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Oxytocin, vasopressin and estrogen receptor gene expression in relation to social recognition in female mice

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Abstract

Inter- and intra-species differences in social behavior and recognition-related hormones and receptors suggest that different distribution and/or expression patterns may relate to social recognition. We used qRT-PCR to investigate naturally occurring differences in expression of estrogen receptor- α (ER α), ER- β (ER β), progesterone receptor (PR), oxytocin (OT) and receptor, and vasopressin (AVP) and receptors in proestrous female mice. Following four 5 min exposures to the same two conspecifics, one was replaced with a novel mouse in the final trial (T5). Gene expression was examined in mice showing high (85–100%) and low (40–60%) social recognition scores (i.e., preferential novel mouse investigation in T5) in eight socially-relevant brain regions. Results supported OT and AVP involvement in social recognition, and suggest that in the medial preoptic area, increased OT and AVP mRNA, together with ER α and ER β gene activation, relate to improved social recognition. Initial social investigation correlated with ERs, PR and OTR in the dorsolateral septum, suggesting that these receptors may modulate social interest without affecting social recognition. Finally, increased lateral amygdala gene activation in the LR mice may be associated with general learning impairments, while decreased lateral amygdala activity may indicate more efficient cognitive mechanisms in the HR mice.

Keywords

Oxytocin; Vasopressin; Social recognition; Estrogens; Individual differences; Progesterone

1. Introduction

Social recognition, the ability to identify or recognize conspecifics, is the basis for complex social relationships [1,2]. In order to facilitate dominance hierarchy and social bond development, one needs to modulate one's behavior based on previous experience with an individual [1,3–5].

Sex differences in sociality in our own and other species, as well as the dramatic sexual dimorphism in social disorders (e.g., 80% of individuals with autism spectrum disorders

(ASD) are male [6]), suggest that gonadal hormones may be involved. The animal literature has supported this, and implicated female gonadal hormones in social recognition and other types of social cognition like social learning (see [7] for a recent review). Typically, rodent social recognition is assessed in the laboratory by measuring habituation (reduction in investigation) to familiar conspecifics and dishabituation (increase in investigation) to novel animals, and/or by examining whether novel conspecifics are preferentially investigated over familiar animals in a choice test (social discrimination [1,4,8]).

Administration of estrogens to ovariectomized (ovx) mice increases long-term social recognition memory [9], and both main intranuclear estrogen receptors, alpha (ER α) and beta (ER β), appear to mediate social recognition, ER α more than ER β [3,4,8,10–12]. Consistently, systemic treatment with ER α or ER β agonists improved social recognition in mice when administered 48–72 h prior to testing [7,13,14], but only the ER α agonist improved performance rapidly (within 40 min [7,15]). Although it has been the subject of little to no research, progesterone may also modulate social recognition. Treatment with estradiol benzoate and progesterone increased habituation rate in ovx rats [12], and progesterone receptor (PR) is expressed in social recognition-related brain areas (e.g., medial amygdala, MA [12]).

Downstream effectors of gonadal hormone action on social recognition may include nonapeptides oxytocin (OT), vasopressin (AVP), and their receptors, which have been shown to mediate social recognition in rodents and are both under the control of sex hormones (see [1] for review). Administration of antagonists or inactivation of the OT gene (“knock out”, KO), its receptor (OTR), or either of the AVP1 receptors (AVPR1a more than AVPR1b) resulted in specific social recognition deficits [4,5,8,16–24]. Exogenous OT rescued social recognition in OTKO mice and improved it in male (but not female) wild type (WT) mice [25] and rats [26], while AVP improved social recognition in both sexes [26–28]. However, AVP antiserum or antagonist administration blocked male but not female social recognition (reviewed in [1]).

While the involvement of these hormones and receptors in social recognition has largely been established, whether their functions and/or distributions are linked to behavior in social situations has received less attention. Species differences exist in both hormones and their receptors in relation to social behavior. Male vole and hamster species that are monogamous and/or paternal express more ER α than polygynous species in socially-related brain regions like the bed nucleus of the stria terminalis (BNST) and MA [29,30]. It is also possible to induce pair bonding in both male mice and promiscuous male voles by introducing the monogamous prairie vole AVPR1a gene into the ventral forebrain or central nervous system [29–32], although variations in the AVPR1a gene may not be sufficient or necessary for this type of mating behavior in voles and deer mice [33]. Within-species variations in hormones and their receptors are also linked to social behaviors; voles that spent more time investigating a novel female, and did not habituate, had more AVPR1a and less OTR in the septum [34], and more social males express less ER α in the BNST and MA [35].

To the best of our knowledge, whether social-recognition-associated behaviors are linked to within-species variation in ERs and PR, or putative downstream effector OT and AVP systems, in female house mice (*Mus musculus*) is unknown. Thus, we tested out-bred female CD1 mice in the proestrous phase of the estrous cycle on the social discrimination paradigm. This phase was chosen partly because it involves high estrogen levels [36] and leads to improved social recognition learning [10,37]. Additionally, social approach and social choice are particularly important at this reproductively active phase, as remembering conspecific and environmental interactions facilitates assessment of the social and physical environment's suitability for reproduction [37,38].

Brain tissue obtained from mice that performed very well (high recognizers, HR) and from those that performed close to chance (low recognizers, LR) was analyzed for levels of ER α , ER β , PR, OT, OTR, AVP, AVPR1a, and AVPR1b mRNA expression. The following brain regions were selected for this analysis, taking into account their role in social recognition and social behavior: BNST, dorsolateral septum (DS), frontal cortex, lateral amygdala (LA), MA, medial preoptic area (MPOA), paraventricular nucleus (PVN) of the hypothalamus, ventromedial hypothalamus (VMH) and hippocampus [2–5,8,25,39–43]. Gene expression was assessed via quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Behaviors in the social discrimination test and gene expression levels were compared between HR and LR mice, and social recognition and social interest were correlated with gene expression.

2. Materials and methods

2.1. Subjects

Fifty-eight adult female mice of the outbred CD1 strain were obtained from Charles River, QC, Canada, and held in the Central Animal Facility at University of Guelph, under a 12:12 h reversed light/dark cycle (lights off at 0800 h) at 21 $\pm$ 1 °C. Mice were kept in clear polyethylene cages (26 $\times$ 16 $\times$ 12 cm), and provided with corn cob bedding, environmental enrichment (plastic containers, paper nesting material), and food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, Madison, WI) and tap water ad libitum.

The 49 experimental mice were individually housed for at least 7 days to develop a home cage territory, with no cage changes taking place for at least 3 days prior to testing, and no experimental mouse was used more than once. Nine ovx stimulus mice were housed in same-sex groups of three, and mice were reused with at least one day between exposures. Ovx stimulus mice, which we have previously shown will be highly investigated by female mice [4,8], were chosen in order to avoid estrous cycle-induced variability and to have more “standardized” stimuli with more consistent levels of circulating gonadal hormones [44]. Brains were collected from experimental mice with an estimated preference for the novel mouse of 85% or higher in the HR group or between 40% and 60% in LR group, which consisted of approximately 16% (n = 8) and 20% (n = 10) of the population, respectively (see Table 1).

2.2. Surgery

Stimulus mice underwent bilateral ovx under isoflurane gas anesthesia (Benson Medical Industries, Markham, ON), with subcutaneous (s.c.) carprofen (50 mg/kg; Rimadyl, Pfizer Canada Inc, Kirkland, QC) in saline at 10 ml/kg as an analgesic and anti-inflammatory. The lower back skin was shaved and cleaned, and a single 1.5–2 cm incision was made in the skin to expose the back muscles. A small 0.5–1 cm incision was made in the muscles overlying the ovaries on each side, and then the ovary and the tip of the uterus were carefully drawn out of the hole. The ovary was removed by cutting just above a clamp on the uterus, and then the uterus was replaced in the abdominal cavity. A few drops of 12.5% marcaine (bupivacaine hydrochloride; Hospira, Inc, Lake Forest, IL) were dripped onto the back incision as a local anesthetic, and the incision was stapled with 1–2 MikRon autoclip 9 mm wound clips (MikRon Precision Inc, Gardena, CA). Mice received a rehydrating 0.5 ml intraperitoneal saline injection, and were allowed 7 days of single-housed recovery; ovx stimulus mice were group-housed at least 7 days before the experiment.

2.3. Apparatus

Trials were run in home cages of experimental mice, and videotaped from above with an 8 mm Sony Handycam (in Nightshot) through clear Plexiglas cage lids. Stimulus mice were

placed into clear Plexiglas cylinders (12 cm high, 7 cm in diameter) that had 36 holes (4 mm diameter) drilled into their bottom third, thus allowing for passage of olfactory cues while preventing direct interactions between stimulus and experimental mice [4]. This ensured that experimental mice did not deposit their own scent onto stimulus mice [45], and also reduced stimulus-induced variability in behavior, while still eliciting high social interest and investigation from experimental mice [4,5,8,46]. Stimulus mice were repeatedly habituated to cylinders prior to testing until they spontaneously entered them with no observable distress [4].

Clean cylinders were used for all trials, while the same empty cylinders were used in inter-trial intervals. Cylinders were washed thoroughly with unscented soap (Alconox, VWR International, West Chester, PA) and baking soda (Arm & Hammer, Church & Dwight Canada Corp., Mississauga, ON), then paper towel- and air-dried.

2.4. Behavioral testing procedure

Gonadally intact female experimental mice were tested during the proestrous phase of the estrous cycle, determined by vaginal cytology as described in Clipperton et al. [47], which was also used to confirm that ovx mice were not cycling. All procedures followed the regulations of the Canadian Council on Animal Care and were approved by the Institutional Animal Care and Use Committee of the University of Guelph.

We followed the procedure described in Choleris et al. [4], with all trials taking place between the second and sixth hour of the dark (active) phase of the light/dark cycle (i.e., between 0900 and 1400). Briefly, both experimental and stimulus mice were moved into an antechamber of the testing room and left undisturbed for at least 90 min. For testing, all mice were moved into the testing room, enrichment was removed from the experimental mouse's cage, and two clean empty cylinders were placed into two corners of her cage for 10 min. Two cylinders, each containing a stimulus mouse from a different cage, replaced the empty cylinders for five 5 min trials, with the empty cylinders returned during the 15 min inter-trial intervals. For the first four trials (T1–T4), the same stimulus mice were used in the same locations, while in the final trial (T5), one of the stimulus mice was replaced with a novel mouse from a different cage than the other two stimuli. The location of the replaced mouse was counterbalanced across subjects. The number and location of stimulus mice was maintained for consistent social stimulation. During all trials, mice were left undisturbed in the room and their behavior was videotaped for subsequent behavioral analysis. During test (T5), the experimenter also sat quietly in an antechamber and manually timed the duration of investigation of the novel and familiar stimulus mice with two Jumbo Digital Traceable Stopwatches (Fisher Scientific, Ottawa, Ontario) to gain a quick preference estimate.

Immediately following T5, any mice with an estimated preference for the novel mouse of approximately 85% or above (HR mice) or 40–60% (LR mice) were sacrificed and brains were extracted as described previously [15]. Briefly, as per institutional Animal Care Committee guidelines, animals were rapidly asphyxiated with CO₂ (typically less than 1 min in duration, thus limiting possible CO₂ effects on brain tissues), cervically dislocated and decapitated. Brains were carefully removed, flash frozen on dry ice and stored at –80 °C.

Videotaped trials were subsequently scored by a trained observer unaware of the initial rough estimate of preference, using The Observer Video Analysis software (Noldus Information Technology, Wageningen, the Netherlands), for eight behaviors based on the mouse ethogram [48] (see Table 2, modified from [4,15]). Social investigation and cylinder investigation were calculated as ratios (investigation of novel mouse or cylinder/

investigation of both mice or cylinders) and totals. Habituation and dishabituation measures of total social behavior were also calculated (see Table 2).

2.5. Quantitative real time reverse transcription polymerase chain reaction

Nine brain regions (BNST, DS, hippocampus, LA, MA, MPOA, PVN, VMH, and a section of frontal cortex as a control region) were isolated as described in Ribeiro et al. [49]. Briefly, frozen brains were sliced coronally at 1 mm intervals, and areas of interest were dissected out using a scalpel or razor blade and fine forceps.

Ribonucleic acid (RNA) from each region was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, San Diego, CA). The amount and quality of total RNA were assayed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) and a Nanodrop UV spectrophotometer. Total RNA from each sample was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. Briefly, 20 μ l of reaction mixture contained 200 or 300 ng of total RNA (see Table 1), 2 μ l 10X Reverse Transcription buffer, 0.8 μ l 25X dNTP mix (100mM), 2 μ l 10X Reverse Transcription random primers, 1 μ l of MultiScribe Reverse Transcriptase, and 4.2 μ l nuclease-free water. The cycling conditions for reverse transcription reaction were: 10min at 25 °C, 120 min at 37 °C, 5 s at 85 °C, and then maintenance at 4 °C until removed from the PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA). For each gene, qRT-PCR was carried out in all brain regions, using Applied Biosystems' gene expression assays (Foster City, CA): ER α (Assay ID Mm00433149_m1), ER β (Assay ID Mm00599819_m1), PR (Assay ID Mm00435628_m1), OT (Assay ID Mm01329577_g1), OTR (Assay ID Mm01182684_m1), AVP (Assay ID Mm00437761_g1), AVPR1a (Assay ID Mm00444092_m1), and AVPR1b (Assay ID Mm01700416_m1). Eukaryotic 18S was used as an endogenous control (Assay ID Mm03928990_g1). Q-PCR reactions were carried out using the Taqman detection system (ABI Prism® 7000 Sequence Detection System, Applied Biosystems, Foster City, CA) and the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. This resulted in each cycle producing a 1.87-fold increase in quantity of cDNA. In some brain regions, the samples for specific animals were of insufficient quality or quantity for qRT-PCR, resulting in smaller sample sizes for these regions.

2.6. Statistical analysis

For all statistical analyses, only significant results are reported, and SigmaStat 3.5 (Systat Software, Inc., Chicago, IL), SPSS 13.0 (SPSS Inc., Chicago, IL), and G*Power 3 [50,51] were used. For the comparisons of the HR and LR groups on behavioral or molecular measures, the alpha level was set at $p < 0.05$. To reduce the number of comparisons, and thus the possibility of type I error, selected mean comparisons were planned a priori. No multiple comparison correction was performed, as Bonferroni and similar corrections are too conservative (i.e., increase the probability of a type II error above acceptable limits). This decision follows recent concerns about the widespread use of such corrections and the consequent increase in type II errors (e.g., [52–54]), and is in line with the statistical analyses in the literature (e.g., [29,35,49,55–57]). We report effect sizes (Cohen's d for t -tests, partial η^2 for analyses of variance, ANOVAs) and power for each significant comparison and allow our readers to interpret the data accordingly.

Due to the large number of behavioral/molecular correlations, a more stringent criterion of $p < 0.01$ was applied to correct for multiple correlations. We believe that this reduces the likelihood of type I error, while maintaining an acceptable power level (greater than 0.80 [58]).

2.6.1. Behavioral analysis—Because ratios violate the homogeneity of variance assumption of parametric statistics, the duration and frequency of the Social Investigation and Cylinder Investigation Ratios were arcsine-transformed prior to analysis [$\arcsin(\text{ratio})$]. Digging and Stretched Approaches were only occasionally observed, so they were not analyzed. For all other behaviors, durations and frequencies were analyzed, and as their results were generally consistent, only the duration results will be reported unless differences were found. Mixed-model ANOVAs assessed the effect of trial number (T1, T2, T3, T4, T5) and group (HR, LR) on the Social Investigation Ratio, the Total Social Investigation and the Total Cylinder Investigation. The Greenhouse–Geisser correction for repeated measures was applied, and Sidak post-hoc tests were performed to assess sources of significance when main effects and/or interactions were found. For each behavior performed in the test trial (T5), group mean comparisons were planned a priori and conducted using independent samples t-tests.

Comparisons between the HR and LR mice were also planned a priori for habituation (Total Social Investigation at T1–Total Social Investigation at T4) and dishabituation (Total Social Investigation at T5–Total Social Investigation at T4), and one-sample t-tests determined if there was a significant preference for the novel mouse or its cylinder during T5 (comparison with chance, 0.50), and if habituation occurred (comparison with no change, 0.00). Assessing effects on habituation/dishabituation was important, as some studies use this as the measure of social recognition (e.g., [8]), and impaired social discrimination may be related to an impairment in habituation and/or dishabituation (e.g., [4]).

2.6.2. Molecular analysis—As all q-PCR reactions were run in triplicate, any cycle threshold (Ct) values for a particular gene in a particular brain region that was more than 0.5 from both other replicates was removed, as per the manufacturer's instructions. Ct values (2 or 3) were averaged for each mouse, and normalized by dividing each subject's average Ct value for each gene product in each brain region by the average Ct for 18S messenger RNA (mRNA) for that subject and region. For each gene product in each brain region, the average amount of gene product in HR animals was set at 1, and the fold difference from average HR was calculated for each mouse using the formula: $F(x) = 1.87^{-(X-Y)}$, where X is the mouse's normalized Ct (nCt), Y is the average nCt for the HR group, and 1.87 is the increase in cDNA quantity per cycle with our equipment (modified from [57]). Higher Cts mean that there was less mRNA in the sample, since more cycles (approximately doubling the amount of cDNA with each cycle) were required to reach threshold. The nCt values for each gene in each brain region were compared between HR and LR groups using independent sample t-tests.

2.6.3. Behavior/molecular correlations—Pearson product–moment correlations were calculated between nCts of each gene product within each brain region and the frequency and duration of the habituation, dishabituation, and the Social Investigation Ratio and Total Social Investigation at T1 and T5.

3. Results

3.1. Behavioral analysis

Mice in HR group had significantly higher Social Investigation ($t(10.09) = 9.95$, $p < 0.001$, Cohen's $d = 4.86$, power = 1.00) and Cylinder Investigation Ratios at T5 ($t(16) = 3.20$, $p = 0.006$, Cohen's $d = 1.53$, power = 0.86) than LR mice (see Fig. 1a). In addition, HR mice preferentially investigated the perforated parts of the two cylinders containing stimulus mice ($m(\text{across trials}) = 109$ s, $m(T5) = 101$ s) over the unperforated parts ($m(\text{across trials}) = 28$ s, $m(T5) = 24$ s), indicating that social investigation and recognition are driven predominantly

by olfactory cues passing through the perforations. Planned comparisons indicated that HR and LR groups differed significantly in social habituation ($t(16) = 2.22$, $p = 0.041$, Cohen's $d = 1.05$, power = 0.54; see Fig. 1b), and one-sample t -tests indicated that HR, but not LR, mice habituated ($t(7) = 3.47$, $p = 0.010$, Cohen's $d = 1.23$, power = 0.84) and had a significant preference for the novel mouse ($t(7) = 25.88$, $p < 0.001$, Cohen's $d = 5.91$, power = 1.00) and its cylinder ($t(7) = 5.39$, $p = 0.001$, Cohen's $d = 2.71$, power = 1.00) during T5. This indicated that only HR mice displayed social recognition.

A mixed-model ANOVA showed a significant effect of trial ($F(2.5,39.9) = 11.874$, $p < 0.001$, partial $\eta^2 = 0.43$, power = 1.00), group ($F(1,16) = 6.352$, $p = 0.023$, partial $\eta^2 = 0.28$, power = 0.66), and a significant trial $\times$ group interaction ($F(2.5,39.9) = 10.753$, $p < 0.001$, partial $\eta^2 = 0.40$, power = 0.99) for the Social Investigation Ratio (see Fig. 1c). Post hoc tests showed that there were significant differences between T5 and all other trials (all $p < 0.002$), primarily due to effects of trial in HR group ($F(1.9,13.2) = 14.056$, $p = 0.001$, partial $\eta^2 = 0.67$, power = 0.99), which also showed a significant increase in preference for the novel mouse in T5 compared with all other trials (all $p < 0.001$). LR mice did not show any significant changes in Social Investigation Ratio across trials.

A mixed-model ANOVA showed a significant effect of trial ($F(2.8,44.2) = 5.769$, $p = 0.003$, partial $\eta^2 = 0.27$, power = 0.92) on Total Social Investigation (see Fig. 1d). Post hoc tests determined that there were significant differences between T1 and T4 and between T1 and T5 (all $p < 0.050$), indicating that Total Social Investigation decreased with repeated trials (see Fig. 1d). There were no significant trial, group or interaction effects on Total Cylinder Investigation. Other behaviors did not differ between HR and LR mice. Importantly, Total Social Investigation was the same for both groups at T1 ($t(16) = 0.986$, $p = 0.339$, Cohen's $d = 0.46$, power = 0.15), showing equivalent initial social motivation.

3.2. Molecular analysis

In the LA, HR mice had significantly higher Cts than LR mice (indicating that they expressed less mRNA) for all gene products except PR and OT (see Table 3 for exact statistics; see Fig. 2a). In the MPOA, HR mice expressed more mRNA for OT and AVP than LR mice, as indicated by HR group's significantly lower Cts (see Table 3 and Fig. 2b). Only significant results are reported.

3.3. Behavior/molecular correlations

3.3.1. Lateral amygdala—Social Investigation Ratio frequency during T5 correlated positively with AVP nCt ($r(13) = 0.729$, $p = 0.005$, power = 0.86; see Fig. 3e), indicating that higher expression of AVP mRNA in the LA is associated with lower Social Investigation Ratio frequency. No significant relationships with the duration measures were found.

3.3.2. Medial preoptic area—Total Social Investigation frequency during T5 correlated positively with ER α nCt ($r(13) = 0.758$, $p = 0.003$, power = 0.91; see Fig. 4a), indicating that higher expression of ER α mRNA in the MPOA is associated with lower Total Social Investigation frequency during T5. Habituation frequency correlated negatively with ER α nCt ($r(13) = -0.769$, $p = 0.002$, power = 0.92; see Fig. 4b), ER β nCt ($r(13) = -0.747$, $p = 0.003$, power = 0.89; see Fig. 4c), OT nCt ($r(12) = -0.832$, $p = 0.001$, power = 0.96; see Fig. 4d), and AVP nCt ($r(13) = -0.761$, $p = 0.003$, power = 0.91; see Fig. 4e) indicating that mRNA expression and Habituation vary together. No significant relationships were found with the duration measures.

3.3.3. Dorsolateral septum—Duration of Total Social Investigation during T1 correlated positively with ER α nCt ($r(9) = 0.860$, $p = 0.003$, power = 0.92; see Fig. 3a), ER β nCt ($r(9) = 0.815$, $p = 0.007$, power = 0.84; see Fig. 3b), PR nCt ($r(9) = 0.818$, $p = 0.007$, power = 0.85; see Fig. 3c), and OTR nCt ($r(9) = 0.796$, $p = 0.010$, power = 0.81; see Fig. 3d), indicating that higher mRNA expression in the DS is associated with lower Total Social Investigation duration during T1.

4. Discussion

Overall, consistent with our selection of HR and LR groups, we found that HR mice scored high on all measures of social recognition (see Fig. 1), while the behavior of the LR mice did not demonstrate any social recognition. We also found that social recognition and levels of social investigation (indicative of social interest) were closely linked to gene expression in the brain.

4.1. Lateral amygdala

Lower expression of mRNA for all genes expressed, except for PR and OT, in the LA of HR mice (see Fig. 2a), taken together with the observed inverse relationship between AVP and the social investigation ratio at test (see Fig. 3e), supports the hypothesis that estrogens may modulate social recognition by acting on the OT and/or AVP systems in this brain region.

The LA has been implicated in both aversive and appetitive associative learning (e.g., [59,60]), and it is possible that the LR mice may have a general learning impairment due to the increased activation of the majority of the genes we studied in this region. Several studies have found that improved learning is associated with decreased gene expression or brain region activation (e.g., [49,61–64]), including, for example, those associated with estrogens administration [49,62]. This suggests that the lower LA gene expression observed in HR mice may indicate increased efficiency of LA-mediated learning mechanisms that would reflect in better performance in the social recognition learning paradigm.

Alternatively, as the LA has been repeatedly shown to mediate fear and anxiety (e.g., [65–68]), the higher expression of genes in the LR group suggests these mice may experience greater anxiety than HR mice. When considered with previous results implying that anxiety may modulate social recognition, affiliation and/or investigation (e.g., [34,69–71]), this suggests that the observed results could be partially explained by differences in anxiety. However, we observed no differences in anxiety-related behaviors, including stretched approaches and amount of investigation at T1, when both animals were novel. Thus, it is unlikely that anxiety was increased sufficiently to impair social recognition by itself.

Most research on social recognition in rodents has focused on the MA [5,18,19], and it is unclear why no differences were found in the current study. Differences in mRNA do not always translate to differences in proteins, and it is possible that had we measured levels of protein, we would have found more ER α and OTR in the MA as predicted by previous studies (e.g., [5,43]). The present results suggest that optimal levels of activation of the genes for estrogen receptors and receptors for their possible downstream targets OT and AVP in the LA may be required for social recognition; hence, the LA may be part of the brain circuit involved in the detection and recognition of social stimuli. Indeed, a general social behavior circuit has been proposed that includes the MPOA, DS, and amygdala, and these structures appear to be involved in key social interactions and their modulation by sex-steroid hormones [72].

4.2. Medial preoptic area

In the MPOA, ER mRNA expression was related to habituation, as were levels of both OT and AVP mRNA (see Fig. 4b–e). Additionally, HR mice expressed more OT and AVP than LR mice in this region (see Fig. 2b), which is a source of both peptides [73,74] and exhibits sexual dimorphism in estrogens, progesterone, OT and AVP [55,75–79].

Increased OT in the MPOA may be related to social interest in HR mice. No Fos activation has been observed in the MPOA of recognition-impaired OTKO males, unlike WT mice, following a brief social interaction, and OT administration to this region rescued social recognition in OTKO mice [25]. In addition, OT administration to the MPOA also prolonged [41,80] and improved social recognition in males [25], while administration of an OT antagonist to the MPOA blocked social recognition in WT mice [25].

AVP antagonist or antiserum treatment only impaired males [25,27,28,40], which suggests that female social recognition does not depend on AVP [1]. However, systemic AVP can improve social recognition in both sexes [1,25,40,81], and although AVP in the MPOA, especially in females, has received little investigation, our results suggest that higher levels of AVP in the MPOA may relate to better social recognition even in female mice. The correlations with ER α and ER β , as well as with OT and AVP mRNA, in our female mice suggest that, as has been proposed for other brain regions (e.g., [4,8]), MPOA OT and AVP involvement in female social recognition may be mediated by ERs.

The involvement of MPOA OT and AVP in social recognition, as well as in numerous social behaviors, including sexual and parental behaviors [82–93] suggests a fundamental role of these hormones in this brain region in the evolution of sociality. Complex social behaviors that depend upon social recognition (e.g., dominance hierarchies) have likely evolved from basic forms of social behavior involved in mating and reproduction [92], probably developing first through philopatry (the delay of dispersal [94]) and cooperation with kin and then expanding to more complex social behavior and social systems [93]. Consistently, AVP and OT are highly involved in maternal behavior: MPOA OTR and AVPR1a binding are higher in lactating than virgin dams, and MPOA release of AVP is increased in the presence of pups; similar results have been observed in the BNST [74,82]. Extension of MPOA social function from reproduction to social recognition may have been a first step in the evolution of sociality. Subsequent involvement of other so-called “social brain” regions would have led to the evolution of complex social behaviors and animal societies [95–97]. The involvement of MPOA OT and AVP in social recognition suggests that LR mice might be generally impaired in social behavior, including more evolutionarily primitive reproduction-related social behaviors from which other social behaviors may have developed [92].

4.3. Dorsolateral septum

While no group differences in behavior or gene expression were observed in the DS, possibly due to small DS sample size ($n_{HR} = 4$, $n_{LR} = 5$), social investigation at T1 had an inverse relationship with mRNA for receptors of both ovarian hormones and for OTR in this region (see Fig. 3a–d). These receptors are known to be related to social behaviors (reviewed in [1]), and our results are consistent with lower OTR levels observed in high investigating prairie voles [34]. Taken together, these results point to the involvement of septal female gonadal hormone receptors and OTR in social interest and approach.

4.4. Overall conclusions

Our results support the idea that variation in ovarian hormones could be related to individual differences in social recognition, at least partly through modulation of the OT and/or AVP

systems, particularly in the DS, LA and MPOA. As mRNA levels do not always reflect differences in protein levels, it should be noted that additional studies are necessary to determine how the observed differences in gene expression relate to levels of receptors and/or hormones, and whether these occur in the same brain regions as the mRNA changes or in regions to which they project.

Receptors for estrogens, progesterone and OT may be important for the regulation of initial social interest in the DS, which could relate to increased attention to social stimuli. Additionally, the LR's increased activation of the majority of gonadal and hypophyseal hormones tested in the LA may relate to general learning impairments, while lower mRNA levels in the HR mice could be indicative of increased efficiency of social recognition learning and/or memory mechanisms. Higher OT and AVP mRNA levels in the MPOA could facilitate social recognition by releasing more OT and AVP into the LS and LA, thus facilitating social recognition, as both the DS and the amygdala receive projections from hypothalamic OT- and AVP-expressing neurons [78,98,99].

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Fig. 1. Behavioural differences between high (HR; filled bars/circles) and low (LR; empty bars/triangles) recognizing mice. a) Ratios of investigating the novel mouse (Social Investigation Ratio) or cylinder containing the novel mouse (novel/(novel+familiar) in the test trial (T5). b) Habituation (Total Social Investigation of both stimuli at T4 – Total Social Investigation of both stimuli at T1). c) Social Investigation Ratio across trials. d) Total Social Investigation of both novel and familiar stimulus mice across trials. * indicates a significant difference between HR and LR mice, $p < 0.05$. † indicates a significant habituation effect (significant difference from 0), $p < 0.05$.

Fig. 2. Levels of mRNA for genes in HR (filled bars) and LR (empty bars) mice, relative to the average amount of gene product in HR animals (set at 1). a) Gene expression in the lateral amygdala. b) Gene expression in the medial preoptic area. * indicates a significant difference between HR and LR mice, $p < 0.05$.

Fig. 3.

Correlations between social behaviour and mRNA expression in the dorsolateral septum (DS) and lateral amygdala (LA). a) Total Social Investigation in the first trial correlated negatively with DS ER α mRNA, ($r(9) = 0.860$, $p = 0.003$). b) Total Social Investigation in the first trial correlated negatively with DS ER β mRNA, ($r(9) = 0.815$, $p = 0.007$). c) Total Social Investigation in the first trial correlated negatively with DS PR mRNA, ($r(9) = 0.818$, $p = 0.007$). d) Total Social Investigation in the first trial correlated negatively with DS OTR mRNA, ($r(9) = 0.796$, $p = 0.010$). e) Social Investigation Ratio at test (T5) correlated negatively with LA AVP mRNA, ($r(13) = 0.729$, $p = 0.005$).

Fig. 4.

Correlations between social behaviour and mRNA expression in the medial preoptic area (MPOA). a) Total Social Investigation in the test trial (T5) correlated negatively with MPOA ER α mRNA, ($r(13) = -0.758$, $p = 0.003$). b) Habituation (Total Social Investigation in T4 – Total Social Investigation in T1) correlated positively with MPOA ER α mRNA, ($r(13) = -0.769$, $p = 0.002$). c) Habituation (Total Social Investigation in T4 – Total Social Investigation in T1) correlated positively with MPOA ER β mRNA, ($r(13) = -0.747$, $p = 0.003$). d) Habituation (Total Social Investigation in T4 – Total Social Investigation in T1) correlated positively with MPOA OT mRNA, ($r(12) = -0.832$, $p = 0.001$). e) Habituation

(Total Social Investigation in T4 – Total Social Investigation in T1) correlated positively with MPOA AVP mRNA, ($r(13) = -0.761$, $p = 0.003$).

Table 1

Quantity of total RNA used in reverse transcription reactions by brain region.

Brain region	Total RNA/20 μl reaction mixture (μg)	High recognition	Low recognition
BNST ^a	1.5	n = 7	n = 7
Dorsolateral septum	1.0	n = 4	n = 5
Hippocampus	1.5	n = 6	n = 9
Lateral amygdala	1.5	n = 4	n = 9
Medial amygdala	1.5	n = 6	n = 9
MPOA ^b	1.0	n = 5	n = 8
PVN ^c	1.0	n = 6	n = 9
VMH ^d	1.5	n = 7	n = 10
Frontal cortex	1.0	n = 6	n = 10

^aBed nucleus of the stria terminalis.^bMedial preoptic area.^cParaventricular nucleus of the hypothalamus.^dVentromedial hypothalamus.

Table 2

Descriptions of behaviors collected during behavioral testing.

Scored Behaviors	
Vertical exploration	Movement to investigate upwards, lifting both front feet off the ground; includes sniffing, wall leans and lid chews.
Horizontal exploration	Locomotor activity; includes active sniffing of air and ground.
Digging	Rapid stereotypical movement of forepaws in the bedding; this behavior was not highly performed.
Self-grooming	Rapid movement of forepaws over facial area and along body.
Inactivity	No movement; includes sit, lie down and sleep.
Stretched approaches	Risk assessment behavior; back feet do not move and front feet approach the stimulus mouse. Only the frequency of stretched approaches was measured, and this behavior was not highly performed.
Social investigation	The experimental mouse actively sniffs the holes at the bottom part of the cylinder containing the stimulus mouse. Investigation of each stimulus mouse was scored separately for all trials.
Cylinder investigation	The experimental mouse sniffs the part of cylinder without holes. Investigations of the two cylinders were scored separately in all trials.
Calculated Behaviors	
Social Investigation Ratio	This ratio was created by calculating the ratio of Total Social Investigation that was focused on the “novel” stimulus, i.e., the cylinder that would contain the novel stimulus mouse in the test trial: (Investigation of “novel” stimulus/Total social Investigation). In the training trials, both stimulus mice should be equally familiar, and thus the ratio is expected to be around 0.5 (random chance). In the test trial, however, if the mouse can discriminate between the novel and familiar stimulus mice, it is expected that the mouse would explore the novel mouse significantly more than chance.
Total Social Investigation	The Total Social Investigation of the two stimulus mice; this was also evaluated as an overall measure of ‘social interest’.
Cylinder Investigation Ratio	The preference for the cylinder that at the test trial will contain the novel mouse was calculated as described for the Social Investigation Ratio above.
Total Cylinder Investigation	The total investigation of both cylinders.
Habituation (Social)	The Total Social Investigation at T1–the Total Social Investigation at T4.
Dishabituation (Social)	The Total Social Investigation at T5–the Total Social Investigation at T4.
Habituation (Cylinder)	The Total Cylinder Investigation at T1–the Total Cylinder Investigation at T4.
Dishabituation (Cylinder)	The Total Cylinder Investigation at T5–the Total Cylinder Investigation at T4.

Table 3

Differences in cycle thresholds (Cts) between High Recognition (HR) and Low Recognition (LR) mice by brain region.

Gene product	T-test	Effect size	Power
<i>Lateral amygdala</i>			
ER α ^a	HR > LR (t(11) = 2.26, <i>p</i> = 0.045)	1.307	0.510
ER β ^b	HR > LR (t(10) = 2.28, <i>p</i> = 0.046)	1.308	0.488
OTR ^c	HR > LR (t(11) = 2.66, <i>p</i> = 0.022)	1.301	0.506
AVP ^d	HR > LR (t(11) = 2.72, <i>p</i> = 0.020)	1.593	0.676
AVPR1a ^e	HR > LR (t(11) = 2.41, <i>p</i> = 0.035)	1.348	0.534
AVPR1b ^f	HR > LR (t(11) = 2.96, <i>p</i> = 0.013)	1.701	0.732
<i>Medial preoptic area</i>			
OT ^g	LR > HR (t(10) = -2.49, <i>p</i> = 0.032)	1.391	0.426
AVP	LR > HR (t(11) = -2.49, <i>p</i> = 0.030)	1.325	0.437

^aEstrogen receptor alpha.

^bEstrogen receptor beta.

^cOxytocin receptor.

^dVasopressin.

^eVasopressin receptor 1a.

^fVasopressin receptor 1b.

^gOxytocin.