Brain aromatase dynamics reflect parental experience and behaviour in male mice

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Abbreviations: BNST, bed nucleus of the stria terminalis; CAM, cortical amygdala; LS, lateral septum; LSD, lateral septum dorsal; LDI, lateral septum intermediate; LSV, lateral septum ventral; MAM, medial amygdala; MPOA, medial preoptic area; NAC, nucleus accumbens; PIR, piriform cortex; VMH, ventromedial nucleus of the hypothalamus.

Abstract

In most mammals, providing paternal care is not automatic. In house mice, experience with pups governs the extent and quality of paternal care. First-time fathers undergo a dramatic transition from ignoring or killing pups to caring for pups. The behavioural shift occurs together with changes in brain estrogen signalling as indicated by changes in estrogen receptor presence and distribution in multiple areas regulating olfaction, emotion, and motivation. Here, we report estrogen dynamics by altered local estrogen synthesis via changes in aromatase, a key enzyme catalysing the conversion of testosterone to estrogen. The amount of paternal experience (5 or 27 days) was associated with increased numbers of immunocytochemically-identified aromatase expressing cells in the medial and cortical amygdala, piriform cortex, and ventromedial hypothalamus. In the lateral septum, and to some extent in the medial preoptic area, parental experience increased aromatase only in fathers with 27 days of experience, and only in the right brain hemisphere, a new case of brain-functional lateralisation with experience. Nuclei/areas associated with maternal care (medial preoptic area, bed nucleus of stria terminalis, nucleus accumbens) exhibited a left-hemisphere advantage in aromatase expressing cells. This newly found lateralisation may contribute to the left-hemisphere dominant processing and perception of pup calls to release parental behaviour.

Introduction

Mammalian paternal care is facultative, but the behavioural and neural dynamics that result in becoming a caring father are less well understood compared to those of obligatory maternal care (e.g., (1–10)). In rodents and primates, it is hypothesised that maternal care is induced by hormonal changes during pregnancy and parturition (onset phase) whereas the continuation of pup-caring (maintenance phase) is governed by sensory stimuli from the pups (2,11,12). In house mice, pup-naïve females, males, and even previous fathers do not experience the onset phase but require various amounts of experience with pups (hours up to several days of contact) for the activation of caring behaviour (13–21). Nonetheless, pup-naïve females engage in consummatory (pup retrieval) and appetitive (preference for pup ultrasounds) behaviours (22,23) after fewer days of experience with pups than either pup-naïve males or fathers (14–16). Pup naïve females thus appear to be more easily sensitised or motivated by pup cues than males and even fathers to provide parental care, suggesting sex differences in neural mechanisms underlying the activation of maternal versus paternal instincts.

Numerous studies have characterised the interactions between limbic system nuclei and areas for the activation and regulation of parental behaviours. Principal nuclei and areas involved in parental care include the medial preoptic area (MPOA), the bed nucleus of the stria terminalis (BNST), the lateral septum (LS), the nucleus accumbens (NAC), nuclei of the amygdala, and the ventromedial hypothalamus (VMH). The main modulatory sensory inputs are thought to be olfaction via the vomeronasal organ to the BNST and amygdala, and via the olfactory bulb either to the amygdala and via the VMH to the LS or via the piriform cortex (PIR) and hippocampus to the LS (e.g., (2,3,7,23–26)). In females, neurons in these regions express estrogen receptors and so may be affected by levels of circulating estrogens during the course of the estrous cycle and during pregnancy and after parturition (12). However, estradiol treatment can activate both maternal and paternal instincts (17,19,27–31). In house

mice, expression of parental behaviours coincides with changes in the distribution of estrogen receptor (ERα) positive cells in limbic brain regions of pup-naïve and pup-experienced females (16,32) and males (14). For instance, in the MPOA of females and fathers, the number of ERα positive cells increased with pup contact and retrieving experience (14,16,32). In pup-naïve male and female voles, animals with spontaneously high levels of pup care showed more ERα positive cells in the MPOA than animals with low levels (33). Qualitatively similar distributions of ERα positive cells were observed in the limbic system between pup-naïve males after five days of experience (14) and pup-naïve females (34), potentially suggesting some levels of convergence in terms of the underlying circuit dynamics.

Unlike in females, low circulating levels of estrogens in males (35) imply that estrogens are primarily synthesised locally in the brain from testosterone by the enzyme aromatase (e.g., (36–38)). Locally synthesised estrogens have been implicated in the onset of paternal behaviours in California mice (39,40) and aromatase activity has been documented in the brains of adult male mice (41–43). Aromatase is expressed in the male mouse limbic system (LS, BNST, MPOA, nuclei of the amygdala, VMH, and PIR), in regions where ERα positive cells have been located in pup-experienced fathers (14). This suggests that estrogen signalling via ERα could be an important regulator of paternal behaviour via an aromatase-dependent estrogen supply. Our objective was therefore to examine changes in aromatase distributions during the onset and maintenance of paternal behaviour of male mice (pup-naïve and fathers) in brain regions known to exhibit changes in ERα positive cells (14).

Materials and methods

Animals

Adult male and female laboratory mice (outbred strain NMRI, 2-3 months old) with no previous sexual or parental experience were housed at the University of Ulm. As previously

(14), mice were randomly assigned to one of three treatment groups consisting of (1) naïve males without any experience with pups (N); (2) males co-caring with their female mate for their first litter for five days (5D); or (3) males co-caring with their female mate for their first litter until weaning and their second litter for five days. The total co-caring time of the latter males (first and second litter) was 26-27 days (27D) since after delivery of the first litter, females in post-partum estrus paired immediately and, while nursing the first litter up to weaning at 21 days, delivered the second litter after 21-22 days of gestation. Mice were housed at 22ºC and a 12 h light-dark cycle (starting at 7.00 h) in plastic cages (26.5 × 20 × 14 cm) either with other naïve males (group N; 4 brothers per cage) or with one female (groups 5D and 27D). Water and food were available *ad libitum*. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the appropriate authority (Regierungspräsidium Tübingen, Germany).

Pup retrieval test

Testing of pup retrieval behaviours was done as previously described (14,17). Male mice (alone or with litter mate and pups) were placed overnight on a running board (length 110 cm, width 8 cm) with a central nest depression (Fig. 1A) suspended in a sound-proof and anechoic room. The running board was supplied with mouse chow and nest material from the cage and covered with a plexiglass hood having an opening for the tip of a suspended water bottle. Retrieving tests were done under dim red light. Before the start of testing, the hood, and the female with the pups (groups 5D and 27D) were removed and placed back to their home cage outside the soundproof room. Seven pups were kept in the room to serve as targets for retrieving. The males were habituated for at least 30 min to the new situation after the hood (group N) or the hood and female with pups (groups 5D and 27D) were removed. Prior to testing males of the N group, five-day old foster pups were rubbed with nest material of the respective males to ensure a familiar scent. During the test, the seven pups (always 1-3 pups

at a time) were randomly placed on the running board, at least 30 cm away from the nest. A male was scored as ‘retriever’ if he retrieved all seven pups to the nest within 10 min. A male was scored as ‘non-retriever’ if he had not retrieved any pups after 10 mins. The distinction between retriever and non-retriever was clear-cut, because once a male had retrieved the first pup, he retrieved all. Retrieving tests were immediately stopped and the pups removed if a male attacked any of the pups (‘aggressor’).

Immunocytochemistry and microscopy

Immediately following the pup retrieval test, males were euthanised by cervical dislocation and their brains quickly dissected and frozen over liquid nitrogen. Brain serial frontal sections (30 µm) were cut on a freezing microtome (Thermo Scientific Microm HM560), mounted on slides, and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH=7.4) for 60 min. Sections were rinsed three times for 5 min with 0.1 M PB in between all subsequent processing steps. Slides were incubated with 0.2% Triton X-100 (Sigma) in 0.1 M PB for 45 min to permeabilise cells and with 1% H_2O_2 (Merck) in 0.1 M PB for 20 min to block endogenous peroxidase. In a humidified chamber, the sections were treated with 2% normal goat serum in 0.1 M PB for 60 min to block non-specific staining, then with the polyclonal rabbit anti-aromatase antibody (Abcam Cat# ab35604, RRID:AB_867729; 0.002 mg/ml in 2% normal goat serum) at 4ºC for 72h. Sections were then incubated with the secondary antibody from goat against rabbit horseradish peroxidase (Dako, Cat# PO448, 0.00125 mg/ml in 0.1 M phosphate buffer) at room temperature for 60 min, followed by staining with 0.015% diaminobenzidine tetrachloride (DAB; Sigma), 0.023% NiCl, and 0.013% H_2O_2 in 0.1 M PB for 7 min. Sections were rinsed in PB, dehydrated in a series of alcohols and xylene, and coverslipped with Entellan (Merck). The specificity of the aromatase antibody has previously been tested and confirmed for the mouse brain (44). We further controlled for non-specific labeling by incubating sections with PB

instead of the primary antibody. In all these control sections only non-specific background labelling was present. Aromatase-specific labelling was absent.

Sections were independently analysed by two experimenters blind to the source of the sections. In view of varying non-specific background labelling, the experimenters started the analysis by checking many sections from all animal groups in order to agree which kind of labelling would be considered aromatase-specific. Cells were considered as expressing aromatase if they were more intensely stained with shades of gray than the background and contained black granulae often grouped around the cell nucleus (Supplement Fig. 1). In cases in which a single or several black granulae without an associated cellular structure in shades of gray was seen, the structure was not counted as an aromatase-positive cell. This procedure led to (a) a high concordance of counting results of the two experimenters, (b) counts of only definite aromatase-positive cells, and (c) relatively low numbers of aromatase-positive cells per evaluated brain area.

Sections were analysed with a light microscope (Axiophot, Zeiss Germany). Brain areas were identified with 10x objective magnification, aromatase-positive cells were identified by focussing with a 40x objective magnification. The brain areas in which aromatase-positive cells were counted are listed in Table 1. These areas largely overlap with those showing ERα positive cell dynamics during the acquisition of pup-caring experience in male (14) and female (16) mice. The Bregma coordinates of the brain areas were from the mouse brain atlas (45) and anatomically matched across all experimental animals. Aromatase-positive cells were counted separately on the right and left hemispheres of the brain. Counts were averaged between two (LSD, LSI, LSV, BNST, NAC, VMH) or three (PIR, MPOA, CAM, MAM) sections for each animal (Supplemental Fig. 2).

Statistical Analyses

All statistical analyses were carried out using R v4.2.0 (46). Male behaviour in response to pup presentation was classified as ignoring, attacking, or retrieving a pup. The outcomes of behavioural trials were analysed as the multinomial probability of exhibiting a given response to pup presentation (aggression, ignore, retrieval) as a function of paternal experience (experience as ordered factor, N < 5D < 27D). To perform pairwise comparisons between the groups for each behaviour, we calculated the probability that the difference in the posterior predictive intervals between groups was significantly directional. We then corrected for multiple comparisons using the false discovery rate.

We combined two partially overlapping datasets of aromatase cell counts from two cohorts of animals to increase our statistical power to identify variation among cohorts and brain regions (Supplemental Fig 2). We observed significant count variation attributable to cohort (e.g., count variation between cohorts for the PIR -1.34 data; Supplemental Fig 2). To address this issue, each dataset was standardised by the mean and variance of paternal groups (5D and 27D; the groups for which the datasets were overlapping). Counts for some brain regions were missing in some mice. Rather than rejecting these mice altogether in our statistical model (described below), we imputed the missing observations based on values inferred from the non-missing entries (R package mice, 47). We generated five such candidate data sets and integrated over the resulting uncertainty.

Our statistical model of aromatase variation was designed to address non-independence arising from correlated variation within individuals among regions, which could be an especially important factor when comparing identifiable subregions of an area, such as in the piriform cortex and each part of the lateral septum. We therefore examined variation in normalised cell counts among all brain regions simultaneously (multivariate ANOVA), treating mouse ID as a correlated group (random) effect. Responses were modelled as *t*-distributed to increase robustness against outlier counts. We examined the interaction of Experience x Hemisphere together with the residual correlations among regions, which

represent relationships among brain regions which are not explained by the other population- and group-effects.

All models were fit using the R package *brms* (48), using four chains of 2500 iterations each, and a thinning rate of 5. We assumed uniform priors throughout. Typically, model convergence would be assessed by $\hat{R}$=1 (49) but because each imputed data set will generate slightly different estimates at convergence, $\hat{R}$ may be misleading. We therefore assured model convergence in a separate analysis of a single data set generated by averaging over the imputed data sets. We calculated the posterior means of each group based on the fitted statistical model (*emmeans* package (50)), and used these to examine the posterior distributions of contrasts between hemispheres (left-right) and between levels of experience (5D-N, 27D-N, 27D-5D) within each hemisphere. Significance of each contrast was judged as a Bayesian probability of direction P>0.975 (51), which is an index of effect existence, i.e., our level of certainty in whether a reported change is positive or negative. This threshold closely corresponds to the frequentist two-sided p-value of 0.05, and so for simplicity, we report the p-value. Plots were generated using the *ggplot2* package (52).

Residual correlations can point to latent (hidden) factors that determine correlated expression across the brain and/or unexpected technical covariation, so inspection of residual correlations can inform biological hypotheses. However, direct interpretation of the residual correlation matrix is challenging because the correlation between two brain regions could be due to mutual covariation with another region. We therefore dichotomised relationships among brain regions by studying the matrix of *partial* correlations (53,54). We constructed an initial graph in which edges were created between all nodes with significant (p<0.05) pairwise correlations. Each edge was iteratively pruned by testing whether the association remained statistically significant after testing it against all other paths between two variables, up to third order. The independence test was determined by the posterior distribution of residual correlation matrices implemented in the R package pcalg (55). The result is an undirected,

acyclic graph representing a network of associations among brain regions. The large number of tests may be conservative and may reject true associations, and thus we also report tentative links observed when setting a less stringent threshold of $p<0.10$.

Results

Male mice retrieve pups after acquiring parental experience

Only males with paternal experience retrieved pups (Fig. 1). Naïve males with no previous sexual experience and pup contact mostly ignored the pups (n = 5/8), although some were aggressive (n = 3/8). Most fathers with 5 days (5D) and 26-27 days (27D) of experience retrieved pups (n = 10/12 and 11/12, respectively). A small number of fathers ignored pups in the 5D (n = 2/12) and 27D groups (n = 1/12) but none were aggressive.

The probability of observing aggressive behaviour toward pups was significantly higher in naïve males compared to fathers regardless of experience (5D and 27D, Fig. 1B). The probability of other non-paternal behaviour like ignoring pups was higher in naïve males compared to 27D fathers. Parental behaviour measured as pup retrieval was significantly higher in fathers (5D and 27D) compared to naïve males. There were no significant differences in the probability of pup-retrieving between the groups of fathers, 5D and 27D (Fig. 1B).

Aromatase expression in the male mouse brain, general picture

We found aromatase-positive cells in each examined region and with each level of experience. In absolute terms, largest numbers of aromatase positive cells per section were seen in PIR, LSI, MAM, and CAM (see unstandardised data in Supplement, Fig. 2). Examples of aromatase labeling in the LSV, PIR, VMH and MAM of experimental groups N and 27D show qualitative differences between the groups and between left and right sides of the brain (Fig. 2). Standardised cell counts together with the group means are shown in Fig 3A along

with our estimates of the posterior differences in aromatase counts by hemisphere (Left – Right, Fig. 3B), and between levels of experience (Fig. 3C).

Lateralised expression of aromatase positive cells

We found evidence for significantly greater numbers of aromatase positive cells in the left hemisphere of the MPOA and BNST in all three experimental groups (Left – Right > 0, Fig. 3B). The NAC exhibited a similar trend to MPOA and BNST, but with a weakly insignificant contrast in the 27D group. We did not detect significant lateralisation of aromatase counts in any of the experimental groups in the amygdala (CAM and MAM), VMH, or posterior PIR (Bregma -1.34) in any groups. In the other regions, the appearance of lateralisation strongly depended on the level of experience. Long-term pup-experience (27D) led to greater numbers of aromatase positive cells in the right hemispheres of anterior PIR (Bregma 0.98), the LSV, and the anterior LSI (Bregma 0.14). Posterior LSI (Bregma 0.98) exhibited a similar trend that was weakly non-significant. In the LSD, pup-experience had a short-term effect (5D) during which counts in the left hemisphere were greater, which was not present in the 27D group (Fig. 3B).

Experience-dependent expression of aromatase

In all cases in which we found significant changes of aromatase counts with experience, the number of aromatase positive cells increased (Fig. 3C). This experience-dependent increase was pervasive for the amygdala (CAM and MAM), the VMH, and the posterior PIR (Bregma -1.34). For these regions, the increase in aromatase positive cells was present in both hemispheres and with increasing experience (i.e., experience contrasts had significant positive values for both brain hemispheres and all experimental groups). In the LSD, LSI, and LSV (both Bregma coordinates), and the anterior PIR (Bregma 0.98), aromatase positive cells increased significantly only in the right hemisphere of 27D males.

Experience-dependent effects were absent in the NAC, the BNST, and the MPOA, with the caveat of a significant effect in the right MPOA of 27D males (27D males had significantly more aromatase positive cells than 5D males). In fact, both BNST and MPOA exhibit trends toward increasing aromatase with experience, but this variation was not statistically significant (Fig. 3C).

Graphical analysis of covariation among brain regions

We examined the unexplained (residual) variation among all brain regions in the study (represented graphically in Fig. 4). Three subgroups of covariation are obvious. First, LS is highly connected (although not in order from dorsal to ventral), and each subregion (D, I, V) is correlated across Bregma coordinates and to at least one other subregion. LS appears to form the backbone of variation, to which other nodes are connected. This includes subgroups comprised of MPOA (connected to LSV), of NAC and BNST (connected to LSI), and of CAM and VMH (connected to LSD). Second, the subgroup of MAM and anterior PIR appears to be weakly connected to other parts of the graph (MPOA and VMH), because the edges are significant only with $p<0.10$. Third, posterior PIR did not appear to be even weakly correlated with any other analysed part of the brain despite an increase in aromatase expression with experience that is similar to CAM, MAM, and VMH (see Fig. 3).

Discussion

Aromatase is expressed throughout the male brain at considerable levels (41,43) , even in pup-naïve male mice (Fig. 3A). Together with this widespread distribution, levels of aromatase vary in an experience-dependent manner (Fig. 3). In mice, fathers that exhibit pup retrieval, have higher levels of aromatase immunoreactivity in certain limbic regions (VMH, amygdala, MPOA, NAC, LSD) after social contact with the female partner following retrieval tests (13). In mice, changes in aromatase expression have been observed in fathers in response

to pup and social experience, e.g. contact with the the mating partner after separation (13). In addition, inhibition of aromatase in fathers from day 1 to day 5 after birth of their first litter significantly inhibited the occurrence of pup-retrieval (13), which needs more than 1 day of pup contact to be securely established in untreated fathers (15). We therefore consider it likely that the presence and increase in brain aromatase expression observed here is integral to the adoption of parental behaviours in house mice. However, we find that the change in aromatase levels varies non-uniformly across limbic brain regions, broadly summarised in Fig. 5. Moreover, the individual expression of parental behaviours will likely depend not only on overall levels but the correlated changes among brain regions. We found significant residual correlations in aromatase counts among brain regions, pointing to intrinsic sources of variation in aromatase among individuals. Such correlations may ultimately contribute to understanding why some fathers ignored pups even though none attacked them. Here, we briefly review the contributions of limbic areas to paternal behaviours and experience.

Amygdala and ventromedial hypothalamus

Three nuclei, the MAM, CAM, and VMH were most affected by pup experience with large increases in the number of aromatase positive cells from N to 5D to 27D animals (Fig. 3 A and C; Supplement Fig. 2). These regions are associated with the regulation of aggression compared to sexual and parental behaviour (e.g., (56–62)). Important changes in aggression in the course of becoming a caring father are mediated by olfactory signals from the vomeronasal organ via the accessory olfactory bulb to the CAM and MAM (e.g., (58)) and concern the switch from aggressive to caring behaviour towards pups (21). Concomitantly, aggressive motivation in fathers increases against male intruders in order to protect their own pups from potential pup killers as shown in Californian mice (63) and prairie voles (64). This increased aggression towards intruders was not mediated by changes in ERα or ERß expression in limbic centers, but reflected in increased neuronal activation (FOS-expression)

in the MPOA, MAM and, potentially the VMH (63). We are not aware of respective tests in male house mice, or whether the decrease in aggression towards pups likewise alters aggression toward potentially dangerous intruders. Such tests combined with aromatase measurements could clarify whether the differentiation of aggression against kin or non-kin depends on aromatase levels in the amygdala and VMH.

Notwithstanding the increased numbers of aromatase-positive cells, a long-term increase in the number of estrogen receptors (ERα) in these nuclei has not been observed (14). This might have been expected because the presence of estrogen can induce the expression of its own receptors, for example in the VMH (65,66), especially in combination with parental experience (32). We suggest that the increase in aromatase activity in the MAM, CAM, and VMH in new (5D) and highly experienced (27D) fathers (Fig. 5) supports the control of instinctive behaviour (67) via increased local estrogen action (e.g., (36,37)).

Piriform cortex

In house mouse mothers, the performance of pup-retrieving does not depend on olfactory signals from the main olfactory bulb and its projections to the PIR (22). Pup-naïve females, however, needed this olfactory information for learning to become as maternal as the mothers (16,22). While learning pup cues for becoming maternal, virgin females with maternal experience have an increase in ERα positive cells in the posterior PIR compared to females without experience (also in the entorhinal cortex; (32)). Similarly, as males became caring fathers, ERα positive cells become visible in the posterior PIR (also in the entorhinal cortex; (14)). In view of the significant increase of aromatase expression in the posterior PIR with increasing paternal experience (Fig. 3C), it is possible that in PIR, unlike MAM, CAM, and VMH, may have induced its own receptors (tested for ERα) and enhanced olfactory learning of pup cues for increased paternal performance. The significant increase of aromatase positive cells only in the right hemisphere in the anterior PIR of 27D fathers does not directly

correlate with any evidence known to us. In studies on ERα expression in the limbic centres of female and male mice under various conditions of the reproductive cycle, experience with pups, and intact or lesioned olfaction, no hemisphere differences have been reported in any centre (14,16,22,32). The splitting of PIR in regions with experience-dependent lateralised (anterior PIR) and non-lateralised (posterior PIR) aromatase change shows compartmentalization of the PIR. Functional relationships need to be elucidated in further studies.

Lateral septum

The LS has a central functional role in the brain integrating cognitive and emotional information in the regulation of social relationships and instincts (68–70) including parental behaviour (71,72). Our analysis of residual correlations likewise (Fig. 4) points to a central role of the LS in the variation of aromatase expression in the mouse brain. Interestingly, these partial correlations reflect main neural connections of the subregions LSD and LSV. For example, there is input from the amygdala to the LS supporting the LSD-CAM partial correlation and there is an output from the LS to the MPOA supporting the LSV-MPOA partial correlation (68–70,73). Thus, at least some of the residual variation in aromatase expression (Fig. 4) appears to mirror functional connections and relationships of the LS.

The main effects of pup-experience on aromatase expression in the LS were changes in the left-right balance (Fig. 3B) and significant increase of aromatase-positive cells only in the right LS of highly experienced (27D) fathers (Fig. 3C). As in the PIR, ERα positive cells in the LS become visible in caring fathers compared to pup-naïve males (14). Since hemisphere differences in ERα positive cells have not been found, we cannot directly relate increased right-hemisphere aromatase expression with non-lateralised increase of ERα expression in the LS of both hemispheres. Lateralised input to the rostral parts of the LSD and LSI comes from the hippocampal CA1 and subiculum region (70,73). In rodents, the CA1

shows lateralisation in synapse morphology and receptor distribution (74,75) and left-right dissociation of memory processes (76) with right-hemisphere dominance of spatial memory in mice (77). Also, androgen receptor levels are higher in the left hippocampus in male rats (75). It is unclear, however, whether and how lateralised hippocampal input to the LS could have caused lateralised aromatase expression in the LS of 27D fathers (Fig. 3C). Right hemisphere lateralisation is associated with the regulation of mood, affect, and stress, and with sustained and global arousal and attention (e.g., (78,79)). Speculatively, our observed right-hemisphere LS bias in 27D fathers could be related to the stress of continuous arousal and involvement in a routine of pup-care.

Medial preoptic area, bed nucleus stria terminalis, and nucleus accumbens

The MPOA together with the BNST and their output via the NAC are essential for controlling parental behaviour in rodents (e.g., (23,24,80)). Significant increases have been observed in the MPOA both of the number of and area occupied by ERα positive cells in one-day pup-experienced virgin female mice (16) and in 5D and 27D pup-experienced father mice (14). The number of ERα positive cells increase in the BNST of one-day pup-experienced virgin females (16) and, although absent in pup-naïve males, ERα-positive cells are present in the BNST of 27D fathers (14,34). We did not observe a statistically significant change in the number of aromatase positive cells in the BNST and NAC and found only a small increase in the MPOA in just the 27D males relative to 5D. This suggests that changes in sensory processing and behavioural expression with experience in MPOA, BNST, and NAC may be more dependent on regulation of estrogen signalling through receptors rather than synthesis. Brain regions that regulate social behaviours, including parental care, are conserved in vertebrates (81). However, neuroendocrine ligand spatial profiles are more variable than receptor distributions within the social decision-making network among vertebrates, although there are regional differences (81). Our work supports experience-dependent regional

differences, and specifically that MPOA, BNST, and NAC may depend on changes in ERα distribution, whereas VMH and amygdala may depend on changes in the synthesis of the ligand. Overall, our results point to a region-specific regulation of receptor and ligand with parental experience.

The brain regions MPOA, BNST, and NAC in N and 5D mice exhibited substantial lateralization of aromatase, biased toward the left hemisphere (Fig. 3B). There is a left-hemisphere dominance for pup-ultrasound perception in mothers (82), a left-hemisphere dominant activation for pup-ultrasound processing in the posterior-dorsal field of the auditory cortex of mothers and pup-experienced females (83), and a left-hemisphere dominant expression of oxytocin receptors and higher rate and precision of neural responding in the primary auditory cortex of mothers and pup-experienced females compared to naïve animals (84). Pup-retrieving behavior was also accelerated by oxytocin action in the left auditory cortex (84). That is, ultrasounds of pups motivate caretaking adults to search for the caller (appetitive behaviour of the parental instinct) in order to be able to perform the consummatory act of pup-retrieving (17). This sound-dependent motivational activation is left-hemisphere dominant suggesting that the acoustic input signals to the MPOA, BNST and NAC are also more relevant when processed via the left hemisphere. Therefore, the greater left hemisphere aromatase expression in the MPOA, BNST, and for 5D and 27D fathers in the NAC, may prepare these centers for a preferred processing of pup ultrasounds in order to activate instinctive parental behaviour. This hypothesis may be tested via unilateral enhancement and/or suppression of neural processing in these neural centres.

Correlated individual variation in aromatase-positive cells between brain regions

The brain's connectivity suggests that cell counts within individuals may be correlated across regions. For example, aromatase could be uniformly lower or higher in some individuals compared to others, or could vary highly non-uniformly across brain regions. We

expect that such variation will contribute to individual differences in behaviour, such as some fathers continuing to ignore pups (Fig. 1). Our study finds significant residual correlated variation among brain regions (Fig. 4) that are not generally depicted in the data shown in Fig. 3. It is noteworthy that the posterior PIR and, to a lesser degree, the anterior PIR and the MAM remained outside this connectivity. Indeed, Supplementary Fig. 2 shows that posterior PIR (Bregma -1.34) is the sole case in which we observed substantial divergence in overall cell counts. Together, these observations suggest to us that there are additional sensory and genetic factors that modulate the numbers of aromatase-positive cells in PIR and MAM, which may integrate other information as brain pathways reorganise to accommodate paternal care, such as stress and arousal status. Our work therefore suggests that going forward, it will be fruitful to consider both the overall regulation of the entire network, along with relative and absolute differences in specific brain regions.

Conclusion

Aromatase expression in the male limbic brain is regulated with parental experience in a region-, hemisphere-, and experience-specific manner. Acquiring paternal pup-caring experience enhanced aromatase expression (number of aromatase positive cells) in brain centres processing olfactory information (PIR, CAM, MAM) and regulating aggression, sexual behaviour and other social relations (MAM, VMH, LS). In centers essential for controlling parental behaviour (MPOA, BNST, NAC), aromatase expression was instead present with left-hemisphere dominance. In general, the aromatase expression dynamics we observed did not mirror the previously reported patterns in estrogen receptors (ERα). This suggests that levels of estrogen receptors are not simply dependent on levels of locally produced aromatase-dependent estrogen through an autoregulatory feedback loop. Rather, paternal behaviour may depend on differential regulation of estrogen signalling through receptor or synthesis in a region-dependent way.

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Table 1. Brain regions analysed for aromatase expression and their Bregma coordinates from the mouse brain atlas (45).

Brain region		**Bregma Coordinates**
Lateral septum dorsal (LSD)	Anterior and posterior	0.98 mm and 0.14 mm
Lateral septum intermediate (LSI)	Anterior and posterior	0.98 mm and 0.14 mm
Lateral septum ventral (LSV)	Anterior and posterior	0.98 mm and 0.14 mm
Piriform cortex (PIR)	Anterior and posterior	0.98 mm and -1.34 mm
Nucleus accumbens (NAC)		0.98 mm
Bed nucleus stria terminalis (BNST)		0.14 mm
Medial preoptic area (MPOA)		0.14 mm
Cortical amygdala (CAM)		-1.34 mm
Medial amygdala (MAM)		-1.34 mm
Ventromedial hypothalamus (VMH)		-1.34 mm

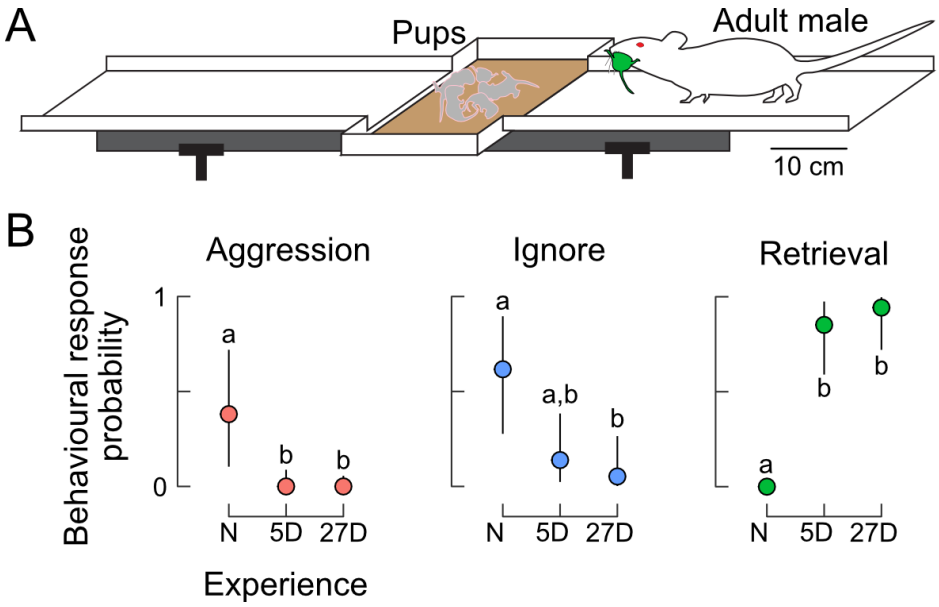


Figure 1. Pup-caring behaviour (retrieving) is determined by paternal experience. **A.** Experimental setting for the pup-retrieving tests on a running board with a central nest depression. Responses were scored as aggression (attacking pups), ignoring, or retrieving (returning pups to the nest). **B.** Probabilities of behavioural expression in naïve (N) males and in fathers with five (5D) or 27 days of experience (27D). Aggression declined immediately from N to 5D fathers. Although fathers occasionally still ignored pups, the probability of retrieval was very high. Letters indicate statistical differences between the groups of males.

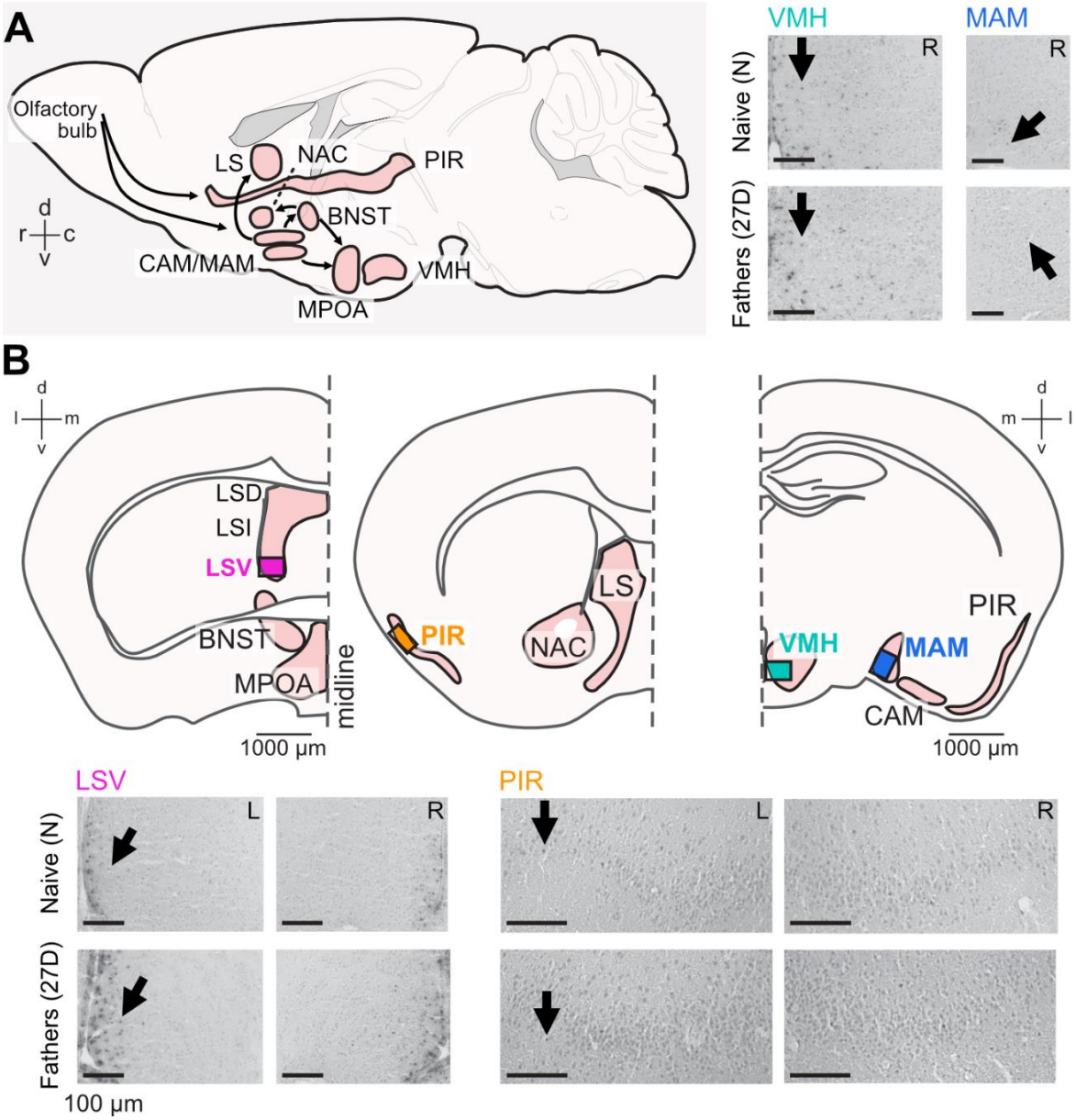
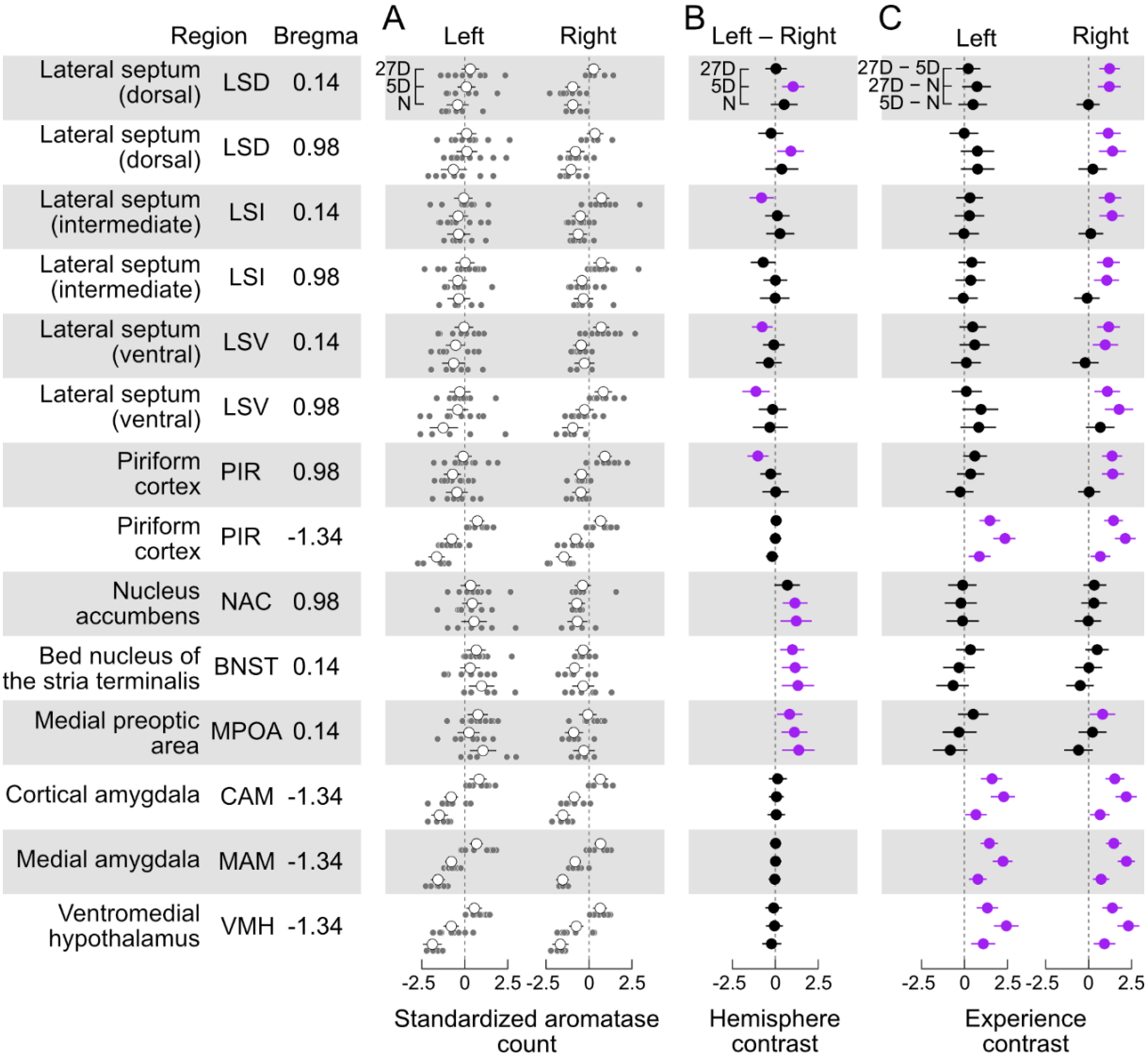


Figure 2. Aromatase expression in the paternal brain. Sagittal view (**A**) and cross sections (**B**) of the brain regions analysed here (Bregma coordinates: 0.98 mm; 0.14 mm; and -1.34 mm). Positions and extensions of nuclei/areas are illustrative only and do not indicate anatomical relationships. The connectivity denoted in (A) shown by the arrows are exemplars for main information pathways (7,85). Representative images from regions highlighted in (B), showing aromatase immunostaining (arrows) in naïve and experienced fathers (27D) in the left (L) and right (R) hemisphere. Scale bars in all images indicate 100 µm.


Region
Bregma
A
Left
Right
B
Left – Right
C
Left
Right
Lateral septum (dorsal) LSD 0.14
Lateral septum (dorsal) LSD 0.98
Lateral septum (intermediate) LSI 0.14
Lateral septum (intermediate) LSI 0.98
Lateral septum (ventral) LSV 0.14
Lateral septum (ventral) LSV 0.98
Piriform cortex PIR 0.98
Piriform cortex PIR -1.34
Nucleus accumbens NAC 0.98
Bed nucleus of the stria terminalis BNST 0.14
Medial preoptic area MPOA 0.14
Cortical amygdala CAM -1.34
Medial amygdala MAM -1.34
Ventromedial hypothalamus VMH -1.34
27D
5D
N
27D – 5D
27D – N
5D – N
-2.5 0 2.5
Standardized aromatase count
Hemisphere contrast
Experience contrast

493

Figure 3. Aromatase expression differentially depends on paternal experience and brain hemisphere. **A.** Standardised cell counts (light grey circles) and posterior means (± 95% confidence interval, white circles) for each investigated nucleus/area separately for the left and right sides of the brain and levels of pup-experience (N, 5D, 27D). **B.** Hemisphere contrast (left – right, with 95% confidence intervals) of the standardised counts of aromatase positive cells for each brain nucleus/area and experimental group. Contrasts with credible intervals that exclude zero are highlighted in purple ($p<0.05$, see text for description). We found evidence for lateralisation of aromatase cell counts both independent of (BNST, MPOA, and most likely NAC) and dependent on experience (most LS and anterior PIR (Bregma 0.98)). **C.** Experience contrast (5D–N; 27D–N, 27D–5D, with 95% confidence intervals) for the standardised counts of aromatase positive cells, separately for each hemisphere and brain nucleus/area. We found aromatase positive cells in posterior PIR (Bregma -1.34), CAM, MAM, and VMH increased with experience level. Expression of aromatase in the LS and anterior PIR increased only in the right hemisphere. No changes were observed in the NAC and BNST. Contrasts with credible intervals that exclude zero are highlighted in purple ($p<0.05$, see text for description). Sample sizes described in text.

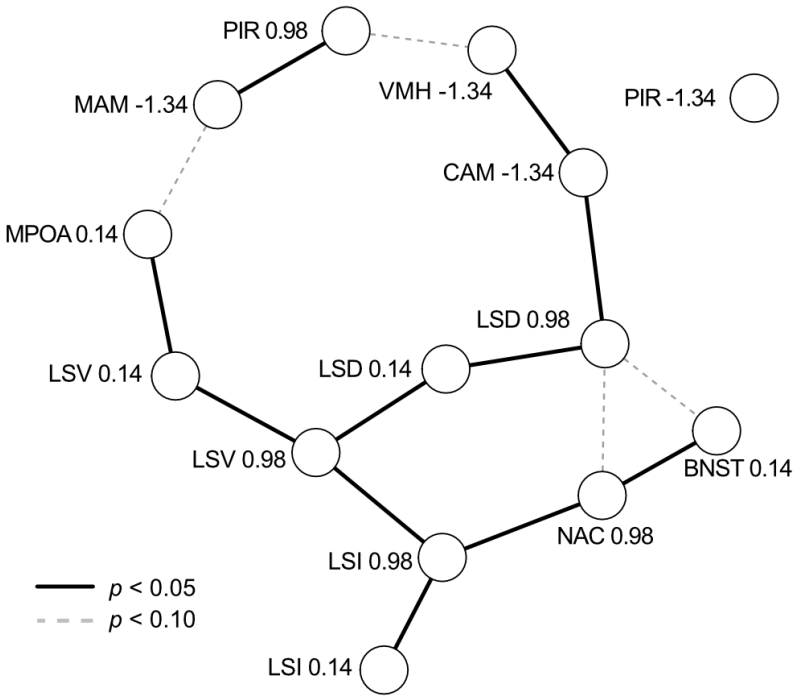


Figure 4. Residual correlations among brain nuclei/areas viewed as a network. Each nucleus/area at each Bregma coordinate (see Table 1) is represented by a node, with edges representing significant partial correlations (solid for $p < 0.05$, dashed for $p < 0.10$).

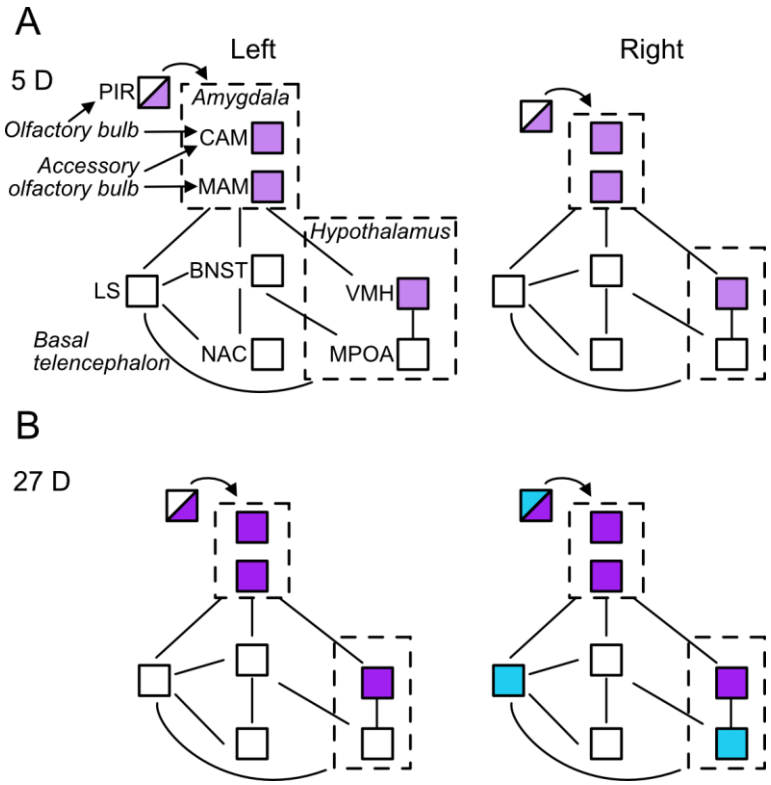


Figure 5. Effects of paternal experience on aromatase expression in areas of the limbic system. Summary of the changes in aromatase expression in 5D **(A)** and 27D **(B)** males in the left and right hemispheres of the brain nuclei/areas analysed in the current study. **A.** Pup experience in first-litter fathers (5D group) increased the numbers of aromatase positive cells (indicated by light purple) relative to naïve males in the amygdala (CAM and MAM), hypothalamus (VMH), and in posterior piriform cortex (PIR, half shading indicates these partial changes). **B.** Second-litter fathers (27D group) had further increased aromatase expression in the same areas as 5D males (indicated as darker purple), i.e., the amygdala (CAM and MAM), hypothalamus (VMH), and posterior piriform cortex (PIR) in both sides of the brain. In the right hemisphere, the number of aromatase positive cells increased (indicated by blue) in the anterior PIR, lateral septum (LS) relative to naïve and 5D males, and potentially in the MPOA relative to 5D males only.

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