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

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Zupanc GK, Ilies I, Sirbulescu RF, Zupanc MM. Large-scale identification of proteins involved in the development of a sexually dimorphic behavior. J Neurophysiol 111: 1646–1654, 2014. First published January 29, 2014; doi:10.1152/jn.00750.2013.—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 activation 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, Apterontus leporhtynchus. In this weakly electric fish, the discharge frequency is controlled by the electrical pacemaker nucleus and is higher in males than in females. After lowering the discharge frequency through chronic administration of sex steroids, 2-dimensional difference gel electrophoresis revealed 62 proteins spots in tissue samples from the pacemaker nucleus that exhibited significant changes in abundance of >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 proteins GFAP and increased gap-junction coupling between astrocytes in females compared with 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 nuclei via a mechanism mediating the uptake of extracellular potassium ions from the extracellular space.

MATERIALS AND METHODS

Experimental animals. Brown ghost knife fish (A. leporhtynchus; 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 to 189 mm, and their weight from 0.9 to 14.7 g. The gonadosomatic index ranged from 0.0006 to 0.0042 in males and from 0.0021 to 0.0599 in females. Animal care and procedures were conducted in accordance with the German Animal Welfare Act (Deutsches Tierschutzgesetz) of 1998 and approved by the local authorities, the Senate of the Free Hanseatic City of Bremen.

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 \( Q_{10} \) of 1.56 (Zupanc et al. 2003).

**Treatment with \( \beta \)-estradiol.** Under general anesthesia with 2% ethyl carbamate (urethane; Sigma-Aldrich) 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; Smiths Medical International) packed with \( \beta \)-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 Pn of other fish, and stored at \(-80°C\) until further use.

2-D DIGE. Protein extraction, protein labeling with CyDye DIGE fluor, and separation of protein extract by 2-D DIGE were performed using the Ettan DIGE system (Amersham Biosciences/GE Healthcare) as described previously (Ilies et al. 2012). Briefly, pooled tissue samples from \( \beta \)-estradiol-treated and control fish, respectively, were homogenized through sonication in lysis buffer (Amersham Biosciences). After centrifugation, supernatant containing 50 \( \mu \)g of protein from each sample were labeled with 400 pmol of Cy3 and Cy5, respectively. For the internal standard, 25 \( \mu \)g of protein from each sample were combined and then labeled with 400 pmol of Cy2. Finally, the two samples and the standard were pooled and used for 2-D-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 \( \mu \)m per pixel. In-gel multidiye codection 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 2-D Software (Amersham Biosciences), separately for the cytosolic and membrane fractions. The matching of all protein spots exhibiting changes in abundance of >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 >1.5-fold in either the cytosolic or the membrane fraction were selected for identification. Preparative gels were run as described above using 500 \( \mu \)g 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, destained, and subjected to in-gel digestion with modified trypsin (Roche Diagnostics) overnight at 37°C. The extracted peptides were desalted using a C18 ZipTip (Millipore) and identified by PMF using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) as described previously (Ilies et al. 2012).

**Immunohistochemistry.** Fish were deeply anesthetized in a 2% solution of MS-222 in aquarium water and intracardially perfused with 2% freshly depolymerized paraformaldehyde (Fisher) in 0.1 M phosphate buffer, pH 7.4. The brain was cryosectioned coronally at a thickness of 16 \( \mu \)m. GFAP, HuC/D, and connexin-43 antigenic sites were labeled using rabbit anti-GFAP (Sigma-Aldrich) or chicken anti-GFAP (Abcam), mouse anti-HuC/D (clone 16A11; Invitrogen), and rabbit anti-connexin-43 (Cell Signaling) primary antibodies followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 or Alexa Fluor 546, goat anti-chicken IgG conjugated to Alexa Fluor 488, and goat anti-mouse conjugated to Alexa Fluor 635 secondary antibodies (all from Invitrogen). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

**Microscopy and image analysis.** Confocal microscopy was performed using Zeiss LSM 700 and Zeiss LSM 710 laser-scanning microscopes equipped with \( \times 25 \) and \( \times 63 \) objectives. Optical sections were taken at a resolution of 0.2–0.5 \( \mu \)m per pixel using ZEN (Carl Zeiss) software. Images were reconstructed in ImageJ (National Institutes of Health) using the Stitching plugin (Preibisch et al. 2009). Background subtraction through top-hat filtering, definition of regions of interest based on HuC/D labeling, and subsequent quantification of GFAP, HuC/D, and connexin-43 immunolabeling were performed in MATLAB (MathWorks) using built-in functions from the Image Processing Toolbox.

**RESULTS**

**Sexual dimorphism in EOD frequency.** To confirm that brown ghost knifefish males and females occupy different frequency domains, the EOD of 83 individuals was recorded, and they 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 $94 \pm 23$ Hz lower than the preimplantation baseline ($P < 0.001$, $z$-test, Bonferroni correction for multiple comparisons; $n = 14$ fish). In all treated fish, both male and female, the observed

![Fig. 2. Analysis of the protein spots that displayed significant increases or decreases in abundance in the pacemaker nucleus (Pn) of β-estradiol-treated fish compared with controls.](image)

The scatterplots show fold change vs. statistical significance ($P$ value, independent-samples $t$-test) based on protein maps from the cytosolic and membrane fractions (A). Horizontal dashed lines indicate the $\pm 1.5$-fold and $\pm 1.5$-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 ($P < 0.05$) $>1.5$-fold in the cytosolic fraction, whereas none of the protein spots of the membrane fraction met these 2 criteria. The abundances of 10- and 38-protein spots (green) were decreased significantly ($P < 0.05$) $>1.5$-fold in the cytosolic and membrane fraction, respectively. The middle (B) shows magnified images of identified protein spots that exhibited significant increases (row 1) or decreases (rows 2–5) in abundance in the Pn of β-estradiol-treated fish (Cy5 conjugation; red) compared with controls (Cy3 conjugation; green). Protein spot labels correspond to those shown in Table 1. 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 with samples from the Pn of control fish (green; $n = 3$ gels; $P < 0.05$ in all cases). Error bars denote SD. 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).
<table>
<thead>
<tr>
<th>Spot No.</th>
<th>GI No.</th>
<th>Protein Name</th>
<th>Fold Change</th>
<th>P Value</th>
<th>Mol Mass, kDa</th>
<th>pI</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 (Jurica et al. 1998; Tolle et al. 1976)</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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<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>
</tr>
<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>
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<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>
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<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 3-Oxoacid CoA transferase 1a</td>
<td>−2.62</td>
<td>0.044</td>
<td>83.1</td>
<td>6.4</td>
<td>Mitochondrial enzyme involved in oxidation of ketone bodies by converting acetoacetate to acetoacetyl-CoA (Robinson and Williamson 1980)</td>
</tr>
<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>See spot c2213, above Actin-cross-linking protein involved in defining morphology of dendrites and axons (Kraft et al. 2006; Nagel et al. 2012)</td>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td>m1318</td>
<td>39794594</td>
<td>Beta-actin-1 (bactin1 protein)</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>
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<tr>
<td>m1319</td>
<td>182889552</td>
<td>Beta-actin-1 (bactin1 protein)</td>
<td>−3.44</td>
<td>0.0004</td>
<td>42.2</td>
<td>5.2</td>
<td>Mitochondrial matrix enzyme that catalyzes the ATP-dependent ligation of succinate and CoA to form succinyl-CoA (Kauffman 1955)</td>
</tr>
<tr>
<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 conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA in fatty acid and amino acid catabolism (Ikeda and Tanaka 1983)</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>
</tbody>
</table>
frequency decrease was proportional to the baseline EOD frequency with higher-frequency EODs showing larger reductions (Pearson $\rho = 0.69$, $P < 0.01$). 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 $23 \pm 16$ Hz lower than the baseline ($n = 17$ fish), a significantly smaller effect than that observed in $\beta$-estradiol-treated fish ($P < 0.001$, independent-samples $t$-test, Bonferroni correction for multiple comparisons).

Protein expression profiles in the Pn: differences between $\beta$-estradiol-treated fish and controls. Eight days after the implantation of $\beta$-estradiol-filled or empty tubings, tissue was collected from whole Pn and processed for 2-D DIGE. An average number of 3,248 ± 199 and 3,006 ± 160 protein spots were detected in the gels run on the cytosolic and membrane fractions, respectively. A total of 1,145 and 819 spots were matched across the 3 2-D gels of the cytosolic and membrane fractions, respectively. In the cytosolic fraction, out of the 1,145 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 $\beta$-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 $\beta$-estradiol-treated fish relative to the Pn of control fish, whereas 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 isoelectric point (pI) range analyzed.

Identification of differentially expressed proteins. Out of the 62 protein spots that exhibited significant changes in abundance of $>1.5$-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. 2, B and 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); beta-actin-1 (bactin1 protein; 2 spots); fascin (1 spot); glutamine synthetase (1 spot); internexin neuronal intermediate filament protein, alpha (1 spot); internexin neuronal intermediate filament protein, alpha b (gefilitin; 1 spot); intraflagellar transport protein 81 homolog (IFT81; 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 $\beta$-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 three males and three females were immunostained against the neuronal marker HuC/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 $\beta$-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 with 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 identi-

### Table 1.—Continued

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>GI No.</th>
<th>Protein Name</th>
<th>Fold Change</th>
<th>$P$ Value</th>
<th>Mol Mass, kDa</th>
<th>pI</th>
<th>Cellular Location and Proposed Function</th>
</tr>
</thead>
<tbody>
<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 (Campellone and Welch 2010; Goley and Welch 2006)</td>
</tr>
<tr>
<td>m1990</td>
<td>47834966</td>
<td>Intraflagellar transport protein 81 homolog (IFT81)</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>
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</table>
fied by immunostaining against HuC/D. The total GFAP labeling associated with the area covered by pacemaker and relay cells was significantly higher in females than in males \( (P < 0.001; \text{Fig. 3C, right}) \). 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 \( (P > 0.10; \text{Fig. 3C, middle}) \).

**Differences between males and females in connexin-43 expression associated with GFAP-labeled astrocytes.** To characterize further 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, plaquelike connexin-43 immunofluorescence was found in close proximity to GFAP-labeled fibers (Fig. 4A). The relative area covered by connexin-43 immunoreactivity in a 20-μm-thick annular region around the HuC/D-immunolabeled pacemaker cells was almost 3-fold larger in females than in males \( (P < 0.01, \text{independent-samples t-test}; n = 4 \text{ males and 4 females; Fig. 4, B–C}) \). The connexin-43-labeled area around relay cells was also increased in females, relative to males, but this difference was not significant \( (P > 0.10) \).

<table>
<thead>
<tr>
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<th>Pacemaker Cells</th>
<th>Relay Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pn Volume, mm³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.20 ± 0.02</td>
<td>120.0 ± 1.8</td>
</tr>
<tr>
<td>Females</td>
<td>0.21 ± 0.03</td>
<td>134.8 ± 1.7</td>
</tr>
</tbody>
</table>

Values represent averages ± SE \( (n = 3 \text{ fish per group}) \). A total of 14–18 transverse brain sections were analyzed per fish. Total cell numbers were estimated using Abercrombie’s method (Abercrombie 1946).
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 for which 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 Fe-S 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 nonneuronal 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. leptorhynchos*. 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 (Dulka and Maler 1994; Dunlap et al. 1998; Zupanc and Maler 1993). Chirps are controlled by a subpopulation of neurons in the central posterior/pacemaker nucleus, the CP/PPn-C (Heiligenberg et al. 1981; Kawasaki and Heiligenberg 1988; Kawasaki et al. 1988; Metzner 1999; Zupanc 2002; Zupanc and Heiligenberg 1992; Zupanc and Maler 1997). The cells comprising this neuronal cluster project to relay cells in the Pn 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; Elekes and Szabo 1985; Moortgat et al. 2000; Tokunaga et al. 1980). 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 mark-
edly higher in females than in males. Notably, this effect did not extend to the third neuronal type in the Ph, 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 Ph is in agreement with prior ultrastructural studies that noted an intimate association of neurons and astrocytes in the Ph, particularly in the region of axon initial segments (Elekes and Szabo 1985; Tokunaga et al. 1980).

Physiological and immunohistochemical studies have demonstrated that three types of ions play important roles in the regulation of the electric activity of the Ph, 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 Ph 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 (Kofuji 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-glial 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 Ph, 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: 1) increase in the size of the glial syncytium; 2) enhancement of the glial coupling through gap junctions; and 3) 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 Ph, 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 (Chen and Roche 2007; Levitan 1994). In the Ph, 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 Ph 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 Ph.

REFERENCES


