with increased abundance (left) from protein spots with decreased abundance (right).

Fig. 3. GFAP expression in the Pn. Images of 4-µm thick optical cross-sections through the Pn of a female (A) and a male (B). Note the high density of intensely labeled GFAP-immunopositive fibers (green) in the Pn of the female, but a markedly lower fiber density and intensity of the immunolabeling in the Pn of the male. The large and medium-sized cells immunolabeled against
Hu C/D (red) are relay cells and pacemaker cells, respectively. The small immunostained cells are small interneurons. Nuclei were counterstained with DAPI (blue). C: Total GFAP labeling was higher in females \(n = 3\) than in males \(n = 3\) in the whole Pn (left; *, \(p < 0.05\)), and when associated with the surface area of pacemaker/relay cells (right; ***, \(p < 0.001\)), but not of small interneurons (middle; n.s., \(p > 0.10\)). Error bars denote standard errors of the means. D-D'':

Three-dimensional reconstruction of GFAP labeling associated with a Hu C/D-immunostained pacemaker cell \(p\) in the Pn. A total of 30 consecutive 0.5-µm thick optical sections were used for the reconstruction. D: Representative optical section. Green and red lines indicate the level of the z-stack cross-section shown on the sides of the panel. D': Surface reconstruction of the z-stack projection showing a dense meshwork of GFAP-positive fibers closely associated with the surface of the pacemaker cell. D'': Perspective view of the reconstruction shown in D'.

Fig. 4. Connexin-43 expression associated with astrocytes wrapped around pacemaker and relay cells in the Pn. A: Three-dimensional shadow projection of a z-stack comprising 22 confocal images spanning a total thickness of 10.5 µm. The Hu C/D-labeled pacemaker cell (blue) is embedded in a dense meshwork of GFAP-expressing astrocytes (green). These astrocytes abundantly express connexin-43 in a punctate, plaque-like pattern (red), indicating the formation of an astrocytic syncytium. B, B': For quantitative analysis of the connexin-43 immunofluorescence in the astrocytic syncytium, a 20-µm-thick annular region around the Hu C/D-immunostained cells was arbitrarily defined. C: The area covered by connexin-43 immunofluorescence in this region was higher in females \(n = 4\) than in males \(n = 4\), but the
difference was statistically significant only for pacemaker cells (**, \( p < 0.01 \); n.s., not significant, \( p > 0.10 \)). Error bars denote standard errors of the means.
Zupanc et al.
Proteins Mediating a Sexually Dimorphic Behavior
Figure 1
<table>
<thead>
<tr>
<th>Spot number</th>
<th>GI number</th>
<th>Protein name</th>
<th>Fold change</th>
<th>p-value</th>
<th>Mw</th>
<th>pl</th>
<th>Cellular location and proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>c623</td>
<td>55716039</td>
<td>NADH dehydrogenase (ubiquinone) Fe-S protein 1</td>
<td>+1.92</td>
<td>0.013</td>
<td>80.4</td>
<td>5.8</td>
<td>mitochondrial enzyme involved in the transfer of electrons from NADH to the respiratory chain (Weiss et al., 1991)</td>
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<tr>
<td>c789</td>
<td>66393075</td>
<td>glial fibrillary acidic protein</td>
<td>+1.96</td>
<td>0.001</td>
<td>42.2</td>
<td>5.0</td>
<td>principal intermediate filament protein in the cytoskeleton of mature astrocytes (Eng et al., 2000)</td>
</tr>
<tr>
<td>c885</td>
<td>40786398</td>
<td>pyruvate kinase isozymes M1/M2</td>
<td>+1.76</td>
<td>0.017</td>
<td>58.6</td>
<td>6.6</td>
<td>cytosolic enzyme that catalyzes the last step within glycolysis, the dephosphorylation of phosphoenolpyruvate to pyruvate (Tolle et al., 1976; Jurica et al., 1998)</td>
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<tr>
<td>c2199</td>
<td>52219194</td>
<td>fatty acid binding protein 11a</td>
<td>+1.70</td>
<td>0.049</td>
<td>15.3</td>
<td>7.8</td>
<td>lipid chaperone involved in lipid transport and metabolism (Flynn et al., 2009)</td>
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<tr>
<td>c727</td>
<td>47087055</td>
<td>protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform</td>
<td>−2.48</td>
<td>0.033</td>
<td>53.7</td>
<td>4.9</td>
<td>Ser/Thr phosphatase involved in a wide range of regulatory cell functions, including cell-cycle regulation, cell-growth and development, cytoskeleton dynamics, and cell motility (Basu, 2011)</td>
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<tr>
<td>c753</td>
<td>41281615</td>
<td>plastin-2</td>
<td>−1.64</td>
<td>0.006</td>
<td>70.4</td>
<td>5.3</td>
<td>actin-binding protein involved in actin filament organization and cross-linking (Delanote et al., 2005)</td>
</tr>
<tr>
<td>c2213</td>
<td>113195584</td>
<td>vesicle-fusing ATPase</td>
<td>−3.72</td>
<td>0.003</td>
<td>83.2</td>
<td>6.4</td>
<td>cytosolic enzyme involved in vesicle-mediated transport and synaptic vesicle fusing/recycling (Südhof and Rizo, 2011)</td>
</tr>
<tr>
<td>c2850</td>
<td>62955673</td>
<td>stathmin 1b</td>
<td>−2.78</td>
<td>0.0002</td>
<td>17.1</td>
<td>6.1</td>
<td>tubulin-binding protein involved in regulation of microtubule assembly/disassembly (Jourdain et al., 1997)</td>
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<tr>
<td>m504</td>
<td>221307553</td>
<td>internexin neuronal intermediate filament protein, alpha</td>
<td>−2.31</td>
<td>0.011</td>
<td>55.7</td>
<td>5.2</td>
<td>neuronal intermediate filament protein that may contribute to axogenesis and cytoskeletal assembly (Lariviere and Julien, 2004)</td>
</tr>
<tr>
<td>m804</td>
<td>113195584</td>
<td>vesicle-fusing ATPase</td>
<td>−2.62</td>
<td>0.044</td>
<td>83.1</td>
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<tr>
<td>m1026</td>
<td>55925442</td>
<td>3-oxoacid CoA transferase 1a</td>
<td>−2.23</td>
<td>0.015</td>
<td>57.6</td>
<td>8.5</td>
<td>mitochondrial enzyme involved in oxidation of ketone bodies by converting acetoacetate to acetoacetyl-CoA (Robinson and Williamson, 1980)</td>
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<tr>
<td>m1130</td>
<td>115494998</td>
<td>fascin</td>
<td>−1.84</td>
<td>0.006</td>
<td>106</td>
<td>8.9</td>
<td>actin-cross-linking protein involved in defining morphology of dendrites and axons (Kraft et al., 2006; Nagel et al., 2012)</td>
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<tr>
<td>m1184</td>
<td>18858755</td>
<td>internexin neuronal intermediate filament protein, alpha b (= gefilin)</td>
<td>−1.64</td>
<td>0.033</td>
<td>54.4</td>
<td>5.2</td>
<td>intermediate neurofilament protein involved in axogenesis (Asch et al., 1998)</td>
</tr>
<tr>
<td>m1229</td>
<td>225579106</td>
<td>glutamine synthetase</td>
<td>−2.51</td>
<td>0.001</td>
<td>42.5</td>
<td>6.0</td>
<td>glia-specific enzyme involved in conversion of synaptically released glutamate to glutamine (Hertz et al., 1999)</td>
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<tr>
<td>m1318</td>
<td>39794594</td>
<td>bactin1 protein (= beta-actin-1)</td>
<td>−2.37</td>
<td>0.007</td>
<td>42.1</td>
<td>5.3</td>
<td>cytoskeletal protein involved in regulation of neuronal morphology and morphogenesis (Luo, 2002)</td>
</tr>
<tr>
<td>GI number</td>
<td>genInfo identifier of the NCBI</td>
<td>protein name</td>
<td>p value</td>
<td>q value</td>
<td>Mw (kDa)</td>
<td>pI</td>
<td>Reference</td>
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<tr>
<td>m1319</td>
<td>182889552</td>
<td>bactin1 protein (= beta-actin-1)</td>
<td>-3.44</td>
<td>0.0004</td>
<td>42.2</td>
<td>5.2</td>
<td>see spot m1319, above</td>
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<td>m1367</td>
<td>37748067</td>
<td>succinate-CoA ligase, ADP-forming, beta subunit</td>
<td>-1.65</td>
<td>0.030</td>
<td>50.4</td>
<td>6.5</td>
<td>mitochondrial matrix enzyme that catalyzes the ATP-dependent ligation of succinate and CoA to form succinyl-CoA (Kaufman, 1955)</td>
</tr>
<tr>
<td>m1436</td>
<td>41393139</td>
<td>isovaleryl-CoA dehydrogenase, mitochondrial</td>
<td>-2.72</td>
<td>0.005</td>
<td>46.3</td>
<td>8.0</td>
<td>mitochondrial matrix enzyme that catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in fatty acid and amino acid catabolism (Ikeda and Tanaka, 1983)</td>
</tr>
<tr>
<td>m1450</td>
<td>47087281</td>
<td>actin-related protein 2-A</td>
<td>-1.50</td>
<td>0.030</td>
<td>44.9</td>
<td>6.3</td>
<td>ATP-binding component of the actin-related protein 2/3 complex involved in initiation of actin-polymerization and in organization of the actin filaments into branched networks (Goley and Welch, 2006; Campellone and Welch, 2010)</td>
</tr>
<tr>
<td>m1990</td>
<td>47834966</td>
<td>IFT81 (= intraflagellar transport protein 81 homolog)</td>
<td>-3.23</td>
<td>0.001</td>
<td>79.2</td>
<td>6.4</td>
<td>together with other proteins of the IFT complex involved in assembly and maintenance of cilia (Taschner et al., 2012)</td>
</tr>
</tbody>
</table>

GI number, genInfo identifier of the NCBI; Mw, molecular weight (kDa); pI, isoelectric point
Table 2. Morphological differences in the Pn of male vs. female *A. leptorhynchus*

<table>
<thead>
<tr>
<th></th>
<th>Pn volume (mm³)</th>
<th>Pacemaker cells</th>
<th>Relay cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of cells</td>
<td>Profile area (µm²)</td>
<td>Number of cells</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>0.20 ± 0.02</td>
<td>25.8 ± 1.3</td>
<td>2086 ± 127</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td>0.21 ± 0.03</td>
<td>24.0 ± 2.2</td>
<td>2036 ± 205</td>
</tr>
</tbody>
</table>

Values represent averages ± standard errors of the means (*n* = 3 fish per group). A total of 14-18 transverse brain sections were analyzed per fish. Total cell numbers were estimated using Abercrombie’s method (Abercrombie, 1946).