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Relevance of c-Src and protein phosphatase 2A to aromatase activity: evidence of an acute self-regulating estrogenic signaling complex in rat central nervous system

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Abstract

We previously reported that aromatase protein levels do not parallel aromatase enzyme activity (Storman, et al., Endocrinology, 2018). This suggests that estrogenic signaling may be modulated via post-translational modification of aromatase protein. The tyrosine and serine phosphorylation state of aromatase are known to influence its activity. To investigate the possible relevance of aromatase phosphorylation to the incongruity observed between aromatase protein and its activity, we explored interactions between aromatase and the tyrosine kinase c-Src and the serine protein phosphatases 2A and 5 (PP2A and PP5), as well as the relationship between levels of tyrosine-phosphorylated aromatase and the extrapolated aromatase activity. We found that (a) hypothalamic aromatase was significantly more heavily tyrosine-phosphorylated than spinal aromatase; (b) aromatase was oligomerized with c-Src and PP2A/PP5, potentially activating aromatase via tyrosine-phosphorylation and serine-dephosphorylation; (c) the associations of c-Src and PP2A/PP5 with hypothalamic aromatase were substantially greater than with spinal aromatase; and (d) aromatase, estrogen receptor α, PP2A, and c-Src were present in a common membrane oligomer. The existence of c-Src and PP2A in an oligomer that also contains aromatase and membrane estrogen receptor α (and presumably other signaling molecules) indicates the presence in the CNS of a potentially self-regulating estrogenic signaling unit. The degree to which such a complex operates autonomously and the regulatory factors thereof are likely to have substantial physiological implications and clinical relevance.

Keywords

hypothalamus; spinal cord; phosphorylation; estrogen; aromatase

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1 INTRODUCTION

Aromatase, a member of the Cytochrome P450 enzyme superfamily, is unique in its ability to catalyze the conversion of androgens into estrogens. Originally discovered in the gonads, aromatase is now known to also be present throughout the central nervous system (CNS) [1, 2]. In the vertebrate CNS, aromatase is not only an integral membrane protein of the endoplasmic reticulum, but is also present in neuronal plasma membranes, particularly in presynaptic terminals [3–7] and possibly dendritic spines [5, 8, 9]. This subcellular localization of aromatase is consistent with the hypothesis that estrogens produced within the CNS can act as neurotransmitters (i.e., synaptocrine estrogenic signaling [10]). Spinal cord and hypothalamic aromatase also localize to multimeric signaling complexes containing membrane estrogen receptor alpha (mERα) and other signaling molecules [11], enabling estrogens to act within the same complex in which they are produced. We have termed this signaling modality oligocrine [11, 12].

This study's predecessor, [12], investigated the differential organization and association of aromatase and mERα between two functionally distinct CNS regions – the spinal cord and the hypothalamus. One of our findings was that while this association between an enzyme catalyzing the formation of estrogen and the receptor upon which it can act exists in both regions investigated, their relative degree of their association differed. Nearly all of aromatase, for example, was associated with mERα in the spinal cord, while that was the case for only 15% of hypothalamic aromatase. Such discrepancy can be potentially explained by different functional tasks of the two regions investigated – hypothalamus acting as an endocrine organ, while the spinal cord acting on a more local (i.e., synaptocrine, oligocrine) level.

Effective regulation and utilization of both synaptocrine and oligocrine estrogenic signaling requires rapid activation/inactivation of aromatase activity on a time scale incompatible with the temporal requirements for *de novo* aromatase protein expression (e.g., as pertains to ovarian aromatase over the reproductive cycle [13]). Instead, alternative strategies are needed for rapid changes in the availability of CNS estrogens. The necessity for acute activation of aromatase is evidenced by a striking incongruity we reported between CNS levels of aromatase protein and its corresponding catalytic activity in [12]. For example, the approximately 40% increment in hypothalamic aromatase activity between proestrous vs. diestrous rats was not accompanied by changes in levels of aromatase protein. Additionally, the detected hypothalamic aromatase activity was robust, while spinal aromatase activity was minimal, despite the spinal cord containing four times the amount of aromatase protein. This suggests that the CNS employs acute, non-transcriptional/non-translational mechanisms to regulate aromatase activity.

There is converging evidence that the phosphorylation state of aromatase is an important determinant of its activity [14–18]. However, the exact site(s) of aromatase phosphorylation, as well as its impact on aromatase activity, is not consistent among studies. For example, conditions promoting global phosphorylation [15] and those promoting global dephosphorylation [19] have both been shown to rapidly downregulate aromatase activity.

This suggests that aromatase activity may be modulated by a complex pattern of phosphorylation/dephosphorylation at multiple sites.

Two phosphorylation sites relevant to modulating aromatase activity have emerged. Tyr361 phosphorylation has been reported to elevate aromatase activity in human breast cancer cells [17]. It is hypothesized to occur via both c-Src phosphorylation of aromatase at Tyr361, and via the impaired ability of the Tyr phosphatase PTP1B to dephosphorylate aromatase at this site [16]. Conversely, phosphorylation of Ser118 has been reported to decrease aromatase activity (decreasing both its specific enzymatic activity and Vmax) while increasing its Km [20].

To follow up on reports that Tyr361 phosphorylation enhances aromatase activity, we tested the hypothesis that the activity of rat CNS aromatase would be paralleled by its Tyr phosphorylation, a deficiency of which could explain the minimal aromatase catalytic activity in spinal cord. Furthermore, since we reported profound differences in aromatase activity between spinal cord and hypothalamus, and between hypothalami of diestrous and proestrous rats [12], hypotheses were tested not only by comparing aromatase phosphorylation between these CNS regions, but also as a function of estrous cycle stage. The latter consideration necessitated the use of female rats, which also enabled comparison of aromatase phosphorylation between two naturally fluctuating physiological states.

Our results suggest that phosphorylation patterns specific to particular CNS regions have the potential to dictate local aromatase activity. Thus, differential phosphorylation/dephosphorylation of aromatase among CNS regions could explain, at least in part, the lack of synchrony in aromatase activity throughout the CNS. Our findings also reveal the presence in both spinal cord and hypothalamus of an aromatase-containing signaling unit in which aromatase, Tyr kinase, and a Ser phosphatase are present along with mERα, providing a subcellular organization for rapid regulation of aromatase catalytic activity (via phosphorylation and dephosphorylation) and localized estrogenic signaling.

2 MATERIALS AND METHODS

2.1 Animals

Experiments used brains and spinal cords from adult female Sprague-Dawley rats (Charles River; 225-275 g). (Female rats allowed us to test the effect of estrous cycle stage on aromatase phosphorylation). Rats were kept on 12-hour light/dark cycle, with food and water available *ad libitum*. All experimental procedures were reviewed and approved by the Animal Care and Use Committees of the State University of New York Downstate Health Sciences University. Estrous cycle stage was determined using vaginal smear histology, proestrus being indicated by large round nucleated cells and diestrus by the predominant presence of small leukocytes. Day 2 of diestrus was used for experiments to minimize variation.

2.2 Tissue isolation

For biochemical experiments, animals were sacrificed by decapitation. The spinal cord was removed from the spinal column, frozen on dry ice, and stored at −80°C. To isolate the

hypothalamus, the brain was removed from the cranium and submerged in an icy slurry of oxygenated artificial cerebro-spinal fluid (aCSF; 124 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 10 mM D-glucose, 1.6 mM $MgCl_2$, and 2.0 mM $CaCl_2$). From 500-μm sections (Leica VT 1000 vibratome) matching plates 17 and 23 of The Rat Brain in Stereotaxic Coordinates [21], the hypothalamus was excised, immediately frozen on dry ice, and stored at −80°C.

2.3 Membrane preparation

Spinal cord and hypothalamus were each homogenized using Wheaton glass homogenizers in Homogenization Buffer (10% sucrose, 20 mM HEPES, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, protease inhibitor cocktail (Roche), PhosSTOP™ phosphatase inhibitor cocktail (Roche), pH 7.4). Following homogenization, and centrifuged at 1,500 x g for 10 mins at 4°C. The supernatant was transferred to a clean tube and kept on ice, while the pellet was resuspended in Homogenization Buffer, homogenized, and centrifuged at 2,500 x g for 10 mins at 4°C. Supernatants from both spins were then merged (and the nuclear/cellular debris pellet discarded) and centrifuged at 31,000 x g for 40 mins at 4°C. The resulting pellet (crude membrane fraction) was resuspended in Storage Buffer (Homogenization Buffer without sucrose), aliquoted, and stored at −80°C until use.

The crude membrane fraction was then solubilized as we previously reported [12, 22]. Crude membrane fraction samples were thawed on ice, centrifuged at 16,100 x g for 15 minutes at 4°C, Storage Buffer discarded, and the pellet solubilized in Solubilization Buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% NP40, 0.25% DOC, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH 7.4) for 1 hour with rotation at 4°C. Following the incubation, samples were centrifuged at 16,100 x g for 15 minutes at 4°C. Total protein concentrations in the clear solubilized membrane supernatants were determined by Bradford dye assay (colorimetric shift measured using DU800 spectrophotometer and BSA standard curve). Equivalent amounts of total protein were used for SDS-PAGE (between 10 and 60 μg) or immunoprecipitation (between 200 and 800 μg), followed by SDS-PAGE.

2.4 Co-immunoprecipitation (co-IP), electrophoresis, and Western blotting

Sequential IP was performed as we previously described [11]. The amounts of IP antibodies used per amount of tissue were empirically determined to ensure quantitative IP of the desired protein (i.e., none of the target protein remained in the supernatant following the immunoprecipitation). Antibodies were cross-linked to Protein A or G agarose beads (Roche 11134515001 or Roche 11243233001, respectively) using DSS (Pierce; final concentration 0.45 mM) according to the manufacturer's protocol. Following the cross-linking procedure, the antibody-protein A/G agarose bead complex was washed with 100 mM Glycine, pH 2.5, to ensure that the antibodies would not elute with the samples during the sequential IP procedure. Following a neutralizing wash of the antibody-agarose bead complex with 20 mM Tris, pH 7.4, samples of equivalent amounts of total protein in Dilution Buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, protease inhibitor cocktail, PhosSTOP™ phosphatase inhibitor cocktail, pH 7.4) were added, and the mixture was gently agitated overnight at 4°C. In experiments requiring isolation of aromatase from co-IPing proteins (pTyr quantification), DTT (0.2 M) was added to the Dilution Buffer to carry out IP under reducing conditions.

Upon completion of the incubation, the beads were washed and gently eluted with 100 mM glycine, pH 2.5. For sequential IP, the eluate was neutralized (1 M Tris, pH 8.8) and incubated with the next antibody (for 3 hours at room temperature or overnight at 4°C). For sequential IP, to ensure an adequate amount of starting tissue, the first IP (using anti-mERα antibody) was performed on 800 μg of total protein x4 tubes (which required pooling of hypothalamic tissues from 3 rats for each of the 3 experiments; spinal cord tissue from one rat per experiment was sufficient). Following elution and neutralization, the second IP (using anti-c-Src antibody) was carried out in 2 tubes (i.e., neutralized eluates from mERα IP were merged), while the third IP (using anti-PP2A antibody) was done in 1 tube (from merged eluates of c-Src IP).

The final IP was eluted with heat (86°C in a heat block for 15 minutes) in LDS Buffer (Novex, NP0007) in presence of 50 mM DTT. The eluate was resolved on 4%-12% plyacrylamide gradient Bis-Tris SDS Gels (Thermo Fisher Scientific) and Western blotted. Chemiluminescence was captured using a G:Box CCD camera (Syngene), and the signals were quantified for comparison using Genetools software (Syngene). Total protein staining was obtained by incubating nitrocellulose membranes with Colloidal Gold (Bio-Rad) for ten minutes, followed by distilled water washes.

2.5 Sequential IP controls

It is possible that elution conditions using low pH glycine buffer could have eluted both the antibodies as well as the proteins, which after neutralization, could result in the initial complex to be re-formed and captured again on the Protein A/G agarose beads in the subsequent step (i.e., antibody-antigen complex from the first IP would be captured by Protein A/G beads of the subsequent IP). This capture would be independent of the antibody, giving an erroneous impression of a sequential IP. However, no IgGs were detected in the Westerns of the glycine wash following the cross-linking procedure, reinforcing our conclusion that the glycine elution procedure following IP did not elute the initial (cross-linked) antibodies, but only the antigens. Moreover, when an unrelated antibody (e.g., anti-serotonin receptor 1A or anti-calcineurin antibodies) was used, aromatase did not co-IP, supporting the notion that interactions between aromatase and c-Src, PP2A, PP5, and ERα are not indiscriminate.

Additional negative data underscore that sequential IP was indeed not spurious but reflected the existence of the concluded complex. These data include the following: (1) sequential IP using antibodies (in order) directed against c-Src, aromatase, and then PP2A, followed by Western blotting for PP5 was negative, notwithstanding our demonstration of PP5's association with aromatase, c-Src, and PP2A individually; (2) PTP1B is present in spinal cord and hypothalamus, but it does not co-IP with c-Src, PP2A or aromatase; (3) sequential IP with antibodies against ERα, c-Src, PP2A, and then aromatase, followed by Western blotting for $mGluR_1$ or KOR failed to reveal the presence of either; (4) aromatase was not present following either sequential IP utilizing antibodies against ERα, PP2A, c-Src, and then MOR or sequential IP utilizing antibodies against ERα, PP2A, c-Src, and then $mGluR_1$, notwithstanding that aromatase was present in Western blots following sequential IP with

antibodies against ERα, PP2A, and then c-Src (i.e., the first three proteins in the sequential IP sequence).

2.6 Antibody and Western signal

2.6.1 Antibodies used

IP: anti-ERα (sc-71064, RRID:AB_1122667; 3 μg per 800 μg of total protein) and anti-aromatase (sc-14245, RRID:AB_2088684; 3 μg per 800 μg of total protein), both purchased from Santa Cruz Biotechnology (SCB); anti-Src (Millipore, OP07L-100UG, RRID:AB_10683060; 1 μg per 200 μg of total protein), anti-PP2A (Millipore, 05-421, RRID:AB_309726; 1 μg per 200 μg of total protein).

Western blot: anti-aromatase (Abeam, ab124776, RRID:AB_10972863; 1:40,000), anti-PP2A (Millipore, 05-421, RRID:AB_309726; 1:2,000), anti-PP5 (Abcam, ab34693, RRID:AB_777387; 1:1,000), anti-Src (Millipore, OP07L-100UG, RRID:AB_10683060; 1:200), anti-PTP1B (Cell Signaling Technology Cat# 5311, RRID:AB_10695100; 1:250) and anti-phospho-Tyrosine (pTyr; Millipore, 06-427, RRID:AB_11211856; 1:1,000).

2.6.2 Antibody specificity—The specificity of each antibody used for Western blots was verified by the absence (or greater than 70% reduction) of signal when preadsorbed antibody flow-through was used. Antibodies were preadsorbed using Affi-Gel 10 slurry (Pierce) as previously described [11]. Specificity of the following antibodies was verified for this study: anti-PP2A, anti-PP5, anti-pTyr, and anti-aromatase (see Figure 1). Specificity of the other antibodies used in this study had been recently demonstrated by us (see [11]) or Abcam for c-Src using knockout animals (see https://www.abcam.com/src-antibody-clone-327-ab16885.html#description_images_1).

2.6.3 Specificity of pTyr aromatase Western signal—Since a general anti-pTyr antibody was employed in these studies, we cannot unequivocally attribute the pTyr Western signal to aromatase vs. other co-migrating proteins. This potential confound was minimized by (a) performing aromatase IP under reducing conditions, thus eliminating co-IP of other proteins, (b) demonstrating that the molecular mass of the anti-pTyr Western signal was indistinguishable from the concomitantly determined molecular mass of IPed aromatase using a pan anti-aromatase antibody, (c) demonstrating the absence of pTyr Western signal when immunoprecipitating with preadsorbed anti-aromatase antibody flow-through and Western blotting with anti-pTyr antibody, (d) the presence of only a single protein in the vicinity of the molecular mass corresponding to aromatase when aromatase was IPed under reducing conditions, utilizing anti-aromatase antibody that had been cross-linked to Protein G agarose beads, subjected to SDS-PAGE and stained with Colloidal Gold, and (e) co-migration of p-Tyr Western signal subsequent to blot stripping and re-probing with anti-aromatase antibodies. Thus, it is highly likely that the pTyr Western signal resulted from phosphorylation of Tyr residue(s) within aromatase as opposed to other proteins.

Admittedly, IP of membrane proteins from detergent extracts can be prone to nonspecific carryover of membrane proteins present in mixed micelles. However, as mentioned above, IPs were performed under reducing conditions, increasing the likelihood that the signal was

derived from aromatase, and not a co-isolated protein. Additionally, anti-aromatase antibody used for IP was raised in goat, while anti-aromatase and anti-pTyr antibodies used for Western blotting were raised in rabbit, requiring anti-rabbit secondary. This difference in the host species of antibodies used for IP and Western blotting precluded contamination of sample with eluted IgGs, and thus eliminated the possible confound of IgG detection by secondary antibodies during Western blotting.

Since rat aromatase does not contain a Tyr at position 361, and not having a basis for predicting the precise location of any hypothesized Tyr phosphorylation, we quantified total aromatase phosphorylated at Tyr (pTyr-aromatase) using a general anti-pTyr antibody. In order to minimize the possibility that a protein other than aromatase was being detected by the anti-pTyr antibody, (a) aromatase was immunoprecipitated under reducing conditions, minimizing potential confounds resulting from Tyr phosphorylation of co-immunoprecipitated proteins, and (b) an aliquot of the IP was Western blotted in the adjacent lane using a pan-anti-aromatase antibody, validating that the band detected with the anti-pTyr antibody corresponded to the band detected with the pan-anti-aromatase antibody. The specificity of the anti-pTyr antibody was authenticated by demonstrating its ability to recognize a peptide containing a pTyr, but not a peptide containing multiple Tyr, none of which were phosphorylated (Figure 1). Quantitative comparisons of pTyr-aromatase between hypothalamus and spinal cord, and between proestrus and diestrus, were made possible by ensuring that aromatase was quantitatively immunoprecipitated (as in [12]). Since the spinal cord contains four times the amount of aromatase protein than the hypothalamus [12], the chemiluminescent Western signal from hypothalamus was adjusted by this factor in order to compensate for the difference in the level of aromatase protein.

2.7 Statistical analysis

Using Prism 6, 2-way ANOVA tests were performed to compare normalized Western blot signals between proestrous and diestrous rat groups and between hypothalamus and spinal cord. Student's t-tests were performed to compare normalized Western blot signals between hypothalamus and spinal cord. $P < 0.05$ (two-tailed) was considered significant.

3 RESULTS

3.1 Effects of stage of estrous cycle on pTyr-aromatase in hypothalamus and spinal cord

To investigate the influence of stage of cycle on spinal and hypothalamic pTyr-aromatase, we compared the levels of pTyr-aromatase within each of the two CNS regions during proestrus and diestrus. Two-way ANOVA detected a significant difference in region (HT vs Sp) ($F_{1,12}$=141.1, p<0.0001, n=4 rats in each group) as well as in cycle stage (P vs D) ($F_{1,12}$=5.43, p=0.038, n=4 rats in each group), but no interaction ($F_{1,12}$=0.17, p=0.69, n=4 rats in each group) (Figure 2).

The finding that significantly more pTyr-aromatase is present in hypothalamus than spinal cord (≈1.7-fold) during both estrous cycle stages could perhaps explain the disproportionate relative abundance of aromatase protein and catalytic activity between hypothalamus and spinal cord we previously reported [12]. Importantly, the molecular mass of the anti-pTyr

Western signal corresponded to the molecular mass of IPed aromatase detected using a pan anti-aromatase antibody. Moreover, only a single protein band was detected when spinal and hypothalamic aromatase was IPed under reducing conditions (utilizing anti-aromatase antibody that had been cross-linked to Protein G agarose beads), subjected to SDS-PAGE, and then stained with Colloidal Gold. Notably, this band was in the vicinity of the molecular mass corresponding to aromatase (~53 kDa).

3.2 Spinal and hypothalamic aromatase associate with c-Src, but not PTP1B

Given the correlation of aromatase activity with its Tyr phosphorylation, we hypothesized a physical association of aromatase with c-Src, which has been reported to Tyr phosphorylate aromatase. We utilized co-IP followed by Western analysis, as previously employed to assess association of aromatase with other signaling molecules [11]. As hypothesized, we found that c-Src co-IPed with aromatase (Figure 3). Interestingly, the phosphatase PTP1B, which has been implicated in dephosphorylating aromatase (deactivating it) in breast carcinoma [16], did not co-IP with aromatase in either the hypothalamus or spinal cord (data not shown). This could indicate that different cells differentially utilize varied phosphatases to regulate aromatase activity. Alternatively, we cannot discount the possibility that PTP1B might associate with aromatase such that the antigenic determinants recognized by the precipitating anti-aromatase antibody are not accessible.

3.3 Spinal and hypothalamic aromatase associate with PP2A and PP5

In contrast to anti-pTyr antibody, we were not able to validate the specificity of any of the five commercially available anti-phosphoSer (pSer) antibodies we tested (i.e., definitive recognition by Western blot analysis of a peptide containing pSer, but not its non-phosphorylated congener, as utilized in [11]). Therefore, instead of directly quantifying aggregate aromatase phosphorylated at Ser (pSer-aromatase), as was done for pTyr-aromatase, we investigated the oligomerization of aromatase with two Ser/Thr protein phosphatases – protein phosphatase 2 (PP2A) and 5 (PP5) – using co-IP and Western analysis as previously employed [11]. We found that aromatase associated with both phosphatases (Figure 3). The physical association of PP2A and PP5 with aromatase suggests that they are well positioned to regulate aromatase activity by de-phosphorylation of its Ser residues.

3.4 Associations of c-Src, PP2A, and PP5 with aromatase over the estrus cycle in hypothalamus and spinal cord

In order to explore if differential phosphorylation of hypothalamic aromatase could explain its increased activity during proestrus vs. diestrus, we investigated the physical association of aromatase with c-Src, PP2A, and PP5, individually, over the estrous cycle (Figure 4, top panels). Additionally, we evaluated if these associations varied between hypothalamus and spinal cord (Figure 4, bottom panels). Three separate 2-way ANOVAs were employed to analyze the variations in the co-IP of each of the three proteins, across the estrous cycle and between CNS regions with the following results: **PP2A** co-IP was significantly different between the CNS regions ($F_{1,16}$=475.9, $p<0.0001$), but neither cycle stages ($F_{1,16}$=0.06, p=0.81) nor interaction ($F_{1,16}$=0.03, p=0.86). **PP5** co-IP was significantly different between

the CNS regions ($F_{1,16}$=206.2, p<0.0001), but neither cycle stages ($F_{1,16}$=0.004, p=0.95) nor interaction ($F_{1,16}$=0.51, p=0.49). **c-Src** co-IP was significantly different between the CNS regions ($F_{1,16}$=180.5, p<0.0001), but neither cycle stages ($F_{1,16}$=0.04, p=0.84) nor interaction ($F_{1,16}$=0.04, p=0.85).

Overall, no difference was detected between the amount of c-Src, PP2A, or PP5 that co-IPed with aromatase, individually, from hypothalami of proestrous and diestrous rats. As was observed for hypothalamus, there was no difference between the amount of c-Src, PP2A, or PP5 that co-IPed, individually, with aromatase from spinal cords of proestrous and diestrous rats (Figure 4, top panels). However, substantially more c-Src, PP2A and PP5 associate with aromatase in hypothalamus compared to the spinal cord, regardless of the estrous cycle stage. On average, 52 times (mean=51.8, SEM=3.97) more c-Src co-IPed with hypothalamic aromatase than spinal aromatase. Furthermore, nearly seven times (mean=6.9, SEM=0.04) more PP2A and approximately five times (mean=5.4, SEM=0.44) more PP5 co-IPed with hypothalamic aromatase than spinal aromatase. Importantly, the ~4x higher content of aromatase in spinal cord vs. hypothalamus [12] did not confound data interpretation since co-IP of c-Src, PP2A, and PP5 was normalized with respect to directly IPed aromatase prior to their comparison between spinal cord and hypothalamus (Figure 4, bottom panels). Normalization with directly IPed aromatase especially highlighted the dramatically more c-Src, PP2A and PP5 that associated with aromatase in hypothalamus than in spinal cord. This observation could potentially underlie the differences in aromatase activity observed between the two CNS regions [12] as a consequence of Tyr phosphorylation and Ser dephosphorylation of aromatase.

3.5 Levels of hypothalamic and spinal PP2A, PP5, and c-Src in plasma membranes

It is possible that the increased association of hypothalamic aromatase with PP2A, PP5, and c-Src was only due to their higher content in hypothalamic membranes compared to membranes obtained from the spinal cord. To eliminate this possibility, we quantified the content of membrane-bound PP2A, PP5, and c-Src in both CNS regions. Membrane levels of PP2A, PP5, and c-Src did not significantly vary between proestrus and diestrus in either CNS region (data not shown). Since no differences were observed as a function of diestrus or proestrus (hypothalamus: t(4)=0.56, −0.30, 1.52, p>0.05, n=3 for PP2A, PP5, and c-Src, respectively; spinal cord, t(4)=1.15, −1.44, 0.67, p>0.05, n=3 for PP2A, PP5, and c-Src, respectively), values were collapsed across diestrus and proestrus for comparison between spinal cord and hypothalamus.

The content of PP2A, PP5, and c-Src was significantly higher in the membrane fraction obtained from the hypothalamus compared to the spinal cord (500±32% for c-Src; 32±8% for PP2A, 45±8% for PP5). However this increment was significantly lower than the observed increase in their association (co-IP) with aromatase in hypothalamus vs. spinal cord from the same cellular fraction, t(9.7)=13.38 for c=Src; t(9.6)=8.78 for PP2A; and t(9.1)=11.74 for PP5, for comparison of the ratio of their co-IPs (n=10) with the ratio of their membrane content (n=6), p<0.001 for all (Figure 5, first panel). This indicates that the magnitude of the increased expression of c-Src, PP2A and PP5 in the membrane compartment of hypothalamus compared to the spinal cord is not sufficient to account for

all of their detected increased association (co-IP) with hypothalamic vs. spinal aromatase, i.e., the differential co-IP of PP2A, PP5, and c-Src with aromatase remained statistically significant even after correcting for differences in membrane content (c-Src: $t(9)=11.78$; PP2A: $t(9)=13.63$; PP5: $t(9)=8.92$, $n=10$ independent determinations; $p<0.001$ for all). Compared to the spinal cord, the relatively higher hypothalamic content of aromatase-associated PP2A, PP5, and c-Src could be responsible, at least in part, for the differences in aromatase activity between the two CNS regions we previously reported in [12] as a consequence of Tyr phosphorylation and Ser dephosphorylation of aromatase.

3.6 A multimeric membrane signaling unit comprised of aromatase, ERα, c-Src and PP2A is present in the CNS

The association (co-IP) of aromatase with c-Src, PP2A, and PP5 indicates that aromatase physically interacts with each, but not necessarily that all are concomitantly present in the same multimeric complex. It is possible that different molecules of aromatase could selectively interact with one or the other individually. To investigate if aromatase, c-Src, PP2A, and PP5 constituted a potential signaling complex, we investigated their presence in a common oligomer with mERα. This was pursued using sequential IP utilizing (in order) antibodies against ERα, c-Src, and PP2A. The final IP was Western blotted using either anti-aromatase or anti-PP5 antibody. A complex containing aromatase together with mERα, c-Src, and PP2A was found in both spinal cord and hypothalamic crude membrane preparation (Figure 6). Since PP5 was found to co-IP with either aromatase, PP2A, or mERα, but not c-Src (data not shown), it was not surprising that we were not able to detect PP5 in the Western blot following ERα, c-Src, and PP2A sequential IP. This suggests that although PP5 might associate with aromatase and mERα within an alternative regulatory complex, PP5 does not appear to function within the complex comprised of mERα, c-Src, PP2A and aromatase.

4 DISCUSSION

This study investigated potential mechanisms of regulation of CNS aromatase activity. We focused on the differences between rat spinal cord and hypothalamus, and between hypothalamus during diestrus and proestrus, since comparisons among them revealed that relative levels of aromatase expression did not predict corresponding enzymatic activity, making them ideal for testing the postulated importance of aromatase regulation by phosphorylation/dephosphorylation. Guided by past reports [17, 20], we focused on Tyr and Ser phosphorylation of aromatase.

The overall findings were: (a) hypothalamic aromatase was more heavily Tyr-phosphorylated than spinal aromatase, providing a basis for the relatively higher aromatase catalytic activity in hypothalamus than spinal cord [12]; (b) aromatase was not only oligomerized with c-Src, PP2A, and PP5 in both the hypothalamus and spinal cord, but (c) their associations with hypothalamic aromatase was substantially greater than with spinal aromatase, suggesting that aromatase is activated potentially via Tyr phosphorylation as well as Ser dephosphorylation, and (d) aromatase is present in a multimeric complex containing

mERα, PP2A, and c-Src in both hypothalamus and spinal cord, suggesting that estrogen production could be acutely regulated within a multimeric complex in which it also signals.

The relevance of phosphorylation to aromatase activity has been demonstrated, albeit indirectly, in the CNS of birds (quail) [14, 23] and directly in human breast cancer cells [17]. Interestingly, phosphorylation of aromatase has opposite effects on CNS aromatase activity in birds vs. breast cancer cells. While these differences could be explained by differential phosphorylation sites, they also suggest caution when extrapolating from the CNS of birds to mammals[24]. This, along with the current demonstration that the magnitude of aromatase Tyr phosphorylation parallels the previously reported aromatase catalytic activity [12], constitutes a potential indication that acute regulation of aromatase activity could also occur in mammalian CNS. Furthermore, these earlier reports notwithstanding, current findings are novel, revealing that aromatase phosphorylation occurs in the mammalian CNS in at least spinal cord and hypothalamus, which could correlate with the previously detected discrepancy in aromatase activity [12].

Since the specific sites of phosphorylation/dephosphorylation relevant to modulating rat CNS aromatase activity have not been established (aromatase has not yet been successfully subjected to mass spectrophotometric analysis, precluding identification of phosphorylation sites), we investigated the overall Tyr phosphorylation state of rat aromatase in spinal cord and hypothalamus. Hypothesizing that c-Src Tyr phosphorylates aromatase, we investigated if levels of pTyr-aromatase and aromatase association with c-Src would potentially influence aromatase activity. Additionally, based on reports that aromatase is also activated by Ser dephosphorylation [17, 20], we tested the hypothesis that the association of the Ser/Thr phosphatases PP2A and PP5 with aromatase would be inversely related to aromatase activity. [We did not directly quantify pSer-aromatase (as we did for pTyr-aromatase) given our inability to authenticate claimed specificity of commercially available anti-pSer antibodies.]

Both hypotheses were validated. The pTyr Western signal corresponding to hypothalamic aromatase was nearly double that of spinal aromatase. Additionally, the pTyr Western signal in proestrus was significantly higher than that of diestrus. It is worth noting that physiological consequences of the admittedly small (10%) increment in aromatase phosphorylation and even the 40% increase in hypothalamic aromatase activity, could be expected to be considerably greater, not just because it impacts catalytic activity, but also because it is envisioned to occur within subcellular microenvironments, which would amplify effects of increased estrogen production by concentrating it. Additionally, when operating outside of the region of first order kinetics, small percentage changes in the concentration/activity of either of the controlling modifying enzymes can give much larger percentage changes in the amount/activity of modified protein. At least a portion of these differences could be generated by the kinase binding to the fully phosphorylated form of the substrate (e.g., aromatase at its docking site) and sequestering it away from phosphatase(s), prolonging the enhancing effect of phosphorylation on enzyme activity. All of these considerations are consistent with a non-linear relationship between phosphorylation and aromatase activity that we are postulating.

Levels of pTyr-aromatase paralleled aromatase activity, underscoring the likely involvement of a tyrosine kinase in regulating aromatase activity. Indeed, c-Src not only co-IPed with aromatase, but the magnitude of co-IPed c-Src with hypothalamic aromatase was nearly 50-fold greater than with spinal aromatase, consistent with the levels of hypothalamic aromatase activity compared to spinal aromatase activity. Interestingly, c-Src co-IP with hypothalamic aromatase did not vary over the estrous cycle, notwithstanding that aromatase activity and tyrosine phosphorylation was greater during proestrus than diestrus. However, it should be noted that c-Src is itself regulated by phosphorylation. Thus, extrapolating changes in associated c-Src kinase activity from the increment or lack thereof in c-Src co-IP with aromatase is likely to be dubious.

Levels of total c-Src protein were approximately 5X greater in hypothalamic than spinal membranes. However, this difference is not sufficient to explain the totality of the disparity in the co-IP of c-Src with hypothalamic vs. spinal aromatase since, even after correcting for differences in c-Src membrane content, the increment in aromatase-c-Src association in hypothalamus vs. spinal cord remained highly significant. This finding suggests that the membrane content of interacting proteins is unlikely to be the sole determinant of the organization of aromatase with other signaling molecules. The specific factors in the local membrane environment causally associated with enhanced interaction of c-Src with aromatase remain unknown.

Since activation of aromatase by Ser dephosphorylation would require Ser phosphatase(s) to be in close physical proximity (ensuring rapid aromatase activation and deactivation), we investigated oligomerization of aromatase with PP2A and PP5. These were selected since estrogens increase PP5 expression [25] and PP2A catalytic activity [26], and both phosphatases physically interact with ERα [26, 27], which oligomerizes with aromatase [11], potentially putting PP2A and PP5 in sufficient proximity with aromatase to regulate its Ser phosphorylation. Indeed, PP2A and PP5 were found to co-IP with spinal and hypothalamic aromatase, underscoring their potential relevance to acute modulation of aromatase activity *in vivo*. The hypothesized relevance of the above Ser phosphatases to regulating aromatase activity was further emphasized by the fact that the magnitude of PP2A and PP5 that co-IPed with hypothalamic aromatase was approximately 5-7-fold greater than that which co-IPed with spinal aromatase, a difference, which, like c-Src co-IP could not be completely explained by the greater content of PP2A and PP5 in hypothalamic vs. spinal membranes. While our interpretation that increased association of hypothalamic aromatase with c-Src, PP2A, and PP5 is a major contributor to the higher aromatase activity in the hypothalamus than spinal cord is inferential, there is undeniably a striking parallel between aromatase regional activity and the magnitude of these associations (along with the known ability of aromatase phosphorylation/dephosphorylation to influence its catalytic activity).

Interestingly, the difference in hypothalamic aromatase activity during diestrus and proestrus was not accompanied by changes in the co-IP of PP2A and PP5 with aromatase. Notably, analogous to c-Src, PP2A activity is itself regulated by phosphorylation, which could be dependent on stage of estrus cycle. Thus, Ser phosphatase activity cannot be inferred from the magnitude of the co-IP of PP2A and PP5 with aromatase.

To test whether aromatase exists within a multimeric complex that not only mediates estrogenic signaling, but also regulates estrogen production, we performed sequential IP of ERα, c-Src, and PP2A, followed by Western analysis for either aromatase or PP5. This revealed the existence of a multimeric complex containing ERα, c-Src, PP2A, and aromatase. One could envision activation of aromatase within this complex via its Ser118 dephosphorylation (by PP2A) and/or Tyr phosphorylation, catalyzed by c-Src. Estrogenic signaling within this complex could be further amplified via c-Src phosphorylation of Tyr537ERα, enhancing ERα binding of estrogens [28]. The amplified estrogenic signaling could be reduced/terminated by PP2A dephosphorylation of Ser118mERα, as well as the dephosphorylation of pTyr-aromatase via an as-of-yet unidentified Tyr phosphatase (we were unable to detect PTP1B in aromatase IP).

It is interesting to note that this multimeric complex was detected in both the spinal cord and the hypothalamus. In our previous study [12], we reported that nearly all spinal cord aromatase was found to be associated with mERα, while ~15% of hypothalamic aromatase was associated with mERα, a distribution that likely speaks to the inherent differences in utilization of estrogens by the two regions. While it would be interesting to speculate on the relevance of the herein described multimeric signaling complex to the functional utilization of estrogens by the hypothalamus and the spinal cord, experimental limitations (e.g., pooling of hypothalamic tissue, inability to evaluate aromatase activity directly in the immunoprecipitated complexes) preclude us from direct comparison between the two regions.

Another level of complexity to consider (but not pursued here) is the variability in expression of mERα between spinal cord and hypothalamus, in addition to determining the fraction of mERα that partakes in the described complex. While the relative abundance of the multimeric complexes we were able to detect in the current study are likely to make up a minute portion of the cellular ERs, and even mERs, the mere existence and presumed functionality of the described multimeric complex is likely to result in significant perturbations of cellular microenvironments via tightly regulated and temporally controlled estrogenic signaling.

The wide spectrum of CNS functional modalities that is influenced by CNS estrogenic signaling (e.g., mood, learning and memory, pain) is enabled by a plethora of estrogenic signaling modalities that function on systemic, synaptic, cellular, and molecular organizational levels [29, 30]. The current study adds a new dimension to CNS estrogenic signaling. The presence of c-Src and PP2A, which are capable of Tyr-phosphorylating and Ser-dephosphorylating aromatase, respectively, in a multimeric membrane signaling complex, which also contains aromatase and ERα (and presumably other signaling molecules), strongly suggests the presence of a potentially self-regulating estrogenic signaling unit in the CNS. Ascertaining the degree to which such complexes function autonomously and the means by which they are regulated is likely to have substantial clinical implications.

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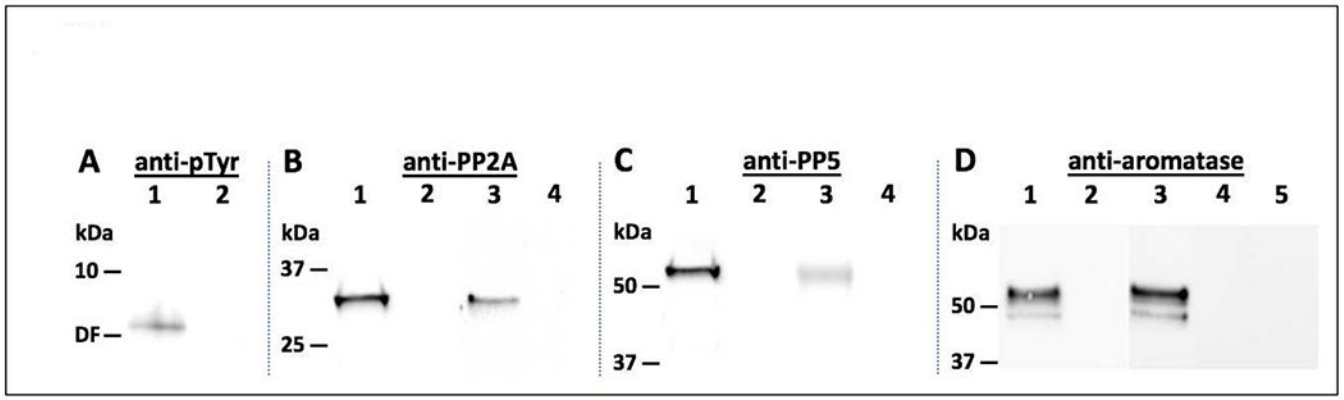

Figure 1.

Antibody specificity. A. The specificity of the anti-pTyr antibody was authenticated by demonstrating its ability to recognize a peptide containing a pTyr (GWB-DC5119, GenWay Biotech), Lane 1, but not a peptide containing two Tyr, neither of which was phosphorylated (sc-542P, Santa Cruz Biotechnology), Lane 2. DF = dye front. B. Specificity of the anti-PP2A antibody was authenticated by demonstrating the absence of detected signal in crude membrane fraction (lane 1 vs. 2) and aromatase IP (lane 3 vs. 4) when Western blotting using untreated or preadsorbed antibody flow-through, respectively. C. Specificity of the anti-PP5 antibody was authenticated by demonstrating the absence of detected signal in crude membrane fraction (lane 1 vs. 2) and aromatase IP (lane 3 vs. 4) when Western blotting with untreated or preadsorbed antibody flow-through, respectively. D. Specificity of the anti-aromatase antibody was authenticated by demonstrating the absence of detected signal in crude membrane fraction (lane 1 vs. 2) and aromatase IP (lane 3 vs. 4) when Western blotting with untreated or preadsorbed aromatase antibody flow-through, respectively. Lane 5 further verified the specificity of anti-aromatase antibody by demonstrating the absence of detected signal following IP with preadsorbed aromatase antibody, with the Western blot probed with anti-aromatase antibody.

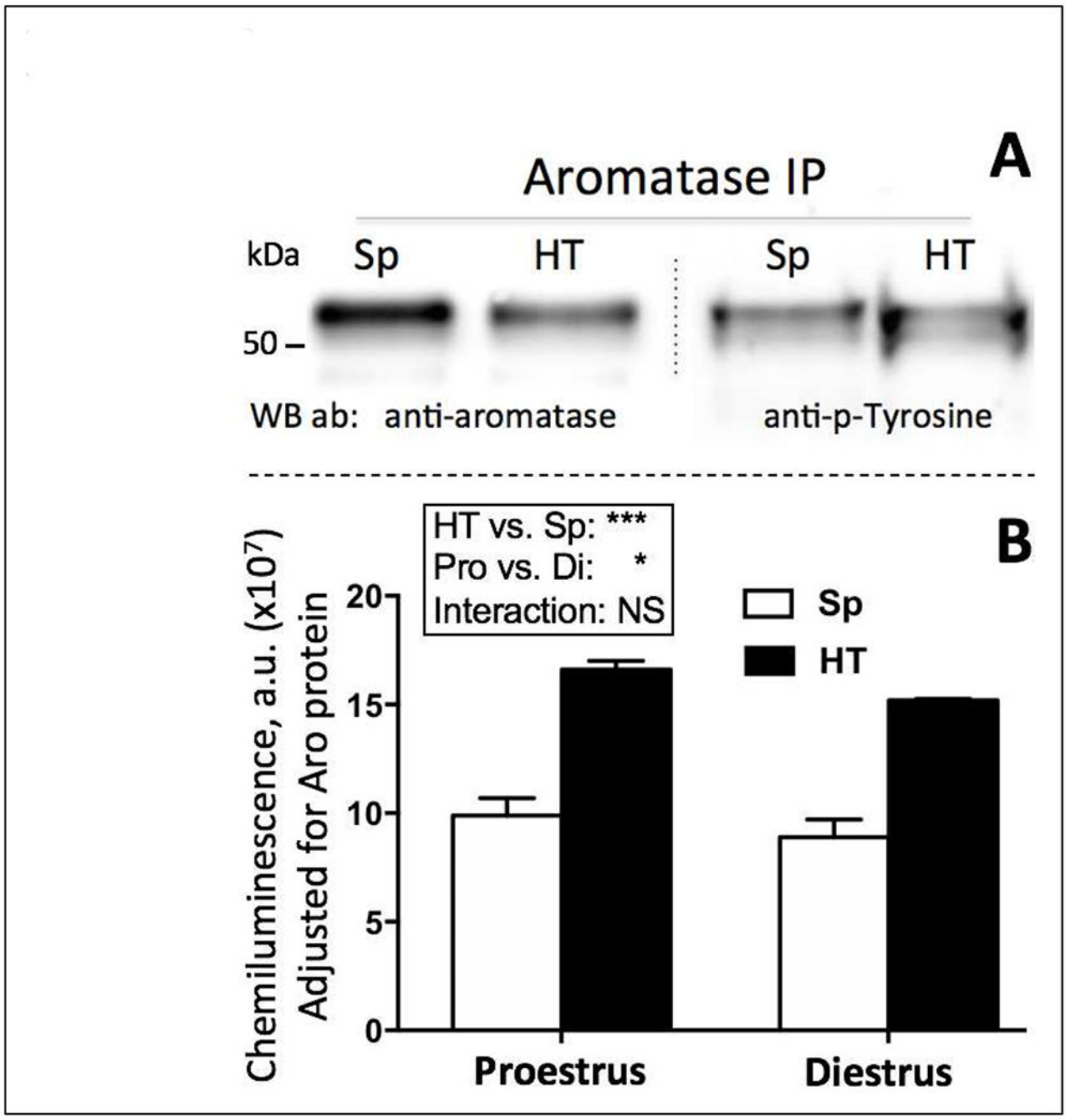

Figure 2.

Levels of pTyr-aromatase are significantly higher in hypothalamic than spinal membranes, independent of stage of estrous cycle. **A.** Aromatase IP was obtained from hypothalamus and spinal cord under reducing conditions. The IP was Western blotted in adjacent lanes using anti-aromatase antibody or anti-pTyr antibody. n=4. Attribution of the pTyr Western signal to aromatase was validated by demonstrating the absence of Western signal when immunoprecipitating with preadsorbed anti-aromatase antibody flow-through (data not shown). **B.** Average chemiluminescence (+SEM) of hypothalamic and spinal pTyr aromatase

obtained from the same proestrous or diestrous animals, adjusted for total aromatase (Aro) that was IPed. n=4 for proestrous and diestrous groups. ***, $p<0.0001$ for spinal cord vs. hypothalamus. *, $p<0.05$ for proestrus (Pro) vs. diestrus (Di). Levels of pTyr-aromatase paralleled the aromatase activity that we recently reported [12] (see Fig. 5, adapted from [12]), being significantly higher in hypothalamus than spinal cord.

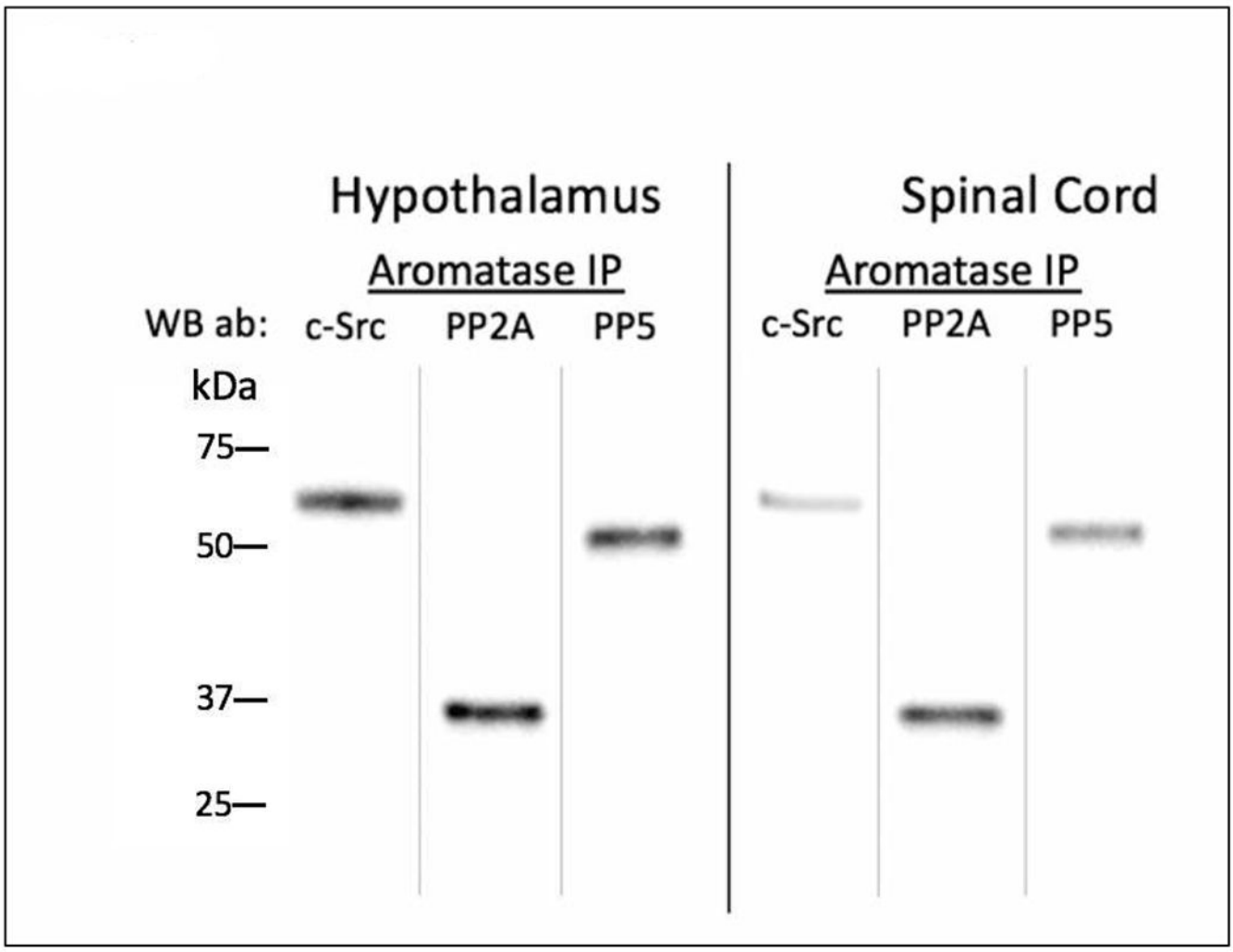

Figure 3.

c-Src, PP2A, and PP5 associated with hypothalamic and spinal aromatase. Immunoprecipitates obtained in parallel from hypothalamus and spinal cord of female rats (mixed cycle stages) using anti-aromatase antibody were Western blotted using antibodies specific for c-Src, PP2A, or PP5. All were found to be present in aromatase immunoprecipitate from both CNS regions, in the expected molecular weight ranges (c-Src ~60 kDa, PP2A ~36 kDa, or PP5 ~55 kDa). n=4.

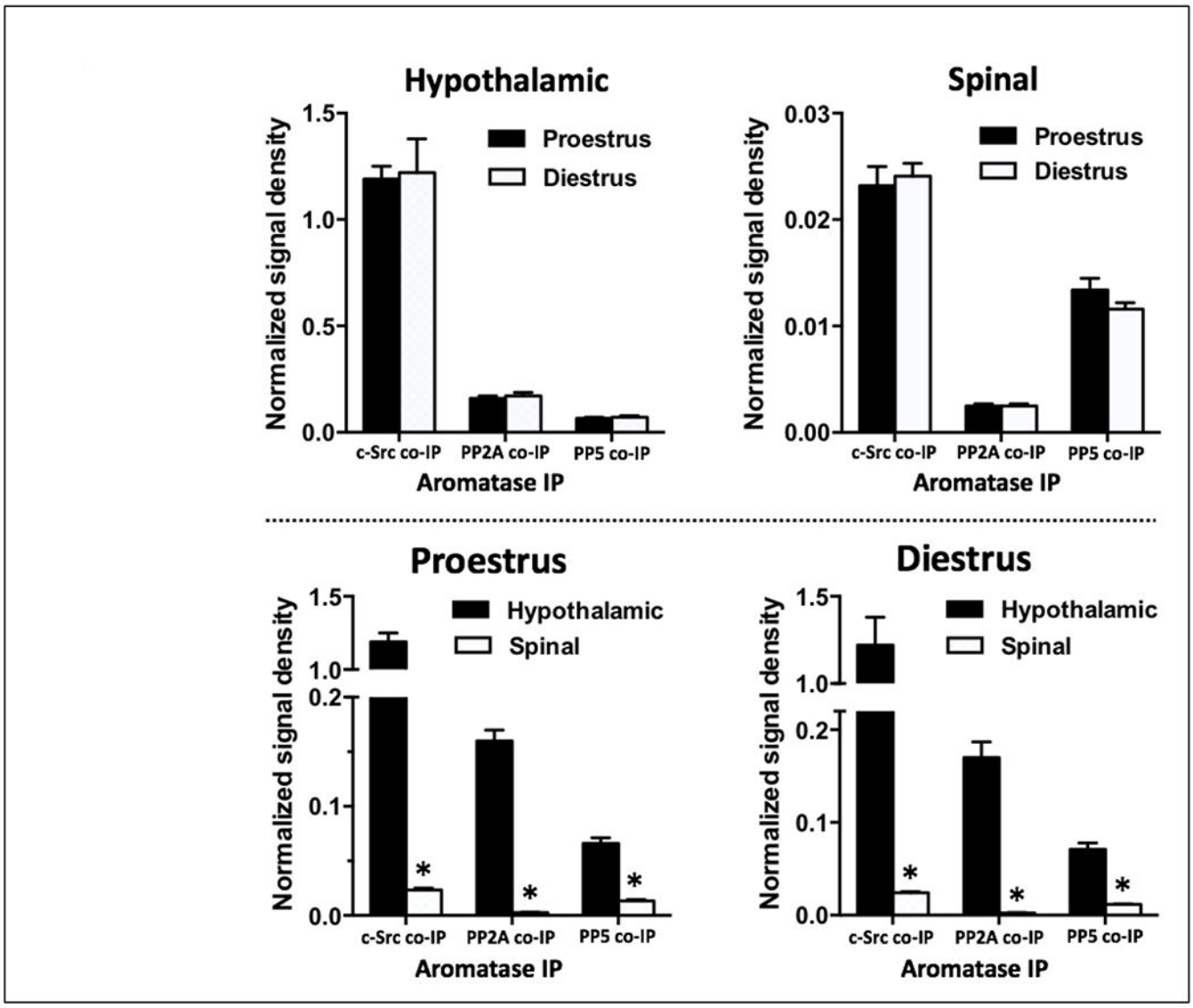

Figure 4.

Association of c-Src, PP2A, and PP5 with hypothalamic aromatase is significantly higher than with spinal aromatase, irrespective of the estrous cycle stage. Figure shows quantification (mean chemiluminescence + SEM) of c-Src, PP2A, and PP5 co-IP with aromatase from hypothalami and spinal cord in proestrous and diestrous rats. Data arranged by CNS region, comparing c-Src, PP2A, and PP5 co-IP across cycle stages in top panels; data arranged by cycle stage, comparing c-Src, PP2A, and PP5 co-IP between hypothalamus and spinal cord in bottom panels. No difference in signal was observed between proestrous and diestrous stages in the co-IP of either c-Src, PP2A, or PP5 with aromatase from either CNS region ($p>0.05$ for all). However, a significant difference was detected in the co-IP of c-Src, PP2A as well as PP5 with aromatase in hypothalamus vs. spinal cord during both cycle stages ($p<0.0001$ for all). n=5 for each estrous cycle stage.

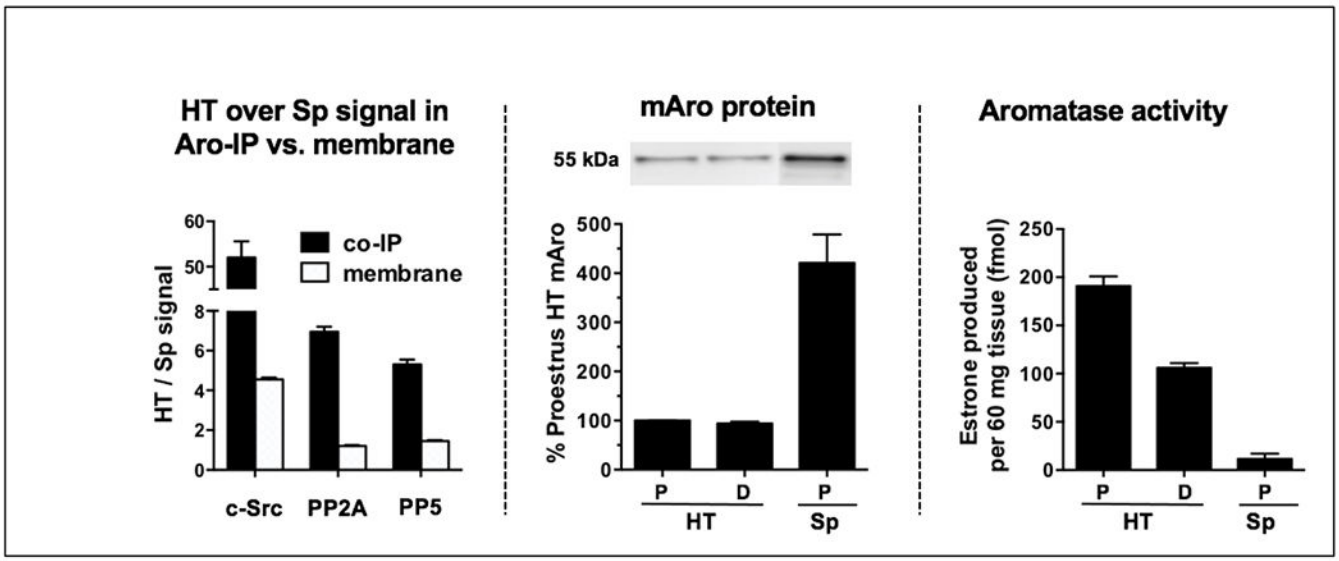

Figure 5.

Substantially more c-Src, PP2A, and PP5 co-IPed with hypothalamic aromatase than spinal aromatase. Left panel shows mean ratio (+ SEM) of hypothalamic to spinal Western signal corresponding to the aromatase co-IP of c-Src, PP2A, and PP5 (black bars) and the ratio of c-Src, PP2A, and PP5 signal in the membrane fraction (i.e., without co-IP) (white bars). n=5 for each group. Aromatase was associated with about 50x more c-Src, 7x more PP2A, and 5x more PP5 in the hypothalamus than spinal cord. The difference in co-IP is disproportionally higher than the difference in the relative abundance of c-Src, PP2A, and PP5 found in the membrane fractions from hypothalamus compared to the spinal cord: 4x more c-Src protein is present in the hypothalamic than spinal membrane fraction, and about 1.5x more PP2A or PP5 in the hypothalamic than spinal cord membrane fraction. The second and third panels re-demonstrate selected data we recently reported [12] related to current work. The second panel shows 4x more aromatase (mAro) protein detected in spinal cord than hypothalami. The third panel shows that (1) there is more aromatase activity during proestrus (P) compared to diestrus (D); as well as (2) there is minimal aromatase activity in the spinal cord compared to the hypothalamus. These differences in activity are in stark contrast to the amount of aromatase protein present, even when correcting for differences in their respective expression levels. Sp=spinal cord; HT=hypothalamus.

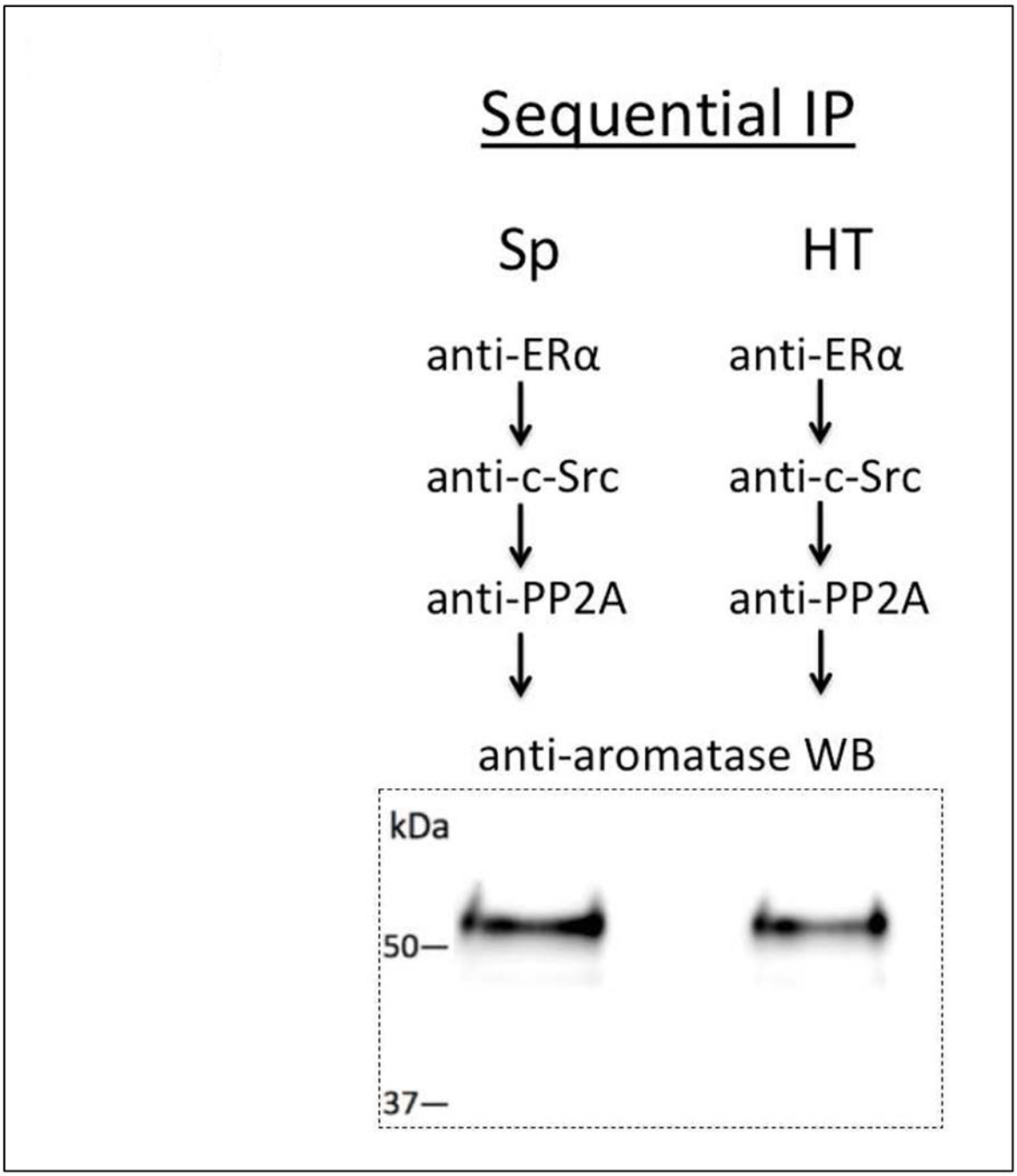

Figure 6.

Aromatase, mERα, c-Src and PP2A are present in a common oligomer in hypothalamus and spinal cord. Sequential IP from hypothalamus and spinal cord was performed using the following antibodies in order: anti-mERα, anti-c-Src, anti-PP2A. The resulting immunoprecipitate was eluted, resolved on SDS-PAGE, and Western blotted for aromatase. n=3 independent determinations. Intensity of the bands cannot be directly compared as

pooling of hypothalamic tissues from 3 rats was required for adequate amounts of starting material. Sp=spinal cord; HT=hypothalamus