Physiology & Behavior 98 (2009) 296–301

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Social environment alters central distribution of estrogen receptor alpha in juvenile prairie voles

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ARTICLE INFO

Article history:
Received 22 April 2009
Received in revised form 3 June 2009
Accepted 4 June 2009

Keywords:
Estrogen receptor alpha
Prairie vole
Social isolation
MPOA
BST
Microtus ochrogaster
Post-weaning social environment
Puberty

ABSTRACT

It is well established that social environment, particularly isolation, has a significant impact on social behaviors and neuroendocrine responses. Estrogen receptor alpha (ERα) expression in limbic structures and associated nuclei is related to the display of social behaviors. We hypothesized that the stress of isolation would cause changes in the pattern of ERα expression in the brain. Using a highly social (typically monogamous and biparental) rodent species, the prairie vole (*Microtus ochrogaster*), we housed juvenile voles with a sibling, stranger or in isolation for either 4 days or 21 days. Housing manipulations began following weaning from parents and group housed siblings. Rodents may be especially sensitive to manipulations of their social environment during this juvenile period. In particular, female prairie voles are induced ovulators, reliant upon exposure to an unrelated male (male urine) to become reproductively active. ERα immunoreactivity was quantified in the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BST), ventromedial nucleus of the hypothalamus (VMH) and medial amygdala (MeA). Significantly fewer ERα immunoreactive (ERα-ir) cells were labeled in the MPOA and BST of females isolated for 21 days compared with stranger housed females. Non-significant differences were shown in the VMH and MeA of females. No differences were found in voles isolated for 4 days. These results suggest that female prairie voles may be more sensitive than males to manipulations of their social environment during the juvenile period.

1. Introduction

Social isolation has profound, and generally detrimental, effects on a variety of mammalian species from primates to rodents [16,21,51]. More specifically, among rodent species isolation can produce impairments in social behavior, stress reactivity and neuroendocrine function [4,27,28]. The developmental timing of isolation is a crucial determinant of the nature and degree of the impairments it can cause an animal [20,48,49]. The post-weaning period is likely to be a particularly sensitive period as in many mammalian species it marks the end of dependence on maternal (or biparental) care and the beginning of a trajectory towards sexual maturation [7]. During this period an animal's social environment may determine future reproductive strategies, social behavior and stress reactivity [31,32]. Social housing conditions during discrete developmental time points around puberty have robust and lasting effects on rodent behavior [22,25].

The post-weaning social environment may represent unique challenges to a highly affiliative (monogamous and biparental) species such as the prairie vole (*Microtus ochrogaster*) [4]. Young prairie voles (unlike less social rodent species including rats and non-monogamous voles), often remain in the natal burrow as an alloparent, rather than dispersing [14,15]. The presence or absence of reproductively active older conspecifics [1,3,43], population density [34], sibling interactions [12,50] and social environment [32] can significantly impact the behavioral strategy a juvenile will pursue. The neuroendocrine mechanisms that modulate behavior in this species, such as estrogen receptor alpha (ERα), should be especially responsive to manipulations of the social environment during the post-weaning period. ERα may be one mechanism that underlies deficits in individual recognition, individual discrimination and exploratory behavior caused by isolation in this species [13,40].

In several rodent species (as well as many other vertebrate species) ERα is concentrated in nuclei associated with social, parental and reproductive behaviors including the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BST), medial amygdala (MeA) and ventromedial hypothalamus (VMH) [10,30,45]. ERα knockout mice show deficits in social recognition (males) [24], social preference (males) [42] and reduced aggression and deficits in sexual behavior (males and females) [38,42]. ERα mediates female rodent sexual [33,37,41] and social behaviors [6]. Female prairie voles are induced ovulators, reaching

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0031-9384/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2009.06.005

sexual maturity (increased ovarian weight and lordosis) following exposure to male urine odor [5]. Therefore, females may be particularly reactive to the stress of isolation during the post-weaning or juvenile period as their reproductive state is directly determined by cues from their social environment. Previous studies on prairie voles demonstrate this heightened sensitivity among females. For example, corticotropin releasing factor (CRF) immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) was significantly elevated and vasopressin immunoreactivity in the PVN was significantly decreased in females isolated for 21 days following weaning [44]. Additionally, isolation has been shown to produce behaviors indicative of depression and anxiety. In particular, isolated females spent less time exploring open arms of the elevated plus maze, reduced sucrose intake and increased immobility in a forced swim test [4,18,19].

Prairie voles are one of a limited number of mammalian species which can display social monogamy and biparental care of offspring [2]. Interestingly, the degree of these social behaviors displayed by males varies between different geographic populations. Males from populations displaying diminished prosocial behaviors have greater ERα expression, particularly notable in the MeA and BST [10]. Additionally, higher levels of ERα expression in discrete nuclei (including the MeA and BST) positively correlate with aggression in male Siberian hamsters and mice [29,46]. A broader comparative study of rodent species demonstrated a similar inverse relationship between ERα and the degree of prosocial behaviors displayed in males [11]. This relationship has been most clearly demonstrated by experimental increases in ERα in the MeA of male prairie voles through viral vector encoding resulting in a decrease in social behaviors [9].

In the present study, we measured ERα immunoreactivity in prairie voles following post-weaning isolation for 4 or 21 days. Given the nature of the deficits in social behavior which occur following isolation, and the relationship between ERα expression and prosocial behavior, we predict that changes in the number of ERα immunoreactive (ERα-ir) cells will be notable in the MPOA, BST, MeA and VMH, and that these changes will be most prominent in females.

2. Methods

2.1. Subjects and groups

Subjects were laboratory-bred male and female prairie voles (*M. ochrogaster*), descendants of a wild stock originally caught near Champaign, Illinois. Our stock was systematically outbred ensuring that all mating pairs did not share any common grandparents. Prairie voles were maintained on a 14:10 light:dark cycle and allowed food (Purina high-fiber rabbit chow) and water ad libitum. Breeding pairs were maintained in large polycarbonate cages (48.2 cm long × 26.6 cm wide × 15.5 cm high) and provided with cotton for nesting material. Within 24 h of birth litters were sexed, weighed and marked for identification. Litters in excess of six were culled and only mixed sex litters were used. Offspring from the breeding pairs (subjects) were housed in these same cages (with dam, sire and littermates) until they were weaned at 21 days of age and randomly placed into one of three housing conditions in smaller (29 cm × 19 cm × 12.7 cm) cages: isolation (singly housed), sibling (housed with a sibling of the same sex and age), stranger (housed with and unfamiliar stranger of the same sex and age). Conditions were maintained for 4 or 21 days. Animals in all conditions were housed in a single-sex colony room. All husbandry and experimental procedures were approved by an IACUC committee, ACC no. 04-078.

2.2. Brain tissue collection and ERα immunocytochemistry

At either 25 (4 day housing conditions) or 42 (21 day housing conditions) days of age animals were anesthetized by intraperitoneal injection with a combination of ketamine and xylazine, followed by cervical dislocation. Brain tissue was processed using spin fixation [8]. Brains were removed and placed in a 4% paraformaldehyde solution for 20 min. Brains were then removed from the solution and blocked into thirds, divided just before the optic chiasm and halfway through the cerebellum, exposing the lateral ventricles in the two rostral divisions. Brains were immersed and gently spun in 4% paraformaldehyde, and 5% acrolein (pH 8.6) solution for 4 h. Individual stir bars were placed in each scintillation vial and spun on a stir plate for 4 h. After fixation, brains were placed in a 25% sucrose solution then changed 24 h later. Tissue was then embedded in a gelatin solution for microtoming. The upper left corner of the embedding mold was notched to discern between left and right sides. Brains were sectioned on a freezing microtome at 40 μm. Alternate sections (every 240 μm) were used for immunocytochemistry.

Sections were incubated in a rabbit polyclonal antibody (anti-ERα C1355, Millipore 06-935) at a concentration of 1:800 for 48 h. Sections were rinsed in KPBS and then incubated in a secondary fluorescent antibody, Alexa-Fluor 546 goat anti-rabbit IgG (Invitrogen A11010) at a concentration of 1:200 for 1.5 h. Sections were rinsed in KPBS and then were mounted and coverslipped using Prolong gold mounting media with DAPI (Invitrogen P36931). Tissue from the animals in the 4 day housing conditions was run separately from tissue from animals

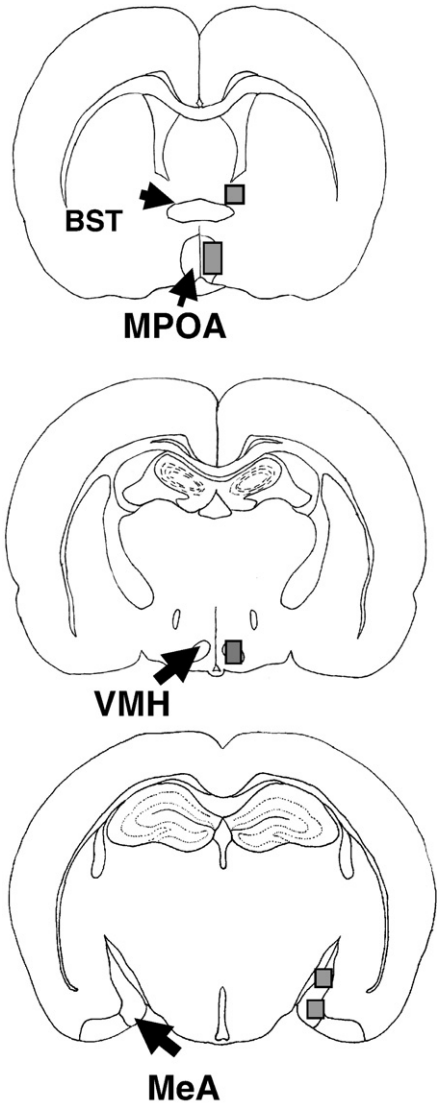

Fig. 1. Measurements of ERα immunoreactivity (ERα-ir). Shaded boxes approximate the areas quantified within the BST, MPOA, VMH and MeA.

in the 21 day housing condition. This can produce interassay variability, therefore these results were not compared statistically. Negative controls of primary and secondary only were run on tissue prior to the collection of any experimental data.

2.3. ERα quantification and statistical analyses

Images were captured using a Nikon 80i epifluorescent microscope and SPOT camera. Analysis of images was performed using Image J/NIH image software. Within each nucleus (MPOA, MeA, VMH, BST) a standardized sampling area was used to count the number of cells immunoreactive for ERα. The sampling grid size for each area (unilateral) is as follows: MPOA 320×320 μm, BST 300×300 μm, VMH 150×150 μm, MeA 250×320 μm (w×h). Sampling grids were used to ensure that the differences were not the result of observer variability that might occur when identifying the borders of each nucleus. Cell counts from each nucleus were bi-lateral and taken from sections matched in rostral–caudal orientation for each subject. Nuclei were identified according to evident landmarks and Paxinos and Watson [39]. Fig. 1 shows the approximate location of sampling grids within each section. DAPI label on each section facilitated the identification of the borders of each area of interest (MPOA, MeA, VMH, BST). The MPOA was identified by a contiguous anterior commissure located above the third ventricle and optic chiasm. Measures within the BST were taken in the more rostal and anterior–medial divisions of the nucleus. The VMH was approximate to Figs. 36 and 37 in Paxinos and Watson [39]. The MeA was identified by the location of the ascending optic tract. The sampling grid was placed twice on each side including measures of the entire MeA (MePD and MePV). In all cases grids were drawn to scale with a pixel to micron conversion within Image J/NIH image. All cells that had identifiable borders within the grid were counted and marked within the image file to avoid double counts. Original and quantified images were saved separately. In all cases cell counts were taken by two observers blind to the condition of the subject and the average was calculated. All quantification was done on grayscale images.

All statistical procedures were performed using SPSS 16.0 and all data were tested for assumptions of normality and equal variance prior to analyses. Data from 4 day and 21 day housed animals were analyzed separately as immunocytochemistry for this tissue was performed at different time points. A significance level of $p<0.05$ was used for all tests; if the significance level was $p<0.01$ this was noted. MANOVA (with sex and housing condition as independent variables) analyses were used on the data. Fisher's LSD post-hoc tests were performed if the overall MANOVA was significant.

3. Results

No statistically significant differences in the number of ERα-ir cells were found between left and right sides of any nucleus, therefore total cell counts from both sides of each subject were added and compared between groups.

3.1. 4 day housing conditions

No significant differences were found among the 4 day housing groups. Within the MPOA, no significant differences in the number of ERα-ir cells were found as a result of housing conditions [$F_{(2,\ 26)} = 1.91$, $p = 0.16$)], sex [$F_{(1,\ 26)} = 3.797$, $p = 0.062$] or their interaction [$F_{(2,\ 26)} = 0.896$, $p = 0.420$]. Within the BST no significant differences were found as a result of housing conditions [$F_{(2,\ 25)} = 2.44$, $p = 0.108$], sex [$F_{(1,\ 25)} = 2.77$, $p = 0.108$] or their interaction [$F_{(2,\ 25)} = 0.339$, $p = 0.716$]. Within the VMH no significant differences were found as a result of housing condition [$F_{(2,\ 25)} = 0.712$, $p = 0.500$], sex [$F_{(1,\ 25)} = 0.955$, $p = 0.338$] or their interaction [$F_{(2,25)} = 0.913$, $p = 0.414$]. No differences were found in the sub-divisions of the MeA (MePD and MePV) so total cell count comparisons are presented. Within the MeA no significant differences were found as a result of housing condition [$F_{(2,20)} = 1.593$, $p = 0.228$], sex [$F_{(1,20)} = 1.085$, $p = 0.310$] or their interaction [$F_{(2,20)} = 0.772$, $p = 0.475$].

3.2. 21 day housing conditions

Statistically significant differences in the number of ERα-ir cells were found in the MPOA, BST and VMH among voles housed in the 21 day housing conditions.

Overall results from the MPOA reveal that housing condition [$F_{(2,28)} = 0.848$, $p = 0.439$] and sex [$F_{(1,\ 28)} = 0.268$, $p = 0.609$] were non-significant but their interaction was [$F_{(2,28)} = 3.163$, $p<0.05$]. Isolate females had significantly fewer ERα-ir cells in the MPOA than stranger housed females ($p<0.05$). The difference between isolate females and sibling housed females was non-significant ($p = 0.72$). Isolate females

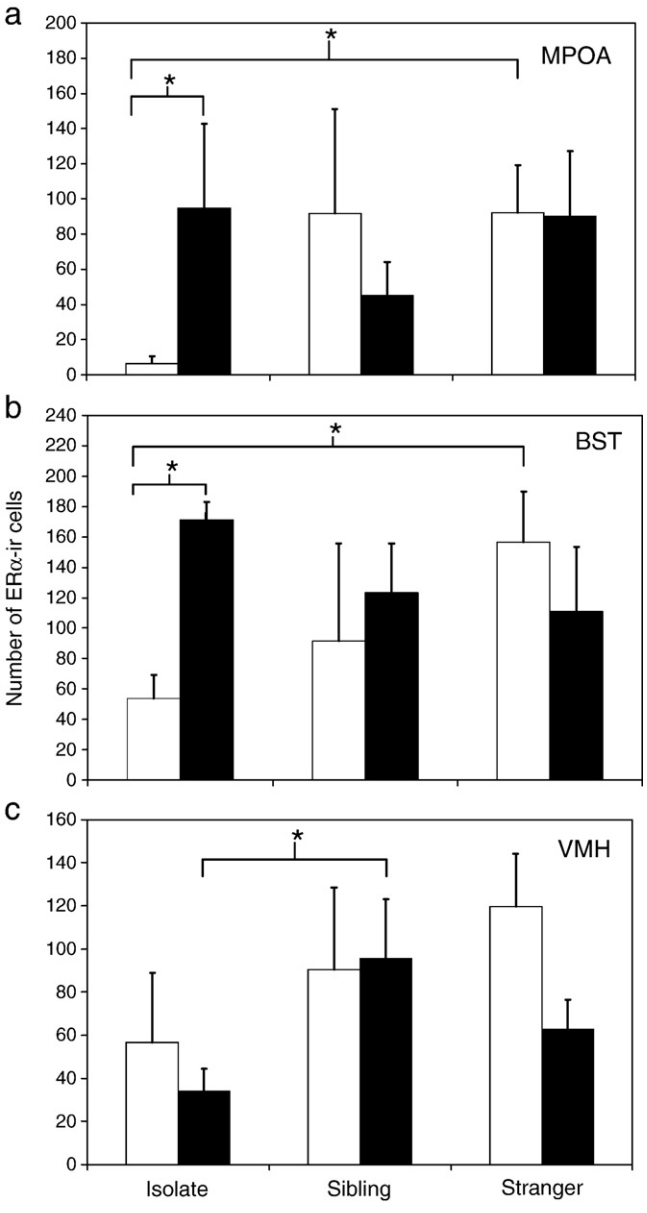

Fig. 2. ERα immunoreactivity (ERα-ir) cell number, 21 day housing conditions. Open bars represent females, closed bar represent males. a. MPOA: Isolate females had significantly fewer labeled cells compared with stranger housed females and isolate males. b. BST: Isolate females had significantly fewer labeled cells compared with stranger housed females and isolate males. c. VMH: Isolate males had significantly fewer labeled cells that sibling housed males. Asterisks designate groups that are statistically different at $p<0.05$. *N*'s are 4–6 per group.

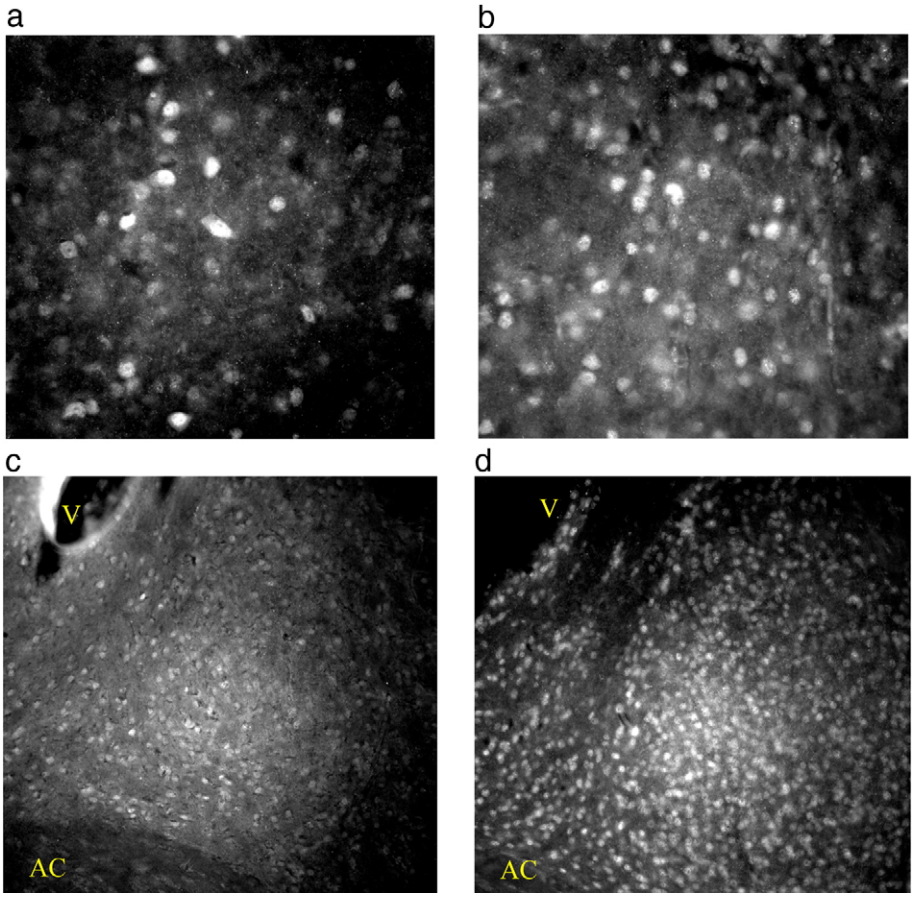

Fig. 3. ERα immunoreactivity (ERα-ir), 21 day housing conditions. a. MPOA, Isolate female, 400× magnification b. MPOA, Stranger housed female, 400× magnification. c. BST, Isolate female 200× magnification. d. BST, Stranger housed female 200× magnification. AC: anterior commissure, V: ventricle.

had significantly fewer ERα-ir cells than isolate males ($p<0.05$) (Figs. 2a, 3a and b).

Overall results from the BST were: housing condition [$F_{(2,17)} = 0.609$, $p = 0.555$], sex [$F_{(1,17)} = 0.514$, $p = 0.483$] and their interaction [$F_{(2,17)} = 0.123$, $p = 0.885$]. In post-hoc comparisons isolate females had significantly fewer ERα-ir cells in the BST than stranger housed females ($p<0.05$). The difference between isolate females and sibling housed females was non-significant ($p = 0.295$). Isolate females had significantly fewer ERα-ir cells than isolate males ($p<0.05$) (Figs. 2b, 3c and d).

Overall results from the VMH demonstrate that housing condition was significant [$F_{(2,18)} = 4.388$ $p = 0.028$]. Sex [$F_{(1,18)} = 2.388$, $p = 0.140$] and the interaction of sex and housing condition [$F_{(2,18)} = 0.161$, $p = 0.853$] were non-significant. Isolate males had significantly fewer ERα-ir cells in the VMH than stranger housed males ($p<0.05$). The difference between isolate males and sibling housed males, was non-significant ($p = 0.053$) (Fig. 2c).

No differences were found in the sub-divisions of the MeA (MePD and MePV) so results from total cell count comparisons are presented. No significant differences were found in the MeA as a result of housing condition [$F_{(2,20)} = 0.200$ $p = 0.820$], sex [$F_{(1,20)} = 0.065$, $p = 0.801$] or their interaction [$F_{(2,20)} = 0.275$, $p = 0.762$].

4. Discussion

Female prairie voles isolated for 21 days had significantly fewer ERα-ir cells in the MPOA and BST compared with stranger housed females and isolate males. No significant differences were found among any groups in the four day housing conditions. These results along with previous studies [4,18,19,44] suggest that females are particularly sensitive to the stress of isolation during the post-weaning period.

The role of ERα in the regulation of female rodent sexual behaviors is well established and has been convincingly demonstrated in knockout mice models, as well as administration of selective antagonists [33,37]. In particular, ERα in the MPOA regulates aspects of proceptive and receptive behaviors [23,26]. The data in the present study demonstrate that ERα immunoreactivity in the MPOA is significantly lower in isolated females than males, whereas a sexual dimorphism (with females having a greater number of ERα cells in the MPOA) in ERα would typically exist in adult animals. Development of this sex difference is a key component of sex specific reproductive behaviors in this species [36]. Isolate females also had fewer ERα-ir cells in the BST. The BST has reciprocal connections to MeA and MPOA with neuropeptidergic fibers projecting to the lateral septum and is another key node in limbic circuitry associated with social behavior [35]. These results demonstrate that multiple nodes in limbic circuitry are sensitive to social isolation.

The MPOA and BST of 21 day isolated females showed significantly less ERα compared to stranger housed females, but not sibling housed females. This is in part due to higher variance within the female sibling group compared with the stranger group. The difference in ERα expression in the MPOA and BST within some pairs of 21 day sibling housed females was fairly large. This pattern was not seen in all sibling housed pairs, however it was not apparent in any stranger housed pairs. Reproductive suppression of younger conspecifics by older reproductively active females occurs in prairie voles [1,3,43]. One possible explanation for the pattern of results we see in the current study

is that mechanisms of reproductive suppression may operate slightly differently in siblings versus stranger housed females. Future studies will investigate if the current patterns of ERα-ir associated with social housing condition correlate with the degree of affiliative and proceptive behaviors (including latency to pair bonding) that females display with exposure to an unfamiliar male and the induction of estrus in this species.

Non-significant differences were found in the MeA and VMH of females isolated for 21 days. One possible explanation for the lack of differences is that the MPOA and BST may be more reactive to social environment, whereas the MeA and VMH are more responsive to actual sexual stimuli and consummatory aspects of sexual behavior. The MeA and VMH show specific increases in activity in ERα containing cells during sexual behavior [17]. Although we did not test these females with any type of male stimuli, we could anticipate that isolation may dampen neural responses in these areas, perhaps related to lower ERα levels. However, we cannot discount the fact that the non-significant differences in these areas may still be functionally significant. Even small reductions in ERα expression may have an impact on female behavior.

Increasing evidence continues to support the concept that the peri-pubertal and pubertal periods are critical periods for the development of social behaviors and the neuroendocrine systems that modulate them, particularly in females [31]. The social environment which an animal is exposed to during this time period can have lifelong effects on future behavioral trajectories [22,25]. Female prairie voles isolated for 21 days following weaning also show significant neuroendocrine changes including increased levels of CRH in the PVN, elevated corticosterone and decreased vasopressin in the PVN [44]. These data, along with the present results, suggest that ERα, vasopressin and the hypothalamic–pituitary adrenal axis may work together to modulate the social stress of isolation in females. The permanence of such neuroendocrine changes and their effects on behavior remains to be determined. Female prairie voles isolated for a similar duration as adults show behaviors that can be interpreted as an index of anxiety or depression [18,19]. Females isolated during the post-weaning period may show similar or exacerbated behavioral deficits.

Although previous research demonstrates an inverse relationship between ERα and the degree of sociality in male rodents, the same relationship has not been demonstrated in females [10,11]. This is likely the result of a variety of factors, the most prominent of which include estrogen's more direct relationship with the reproductive physiology of females, sexual maturation and adult sexual behavior. The current data certainly do not discount the role of ERα in the modulation of social behavior of females, but they suggest that the relationship between ERα and sociality is quite different than it is in males. The established inverse relationship between ERα and male rodent social behavior is based upon comparisons of adult males, rather than measures of reactivity of juveniles to a particular social environment. This relationship between ERα and male social behavior has been shown in the MeA and BST, but not the VMH [10,11]. However, in the present study, the only difference found in males was in the VMH with 21 day isolate males showing less ERα than siblings. The VMH's role in female sexual behavior is well established, but it appears to play a less central role in male sexual or social behavior. Understanding the functional significance of this pattern in males will be facilitated through behavioral studies which measure their affiliative behaviors following isolation.

The relationship between the stress of isolation and its effects on ERα in females may have broader implications for the understanding of the neurobiology of stress. Isolation is frequently used as a model of chronic stress [19,21]. Variability in ERα gene polymorphisms is associated with major depressive disorders in humans [47]. ERα may be a mechanism that underlies social impairments brought on by chronic stress and possibly depression.

Acknowledgements

This research was supported by NIH MH 072935 and NAAR to CSC. Additional support was provided by the College of Charleston, Small Foundation Grant and Undergraduate Research and Creative Activities Program. We wish to thank Chantel Roedner and Michael G. Miner for assistance with immunocytochemistry and quantification of cells.

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