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Regulation of progestin receptors in medial amygdala: Estradiol, phytoestrogens and sex

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ABSTRACT

Exposure to estrogens during critical developmental periods and in adulthood affects sex differences in the brain. We examined the roles of estradiol (E2) and phytoestrogens, and their interactions, on potential sex differences in brain. We used aromatase knockout (ArKO) mice, which cannot produce endogenous estrogens, along with wild type (WT) littermates. Mice were gestated, raised and maintained on a diet either rich in phytoestrogens or a diet virtually void of soy-derived phytoestrogens. Adult males and females were gonadectomized and received implants filled with 17-β-estradiol to induce progestin receptors (PR), while controls received empty implants. Mice were sacrificed five days later and brain sections containing the posterodorsal medial amygdala (MePD) were processed for PR immunoreactivity. Activation of sex differences in PR required adult E2 treatment. A diet high in phytoestrogens was required for expression of sex differences in PR after E2 treatment. Our data underscore the important contribution of dietary phytoestrogens for the development of sex differences in PR-ir in the adult mouse medial amygdala. We hypothesize that both aromatization of androgens to estrogens and dietary sources of additional estrogens are part of the normal requirement for sex differences in the rodent brain.

Endogenous estrogens act during development and in adults to activate expression of reproductive and other social behaviors [1–3]. In contrast, phytoestrogens are biologically active non-steroidal molecules found primarily in soy products, legumes and whole-grains, they have been show to modulate aspects of reproduction including behavior [4–8]. Phytoestrogens act via estrogen receptors (ERα and β) and thus activate many of the same neural circuits activated by endogenous estrogens [4,5,9]. In recent years, increasing numbers of experiments have examined neuroendocrine effects of phytoestrogen intake as high concentrations of these compounds are increasingly being used as supplements in many consumer products [10,11]. In addition, standard soy-derived commercial laboratory rodent diets contain high concentrations of isoflavones [12]. Therefore, in addition to endogenously produced estrogens, phytoestrogens are present in both human and animal diets during development and adulthood and need to be considered in studies of hormone actions.

To study phytoestrogens in an animal model that should be exquisitely sensitive to estrogens, we utilized the aromatase knockout (ArKO) mouse [13]. The mutants carry targeted disruptions in both the transcriptional and translational sites of the *Cyp19* gene, do not produce aromatase cytochrome *P450* and are unable to convert testosterone to estradiol. The ArKO mouse does, however, expresses the classic estrogen receptors (ERα, ERβ) and responds to exogenous estrogen administration [13]. Subtle disruptions to sperm production are noted in the ArKO testes and can be rescued by a diet high in phytoestrogens [7]. In addition ArKO females treated with hormones to prime lordosis in adulthood show lower levels of behavior than wild type littermates, but only when they are raised on a phytoestrogen rich diet [14]. To separate effects of phytoestrogens and endogenous estrogens, we selected two laboratory chows. One has been shown to result in plasma phytoestrogen levels in rodents equivalent to those seen in human populations that consume large amounts of soy (phyto-rich) the other diet contains little soy (phyto-free) [12,15].

Here we test the hypothesis that endogenous estrogens and phytoestrogens have synergistic actions on estrogen-induced progestin receptor in the mouse brain. We focused our analysis on the medial posterodorsal amygdala (MePD) for three reasons. First, this region contains PR, ERα, and ERβ [16,17]. Second, the MePD volume is sexually dimorphic and highly plastic [18–20] and lastly, this region is important for a number of hormone-dependent social behaviors in

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several rodent species [19,21,22]. Our results show that the ability of E2 to induce PR immunoreactivity in this region is influenced by sex and E2 exposure prior to adulthood, however, both factors interact with dietary phytoestrogens.

1. Materials and methods

1.1. Animals and treatments

Mice were produced using heterozygous breeding pairs. Each member of the pair had one normal and one disrupted *Cyp19* gene [13]. At the time of these studies mice had been backcrossed for at least four generations with C57BL/6J mice. Mice were housed in a 12:12 light:dark cycle and given food and water *ad libitum*. One group of animals (and their dams) was fed a phytoestrogen-free diet (phyto-free; Harlan Teklad Global Diet 2014) that does not contain alfalfa or soybean meal, sources of coumestrol and isoflavones, respectively. The second group of animals was raised on a diet containing 600 μg of total dietary phytoestrogens/g of food (phyto-rich; Harlan Teklad Global Diet 8604; [15]. By monitoring food intake in a separate group of WT animals, we found that on average males ($n=9$) consumed 3.43 ± 0.03 g food/day. When isoflavone consumption was calculated for animals eating the phyto-rich diet, using the range supplied by the manufacturer (427–565 μg/g food), intake ranged from 1.465–1.938 mg isoflavone/day. Using the range provided for the phyto-free diet (5–15.5 μg/g food) isoflavone intake was calculated at 0.0171–0.0532 mg isoflavone/day.

Mice were genotype by PCR amplification of tail DNA [14]. Adult mice (age 45–90 days of age) were used in this study. Each mouse was gonadectomized one week prior to receiving hormone treatment. Mice received Silastic implants (1.98 I.D.×3.17 mm O.D.) containing either 50 μg 17-β-estradiol dissolved in 25 μl sesame oil, or empty implants. Implants were positioned subcutaneously in the midscapular region and animals were sacrificed five days after implantation. The following 16 groups were formed. WT mice on phyto-free diet with blank implants ($n=8$ males, $n=8$ females), WT mice on phyto-rich diet with blank implants ($n=11$ males, $n=7$ females), WT mice on phyto-free diet with E2 implants ($n=9$ males, $n=9$ females), WT mice on phyto-rich diet with E2 implants ($n=9$ males, $n=7$ females), ArKO mice on phyto-free diet with blank implants ($n=9$ males, $n=6$ females), ArKO mice on phyto-rich diet with blank implants ($n=10$ males, $n=9$ females), ArKO mice on phyto-free diet with E2 implants ($n=11$ males, $n=8$ females), and ArKO mice on phyto-rich diet with E2 implants ($n=12$ males, $n=11$ females).

At the time of sacrifice, mice were deeply anesthetized with an overdose of sodium pentobarbital and brains were rapidly removed and fixed via immersion in 5% acrolein [23]. Following overnight immersion in 0.1 M phosphate buffer containing 30% sucrose, brains were frozen and serial coronal sections (30 μm) collected through the forebrain and stored in cryoprotectant at −20 °C. Consecutive sections were divided into four vials, one vial (1/4 of the brain) was processed for immunocytochemical analysis of PR.

1.2. Immunocytochemistry

Sections were removed from cryoprotectant and rinsed in 0.2 M Tris Buffered Saline (TBS, 5×10 min) prior to a 30 minute incubation in $NaBH_4$ and a 10 minute incubation in 0.3% H_2O_2, with 3, 10 minute rinses in between. Tissue was incubated at 4 °C for 48 h in a primary antibody directed against the hinge region of the progestin receptor (H-928, 0.2 mg/ml; StressGen Biotechnologies Carp., Victoria, British Columbia, Canada). Next, brain tissue was incubated in biotinylated horse anti-mouse secondary antibody (Vector Laboratories, 1:500). Following additional rinses and a one-hour incubation in an avidin–biotin complex (Vector, 1:1000), tissue was stained using a nickel intensified diaminobenzidine (DAB) solution (0.25% nickel ammonium sulfate and 0.05% DAB) activated by 0.001% hydrogen peroxide. Brain sections from each group were included in every run to control for intra-run variability. All incubation times were held constant between runs.

1.3. Image analysis and statistics

Immunoreactivity was quantified in the best matched, unilateral section from each brain using Metamorph Image Analysis (Universal Imaging West Chester, PA). As illustrated previously [24], the area quantified was the posterodorsal medial amygdala (MePD, −1.82 mm; Fig 46 in [25]. The observer was "blind" to the group designations of the sections used for the analysis. Relationships among groups were assessed first with a four-way analysis of variance test (ANOVA) with genotype, hormone treatment, sex and diet as factors. Since it is well known that E2 is required to induce PR in the brain, and no differences between any other factors were noted in brains from control (blank implant) mice, we conducted further analyses on brains from mice exposed to E2 in adulthood. When main effects or interactions were noted, differences among groups were analyzed using Fisher's LSD Multiple-Comparison tests. Significance was reported at $p<0.05$ or less.

2. Results

Analysis of all four factors (implant, sex, food, and genotype) revealed a strong main effect of implant. Animals that received E2 for five days before brains were collected had significantly more PR-ir cells in the MePD than gonadectomized controls ($F(1,142)=173.01$, $p<0.00001$; Table 1 and Fig. 1). A trend was noted for a sex difference with males having more PR-ir than females ($F(1,142)=3.61$, $p<0.06$). No main effect of food or genotype were detected ($F(1,142)=3.19$, 0.22, respectively). Interactions between sex and food ($F(1,142)=3.98$, $p<0.05$) and genotype and food ($F(1,142)=4.78$, $p<0.03$) were detected. Males consuming the phyto-rich chow had more PR-ir cells than animals in any of the other groups and WT mice of both sexes had more PR-ir when they consumed phyto-rich versus phyto-free chow ($p<0.05$). No sex by genotype interaction was detected ($F(1,142)=1.68$).

To isolate the roles of genotype, sex and food we asked if these factors had any influence on baseline levels of PR-ir. A three-way analysis of these variables restricted to brains of control animals (no E2 implants) revealed no effects of sex or genotype ($F(1,65)=0.1, 1.33$ respectively). We noted a trend for an effect of diet ($F(1,65)=3.41$, $p<0.07$) with animals on the phyto-rich diet having more PR-ir cells than animals on the phyto-free chow. We did not find any interactions between sex and food, genotype and food or sex and genotype ($F(1,65)=1.68$, 0.02, 2.67 respectively).

In contrast, all the same significant effects that we noted in the full data set were present in the analysis of data from the E2 implanted mice only. This demonstrates that the effects of sex, food and genotype are enabled when E2 is given. A main effect of sex ($F(1,75)=4.87$, $p<0.03$) was due to more PR-ir cells in the MePD of males than females (Table 1 and Fig. 2). No main effects of genotype or food were found ($F(1,75)=0.31, 1.52$ respectively). An interaction between sex and food and another between genotype and food were observed

Table 1
Mean ± SEM PR-ir neurons (*N* per group) in the MePD of mouse brains.

Diet	Phytoestrogen rich diet		Phytoestrogen free diet	
Treatment	Blank	E2*	Blank	E2*
WT Males	37.9 ± 14 (11)	390.1 ± 58 (9)	10.4 ± 4 (8)	226.4 ± 28 (9)
WT Females	10.1 ± 3 (7)	253.6 ± 50 (7)	6.6 ± 3 (8)	173.7 ± 46 (9)
ArKO Males	31.6 ± 11 (10)	274.9 ± 44 (12)	9.0 ± 3 (8)	227.4 ± 42 (11)
ArKO Female	33.2 ± 17 (9)	168.4 ± 34 (11)	28.5 ± 10 (6)	270.9 ± 44 (8)

* Significant main effect of hormone treatment, E2 > Blank.

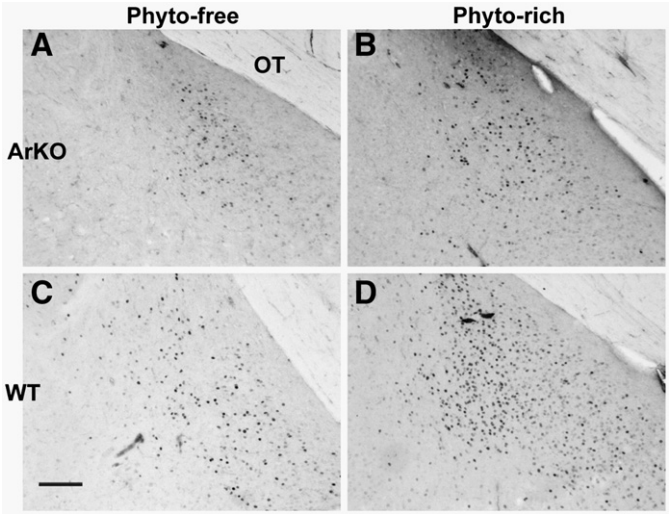

Fig. 1. Photomicrographs of PR-ir cells in the medial posterodorsal amygdala of aromatase knockout (ArKO) male mice (A, B) and WT male littermates (C, D). Males were castrated and one week later treated with estradiol for five days prior to sacrifice. Panels A and C show tissue from mice fed Phytoestrogen (Phyto)-free chow. Panels B and D illustrate tissue from males fed a Phyto-rich chow. Scale bar = 100 μm. OT = optic tract.

($F(1,75) = 4.25$, 4.35, respectively $p<0.045$). As described above, the sex by food interaction was due to males consuming the phyto-rich chow which had more PR-ir cells than animals in any of the other groups. The genotype by food interaction was caused by the significant difference between WT mice that consumed phyto-rich versus phyto-free chow ($p<0.05$). No interaction between sex and genotype was detected ($F(1,75) = 1.50$).

3. Discussion

Here we report a sex difference in PR-ir in the mouse MePD. As in many other regions, this dimorphism in the MePD depends on developmental and/or activational E2 [18,26–32]. In addition we now show that dietary phytoestrogens are involved. The sex difference in PR-ir is eliminated by either depriving males of phytoestrogens via a diet low in these compounds, or by disruption of the aromatase enzyme gene, *Cyp19*. Estradiol-regulated PR expression has previously been shown to be sexually dimorphic in several rodent species [32–34]. The direction of the sex difference varies with the age of the animal and the region of the brain examined. Neonatal male rats and mice express higher perinatal PR-ir cell numbers in the medial preoptic area (mPOA) than females [30,35]. This sex difference may be caused by higher circulating levels of testosterone in male as compared with female neonates [36]. The sex difference in mice is eliminated by disruption of the ERα gene [35]. In adults, E2-mediated PR induction is greater in females than males in the ventromedial nucleus (VMN) and the medial preoptic area (mPOA) [32,33]. This report is the first we know of to document a sex difference in the MePD in PR-ir cells in response to adult treatment with E2. However others have shown elegantly in several rodent species that the MePD is highly plastic during puberty and in adulthood; responding to changes in photoperiod and behavioral interactions [37–43].

Dietary phytoestrogens enhance the effects of E2 on PR protein induction; this effect was noted in WT males, but not in the ArKO males. Since E2 treated female mice in all genotype and diet groups showed statistically equivalent levels of PR induction, which were comparable to the ArKO males, we speculate that the ability to respond differentially to the phyto-rich versus phyto-free diet requires exposure to E2 some time prior to adulthood during development. It is at this time that ERβ activation can have defeminizing actions in brain [44]. The maximal PR induction response is only observed in adult WT males in which dietary phytoestrogens were available throughout life. This suggests that phytoestrogens cannot completely compensate for endogenous E2 produced by aromatization in the male brain and vice versa. Partial compensation is also noted in males. When E2 treatment is given to ArKO male mice fed the phyto-rich diet their PR-ir cell numbers are similar to WT males fed the phyto-free chow. This result suggests that phytoestrogens and E2 work synergistically to maximize plasticity in response to E2 in the adult male MePD. This finding is similar to a study done in the ArKO testes in which maintenance on a phytoestrogen rich diet was able to partially reverse infertility [7].

The MePD is an interesting area to examine. Recent studies demonstrate that it is highly plastic, sexually dimorphic, and more

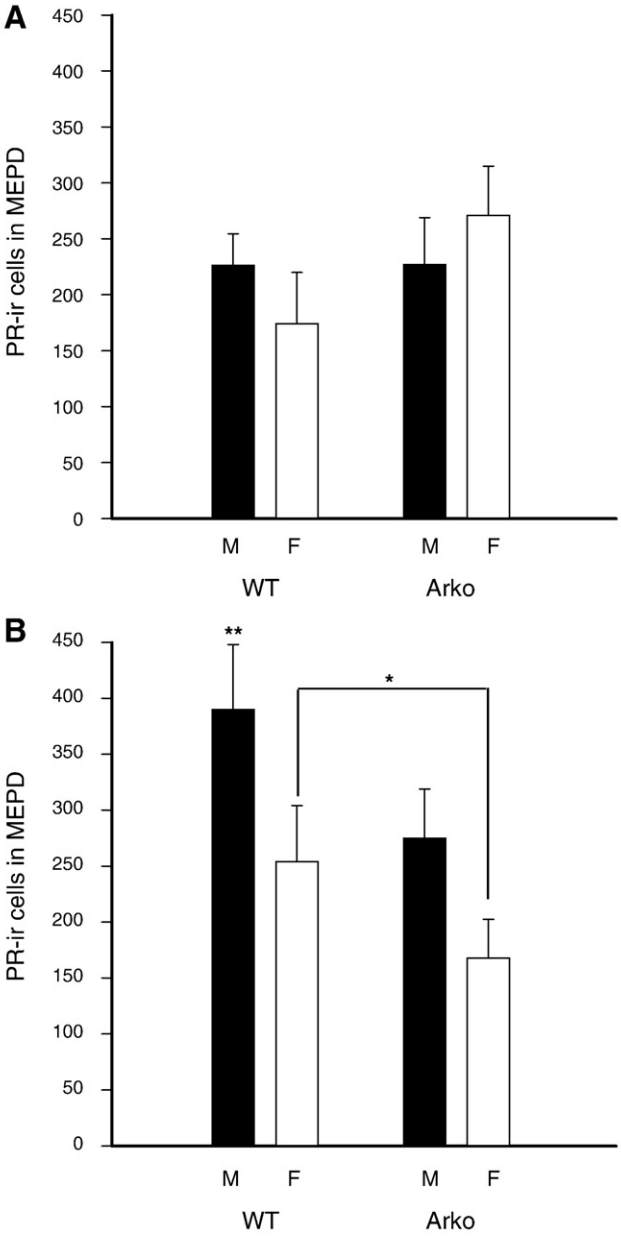

Fig. 2. Mean (+SEM) PR-ir cell numbers with in the medial posterodorsal amygdala (MePD) of wild type (WT) and aromatase knockout (ArKO) mice. All mice were gonadectomized and one week later treated with estradiol for 5 days prior to sacrifice. In panel A data from mice on a phytoestrogen reduced diet are shown. In panel B are data from mice consuming a phytoestrogen rich diet. Black histograms represent males (M) and white histograms represent females (F). *When consuming phyto-rich food females have significantly fewer PR immunoreactive (-ir) neurons induced by estradiol than do males, $p<0.05$. **Significantly more PR-ir neurons were present in MePD of the WT males on phytoestrogen rich chow as compared with all other groups.

asymmetric in males than females [40,43,45,46]. We did not count neurons in our study by any method other than PR-ir. Data from rats have demonstrated more neurons and glia in the male MePD versus the females [43]. It is possible that the enhanced PR-ir we noted in male brains was simply a reflection of more neurons in the male MePD as compared with the female. However in a strain of mice which we did not use here, no sex differences in cell numbers were noted, but the volume of the MePD was greater in males likely due to large cell somata [42]. Photoperiod, a predictor of the breeding season for Siberian hamsters, can modulate both androgen levels and the size of the MePD however co-habitation with females can block the effect of short days on the MePD [47]. Laboratory mice are not photoperiodic, however the presence of phytoestrogens in food may likewise signal the spring and suitable breeding conditions. Only a few studies have assessed variability in phytoestrogenicity over time in plants but they do document seasonal changes [48–50].

The role of phytoestrogens in the MePD has not been examined, but other brain regions have been studied. In rats, volumes of the sexually dimorphic nucleus of the preoptic area (SDN-POA) are equivalent in males and females when the animals are raised on a phytoestrogen low chow, but a sex difference is present in adulthood in rats on a standard chow (containing a high level of phytoestrogens) [51]. In addition females have a larger anteroventral periventricular (AVPV) nucleus than males. In males diet significantly affects this region with males on low phytoestrogen food having greater volumes than males on phyto-rich diet. In addition to neural effects, phytoestrogens affect behavior. Male rats typically perform better than females at visual spatial memory (VSM) tasks requiring the use of reference, but not working, memory. Dietary phytoestrogens reverse the direction of this sex difference [15]. Phytoestrogens produced anxiolytic effects in both male and female rats [52]. Furthermore, female rats treated with an isoflavone supplement display significantly reduced female receptivity [5]. Taken together, these data show that phytoestrogen consumption can result in a physiological change in both brain and behavior.

The MePD is part of the chemosensory pathway that transduces sexually relevant olfactory information [53]. Male rats exposed to an anti-aromatase drug during perinatal development do not display their typical olfactory preferences for females [54]. In ArKO mice olfactory abilities detected with a liquid olfactometer are comparable to WT males, but ArKO females have enhanced abilities to distinguish between urine from females primed with E2 and progesterone from those primed with E2 only [55]. While the direct connection between these behaviors and the MePD has not been shown, it is known that exposure to opposite sex chemosignals stimulates c-*fos* immunoreactivity in the MePD [56,57]. It is not known if these responses are affected by phytoestrogen consumption.

Phytoestrogens can also affect receptivity in adult female mice. Female ArKO mice had previously been shown to express lower levels of receptivity than WT littermates, and this led to the hypothesis that prepubertal exposure to estradiol is needed for complete feminization of this behavior [58]. However when females raised in a manner identical to the present experiment, consuming phytoestrogen-rich versus phyto-free diets, were tested for receptivity only ArKO females on phyto-rich chow had impaired receptivity [14]. Thus phytoestrogens may defeminize female behaviors. Moreover this action is likely mediated by ERβ. Estrogen receptor β knockout males treated in adulthood with priming steroids display higher levels of lordosis than wild type control males [59]. Female mice treated for the first three days after birth with either estradiol or a specific ERβ agonist, but not an ERα agonist, have lower lordosis quotients in adulthood than controls [44]. Interestingly when adjacent sections from the male brains used in this study were examined for numbers of ERα and ERβ-ir cells in the MePD an effect was seen only for ERβ. Males treated with estradiol and raised on a phytoestrogen rich chow had elevated ERβ positive cells as compared with males that received the low phytoestrogen chow (Kudwa and Rissman, unpublished). Taken together these results suggest that phytoestrogens affect ERβ dynamics in this region.

In humans, the amount of soy supplements routinely ingested by mothers, and subsequently by their infants, is increasing. It is likely that these soy supplements are delivering phytoestrogens to developing infants and in particular to the central nervous system [6]. Considering that phytoestrogens can affect neural development and plasticity in adult rodents it is important for us to determine how these dietary supplements act in humans.

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