Becoming a mother shifts the activity of the social and motivation brain networks in mice

Highlights
- Pups activate the sociosexual brain network of females more than nonsocial objects
- Pregnancy boosts motivation for pups and reduces incentive salience of buttons
- During pregnancy, specific circuits govern decision of caring or attacking pups
- The socio-motivational brain works as a network rather than a labelled-line circuit
Becoming a mother shifts the activity of the social and motivation brain networks in mice

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SUMMARY
During pregnancy hormones increase motivated pup-directed behaviors. We here analyze hormone-induced changes in brain activity, by comparing cFos-immunoreactivity in the sociosexual (SBN) and motivation brain networks (including medial preoptic area, MPO) of virgin versus late-pregnant pup-naïve female mice exposed to pups or buttons (control). Pups activate more the SBN than buttons in both late-pregnant and virgin females. By contrast, pregnancy increases pup-elicited activity in the motivation circuitry (e.g. accumbens core) but reduces button-induced activity and, consequently, button investigation. Principal components analysis supports the identity of the social and motivation brain circuits, placing the periaqueductal gray between both systems. Linear discriminant analysis of cFos-immunoreactivity in the socio-motivational brain network predicts the kind of female and stimulus better than the activity of the MPO alone; this suggests that the neuroendocrinological basis of social (e.g. maternal) behaviors conforms to a neural network model, rather than to distinct hierarchical linear pathways for different behaviors.

INTRODUCTION
Maternal behaviors are strongly adaptive, as they promote survival of the offspring until reproductive age and ensure proper neurodevelopment of pups (Curley and Champagne, 2016). Conversely, parental neglect or mistreatment has devastating effects on development and health of the offspring (Mehta et al., 2021). Understanding the neuroendocrine mechanisms of maternal care would therefore help avoiding maternal neglect and ensuring proper maternal care, promoting mental and physical health of both mothers and future generations (Kundakovic and Champagne, 2015).

Motherhood constitutes a special period in the lifespan of a female mammal. As observed in rodents, by the end of pregnancy females radically change their behavior, as they start building a nest where pups may be kept warm and safe (see Numan and Woodside, 2010), and furiously attack unknown adults approaching it. This phenomenon is known as maternal aggression (also called prepartum aggression in late-pregnant females; Mann and Sare, 1982; Mayer and Rosenblatt, 1984). After delivery, rodent females stop interactions with adult conspecifics and their social life becomes virtually restricted to dedicatedly taking care of their offspring. Thus, dams spend hours per day nurturing and licking-grooming pups while covering them with their own body to keep them warm. These changes in behavior are not merely due to the presence of pups, as virgin females either avoid pups (rats; Fleming and Rosenblatt, 1974) or show little motivation to take care of them (as in mice; Salais-López et al., 2021). In addition, even pup-sensitized virgin female mice displaying frequent pup care show no nest defense (Martín-Sánchez et al., 2015b). These data suggest that hormones acting on the brain of females during pregnancy and postpartum (steroids and lactogens; Salais-López et al., 2017) contribute to changes in their response to pup stimuli, including chemosignals (Navarro-Moreno et al., 2020), and other social cues, such as male pheromones (Martín-Sánchez et al., 2015a, 2015b). These changes promote a strong motivation to take care of pups and to defend the nest from putatively infanticide intruders. Where and how these changes in brain activity occur is still largely unknown.

Based mainly on early studies using lesion experiments in rats (Lee et al., 2000; Numan et al., 1990; Numan and Numan, 1996), there is a quite generalized view attributing a fundamental role in the expression of

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https://doi.org/10.1016/j.isci.2022.104525
maternal behaviors during postpartum to the medial preoptic region and parts of the adjoining bed nucleus of the stria terminals. Some later findings have further emphasized this view (Wu et al., 2014). In fact, pup-induced activity specifically occurs in galanin-expressing (Gal+) cells of the preoptic region in males and females expressing parental care (but not in males attacking pups). In addition, MPO-Gal+ cell's ablation impairs maternal behavior in virgin and lactating females and facilitates pup attack in (otherwise parental) virgin females and fathers. Finally, optogenetic activation of Gal+ preoptic cells promotes pup care and suppresses pup attack in virgin male mice.

Taken together, these pieces of evidence led to a “linear view” of the neuroanatomy of maternal care, according to which, activation of the medial preoptic area (MPO), or even a specific cell population within it, would generate parental care and suppress pup neglect or attack (Wu et al., 2014). The activity of the MPO as the “maternal brain center” would be boosted by hormones (estrogens and prolactin/lactogens) (Numan and Woodside, 2010; Numan and Young, 2016) during pregnancy and postpartum. Other nuclei might influence the activity of the MPO, such as serotonergic inputs from the raphe (Lu et al., 2001), vasopressinergic inputs from the paraventricular hypothalamus (Kohl et al., 2018), or dopaminergic afferents from anteroventral periventricular nucleus (Scott et al., 2015).

By contrast to this linear view, recent literature highlights that maternal allostasis depends on multiple systems affecting the activity of many brain centers, such as neuroendocrine systems involved in food intake, energy expenditure, and stress (Russell and Brunton, 2019), as well as oxytocin and endogenous opioid systems (Wallin et al., 2021). Based on this and other lines of evidence, Swain and Ho (2019) propose the concept of maternal behavior neurocircuit, an evolutionarily conserved, widespread neural network controlling parenting under the influence of hormones and opioids; this recalls the socio-sexual brain network (SBN) concept, coined by Sarah Winnans Newman two decades ago (Newman, 1999), a network of interconnected brain centers whose activity profile would allow expression of different social behaviors (sexual, agonistic, affiliative; we must add parental conducts) under the influence of steroid (and maybe other) hormones.

Motivational aspects of social behaviors have deserved specific attention (O’Connell and Hofmann, 2011), and this aspect of maternal behavior has also been linked to the theory of the MPO as the maternal brain center. Thus, it has been shown that, through descending projections to the ventral tegmental area (VTA), the MPO would enhance activity of the tegmento-striatal pathway in response to pups (Fang et al., 2018; Numan et al., 2009; Numan and Smith, 1984; Numan and Young, 2016), thus leading to pup-directed motivated behaviors typical of motherhood (Pereira and Morrell, 2011).

Despite this, several data support important participation of other nuclei (besides the MPO) in the control of motivated maternal behaviors. Thus, the basolateral division of the amygdala has also been implicated in motivated maternal behaviors through direct amygdalo-striatal pathways (Lee et al., 2000; Numan and Woodside, 2010). Recent work has also shown how increased motivation of females for pups during motherhood is associated with prolactin signaling in several locations of the brain, not just the MPO (Salais-López et al., 2021). In fact, mapping the action of prolactin and placental lactogens in the brain of female mice (Salais-López et al., 2017, 2021), as well as the distribution of estrogen receptors in the brain of rodents (Mitra et al., 2003; Simerly et al., 1990) indicate that many centers, besides the MPO, are targeted by lactogens and steroids. Thus, the linear view of maternal care does not fit the widespread action of these hormones in the brain of females. In addition, recent evidence indicates that sensory centers not directly linked with the MPO also change their activity profile in response to pups during late pregnancy (Navarro-Moreno et al., 2020). However, how and where the combined action of lactogens (placental and hypophyseal, e.g. prolactin) and steroids during pregnancy might change the response to pup-derived and other social stimuli during motherhood is still largely unknown.

Trying to fill this gap, in the present work we have exposed adult virgin and late-pregnant (LP) females to pups or to a nonsocial control stimulus (buttons), and we compare the activity of several socio-sexual and motivational centers of their brain by analyzing the density of cells expressing cFos protein in these brain nuclei. The advantage of using LP instead of postpartum females to study the neurobiology of motherhood is that they have already undergone the effects of pregnancy hormones but they are pup-naive, so that pups constitute an equally novel stimulus for LP and virgin females. Therefore, differences in pup-directed behaviors and pup-induced brain activity between females can be safely attributed to the effects of
pregnancy hormones but not to previous experience with pups (important in postpartum females). In fact, pups induce similar behaviors in LP and virgin female mice, except for pup-directed attacks, which are exhibited by some LP but not virgin females (Navarro-Moreno et al., 2020; Table S1).

Here, we focus on the brain centers and circuits directly involved in the expression of social behaviors, the socio-sexual brain network (Newman, 1999), as well as the main brain centers involved in motivated and goal-directed behaviors. Specifically, we aim to check whether changes in brain response to pups induced by pregnancy hormones are restricted to the MPO or, alternatively, a network view may better explain motherhood-associated changes in brain activity elicited by pups and parenting/pup attack. To do so, we combine classic statistics (ANOVA analysis of data from individual nuclei or equivalent nonparametric tests) with a somewhat innovative statistical approach consisting of a global (data of all the brain nuclei under scrutiny) analysis of principal components and linear discriminant analysis.

Together, our results confirm important pregnancy-related changes in the response to pups and buttons in the SBN and, even more pronouncedly, in the motivation brain circuitry. Moreover, the activity of the MPO alone has a poor predictive power of whether a female is pregnant or not and whether it was exposed to pups or buttons. By contrast, considering the data of the whole system (SBN and motivation circuitry) allows classifying animals with a high accuracy and sensitivity. These findings strongly recommend the use of a network, rather than hierarchical perspective, to understand the neurobiology of social conducts, including maternal behaviors.

RESULTS

To investigate this issue, we exposed pup-naive late-pregnant females (LP) and virgin females (V) to three- to four-day old pups (P) or to a novel, nonsocial control stimulus, buttons (B) of a size similar to pups. We recorded interaction of the females with pups during the first 30 min after exposure and, 1 h afterward, females of all four groups (LP-B; LP-P; V-B, V-P) were perfused with fixative solution under deep anesthesia and their brains sectioned and processed for cFos immunohistochemistry.

The data on behavior of late pregnant and virgin females exposed to pups (LP-P and V-P, respectively) were reported in a previous paper (Navarro-Moreno et al., 2020), and a summary of the results is shown as supplemental information (Table S1). In the present study, we analyzed the density of cFos immunoreactive cells in specific frames of brain centers belonging to the socio-sexual brain network (Figures 1 and 2) and the centers involved in motivated behaviors (Figure 3).

The SBN shows a differential response to pups in virgins and pup-naive LP females

We compared the expression of cFos in four nuclei of the SBN of both virgin and LP pup-naive female mice, e.g. lateroventral septum (LSV), medial amygdala (posteriorodorsal division, MePD), medial preoptic nucleus (MPO) ventromedial hypothalamic nucleus (ventrolateral division, VMHVL), and the lateral columns of the periaqueductal gray (PAG). Data on the MePD were already reported in our previous paper (Navarro-Moreno et al., 2020) devoted to the impact of pregnancy on chemosensory processing. In these nuclei, a two-way ANOVA with FEMALE (LP versus V) and STIMULUS (P versus B) as the main factors (raw or log-transformed data when needed to achieve normality), revealed a significant effect of STIMULUS, with exposure to pups eliciting higher levels of cFos expression than buttons in the MePD (F1,26 = 81.312, p < 0.001) (Figure 1A), LSV (U = 15, p < 0.001) (Figure 1B), MPO (t = -4.17, p < 0.001) (Figure 1D), and the VMHVL (F1,26 = 48.966, p < 0.001) (Figure 2B). In addition, in the MPO and LSV there is a surprising significant effect of FEMALE (p < 0.03 in both nuclei) with virgins showing globally (pooling females exposed to both stimuli) higher cFos immunoreactive (cFos-ir) cell density than LP females.

By contrast, the paraventricular hypothalamic nucleus (anterior part, PaA), whose oxytocinergic cells have been involved in some aspects of maternal behavior (e.g. anxiety suppression; Knobloch et al., 2012), showed no significant differences in the density of cFos-ir cells between stimuli (two-way ANOVA, F1,26 = 2.749, p = 0.11; see Figure 2A) or females (p = 0.588). Given the preeminent role attributed to oxytocin (OT) in behavioral changes associated with motherhood (e.g. Numan and Young, 2016; Tsuneoka et al., 2013), we specifically analyzed an area of the preoptic region that is enriched in OT-immunoreactive cells, the so-called anterior commissure nucleus/antero-dorsal preoptic area (AC/ADP; Otero-García et al., 2016). Here, there is not just increased activation by pups as compared with buttons (F1,26 = 37.623, p < 0.001) but also a significant FEMALE×STIMULUS interaction (F3,26 = 4.789, p = 0.038) that indicates...
Figure 1. Expression of cFos (immunohistochemistry) in some nuclei of the socio-sexual brain network (SBN)

Expression of cFos (immunohistochemistry) in some nuclei of the socio-sexual brain network (SBN) of late-pregnant (LP) and virgin (V) females exposed to pups (P) or buttons (B). The column at left shows low-power pictures of the sections with indication of their approximate antero-posterior level relative to bregma (according to Paxinos and Franklin, 2004) and frames or dotted lines (regions-of-interest, ROI) delineating the location of the nuclei analyzed. The nuclei sampled are the posterodorsal medial amygdala (MePD; A), the ventrolateral septum (LSV; B), the region of the nucleus of the anterior commissure/anterdorsal preoptic nucleus (AC/ADP; C), and the medial preoptic area (MPO; D). The central column shows examples of microphotographs of the brain regions analyzed for each experimental group: virgins exposed to buttons (V-B), virgins exposed to pups (V-P), late-pregnant exposed to buttons (LP-B), and late-pregnant exposed to pups (LP-P). Scale bars correspond to 250 μm for the first column and 100 μm for the second one. The column at right shows bar histograms illustrating the density of c-Fos positive cells (mean ± SEM; log-transformed data for those nuclei not showing a normal distribution of data) of each nucleus in each group of females (V-B, green; V-P blue; LP-B yellow; LP-P red). Individual data of each animal are also represented (circles; open circles correspond to females showing pup-directed attacks). When significant, the main effects revealed by the ANOVA are indicated on top of each histogram. Where there is a significant FEMALExSTIMULUS interaction, significant post-hoc comparisons (with Bonferroni corrections) are also illustrated on the histogram (***p < 0.001, **p < 0.01, and *p < 0.05). Other abbreviations: 3V, third ventricle; acp, anterior commissure, posterior; BMA, basomedial amygdaloid nucleus, anterior part; BST, bed nucleus of the stria terminalis; BSTIA, intra-amygdaloid division of bed nucleus of the stria terminalis; cc, corpus callosum; CPu, caudate putamen; D3V, dorsal third ventricle; ec, external capsule; f, fornix; ic, internal capsule; LSD, lateral septal nucleus, dorsal part; LV, lateral ventricle; MePV, medial amygdaloid nucleus, posteroverentral part; MS, medial septum; opt, optic tract; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus; PVA, paraventricular thalamic nucleus, anterior part; sm, stria medullaris of the thalamus; st, stria terminalis; VP, ventral pallidum.
sharper differences between stimuli in LP as compared with virgin females (Figure 1C). Similar findings are observed in the MePD (significant FEMALExSTIMULUS interaction; $F_{2,25} = 15.277, p = 0.001$; see Figure 1A), a nucleus that is usually considered the chemosensory interface of the SBN. In both nuclei, AC/ADP and MePD, analysis of this interaction using post-hoc tests revealed that the response to pups is similar in LP and virgin females ($p = 0.57$ for AC/ADP, Figure 1C; although there is a trend in MePD, $p = 0.055$, Figure 1A), but there is a significant decrease in the response to buttons in LP females as compared with virgins ($p = 0.018$ for AC/ADP; $p = 0.002$ for MePD); this suggests that LP females may partially ignore nonsocial salient stimuli, such as buttons.

Finally, we also analyzed the cFos-ir cells in the lateral column of the periaqueductal gray (PAG) (Figure 2C), a brain region modulating social and motivated behavioral responses and known to be involved in...
Figure 3. Expression of cFos (immunohistochemistry) in nuclei of motivation brain circuitry
Expression of cFos (immunohistochemistry) in nuclei of motivation brain circuitry of late-pregnant (LP) and virgin (V) females exposed to pups (P) or buttons (B). The column at left shows low-power pictures of the sections with indication of their approximate antero-posterior level relative to bregma (according to Paxinos and Franklin, 2004) and frames or dotted lines (regions-of-interest, ROI) delineating the location of the nuclei analyzed. The nuclei sampled are prefrontal and infralimbic cortical areas (PrL-IL; A), the anterior part of the basolateral amygdaloid nucleus (BLA; B), the nucleus accumbens shell and core (AcbSh and AcbC respectively, C and D), and the anterior and posterior parts of the ventral tegmental area (VTaA and VTAp; E and F). The central column shows examples of microphotographs of the brain regions analyzed for each experimental group: virgins exposed to buttons (V-B), virgins exposed to pups (V-P), late-pregnant exposed to buttons (LP-B), and late-pregnant exposed to pups (LP-P). Scale bars correspond to 250 μm for the first column and 100 μm for the second column. The column at right shows bar histograms illustrating the density of c-Fos positive cells (mean ± SEM; log-transformed data for those nuclei not showing a normal distribution of data) of each nucleus in each group of females (V-B, green; V-P, blue; LP-B, yellow; LP-P, red). Individual data of each animal are also represented (circles, open circles correspond to females showing pup-directed attacks). When significant, the main effects revealed by the ANOVA are indicated on top of each histogram. Where a significant FEMALEXSTIMULUS interaction was present, significant post-hoc comparisons (with Bonferroni corrections) are also illustrated on the histogram (**p < 0.01, *p < 0.05). Abbreviations: ac, anterior commissure, anterior part; Aq, aqueduct (Sylvius); BMA, basomedial amygdaloid nucleus, anterior part; cp, cerebral peduncle, basal part; CPu, caudate putamen, ec, external capsule; EW, Edinger-Westphal nucleus; fmi, forceps minor of the corpus callosum; gcc, genu of the corpus callosum; ic, internal capsule; IPC, interpeduncular nucleus, caudal subdivision; IPR, interpeduncular nucleus, rostral subdivision; lo, lateral olfactory tract; LS, lateral septal nucleus, LV, lateral ventricle, MePD, medial amygdaloid nucleus, posteroventral part, MePV, medial amygdaloid nucleus, posteroverentral part; ml, medial lamniscus; opt, optic tract; PAG, periaqueductal gray; Pir, piriform cortex, PLCo, postrolateral cortical amygdaloid nucleus; PP, peripeduncular nucleus, RMC, red nucleus, magnocellular part; RPC, red nucleus, parvicellular part; SN, substantia nigra; VDB, nucleus of the vertical limb of the diagonal band.

The motivational circuitry changes its response to pups/buttons during pregnancy
We also analyzed cFos-ir cell density in the main nuclei of the motivational brain circuitry in female mice: ventral tegmental area (anterior, VTaA; and posterior division, VTAp); nucleus accumbens (core, AcbC; and shell AcbSh); anterior basolateral amygdaloid nucleus (BLA); and prefrontal/infralimbic areas of the prefrontal cortex (Pr/L/IL) (Figure 3). Two-way ANOVAs revealed a highly significant FEMALEXSTIMULUS interaction (F3,26 = 13.170, p = 0.003). The post hoc analysis reveals that pups induce higher response in LP compared with virgin females (p = 0.016), whereas buttons induce higher response in virgins than in LP females (p = 0.047). Moreover, pups raise higher response than buttons in LP females (p < 0.001), but no differences between stimuli are observed in virgin females (p = 0.6). In other words, pups activate the PAG more than buttons only during pregnancy.
Pregnancy reduces salience of nonsocial objects

As indicated earlier, in several nuclei of the SBN and motivation brain circuit, pregnancy seems to reduce the activity induced by nonsocial control objects, e.g. buttons; this can be due to a pregnancy-induced insensitivity to button-derived stimuli, a decreased salience of those objects resulting in reduced exploration, or both. To study this, we performed an additional experiment in which we analyzed the behavior of two groups of females identical to those of the cFos experiment (virgins and LP; pairs of same-condition females) for 5 min after introducing eight buttons in their home cage.

The results (Figure S1) indicate that virgins explore buttons significantly more than LP females (more episodes of button sniffing; more time engaged in sniffing buttons); this is not a general effect of pregnancy on mobility or on interest on exploring objects in their environment, as inter-female interactions (sniffing to the other female in the same cage) do not differ between virgin and LP females.

As a conclusion, it seems that pregnancy reduces specifically interest in nonsocial objects such as buttons; this might have resulted in reduced cFos activity in many nuclei of the brain in LP females exposed to buttons (as compared with button-exposed virgin females).

Correlation between brain activity and maternal behaviors

Using the same strategy as in our previous report (Navarro-Moreno et al., 2020), here we explore anatomo-functional relationships within the SBN and motivation circuits of the brain based on analysis of correlation between cFos-ir cell density and behavior in pup-exposed females, both virgins and LP. There are significant correlations between brain activity and a few behaviors, but those differ between LP and virgin females.

First, cFos-ir cell density and pup aggression score showed a highly significant, strong, and negative correlation in the VTAp (Rho = 0.925; p = 0.003; see Table S2 and Figure S2A), e.g. the higher the activation of this brain region, the lower the likelihood that the female attacks pups. By contrast, the cFos-ir cell density in the PAG shows a positive correlation with pup aggression (Rho = 0.964; p < 0.001; see Table S3 and Figure S2I). Virgin females do not show pup attacks and, therefore, there is no correlation of this behavior with brain activity in virgins.

Another behavior displayed by females in the presence of pups is what we call “approach-and-retreat,” a reaction similar to risk assessment: the female approaches a pup, sniffs at it for a while, and then retreats without retrieving the pup, e.g. the female takes the decision of not to pick up a pup after exploring it. Although virgin and LP females do not differ in the expression of these approach-and-retreat responses (Table S1), in LP females this behavior is significantly and negatively correlated with cFos-ir cell density of two nuclei of the motivation brain circuitry, the BLA (Rho = −0.852; p = 0.015) and the AcbC (Rho = −0.778; p = 0.039) (Table S2 and Figures S2B and S2C). Although causal relationships cannot be established through correlational analyses, the activation of these two connected centers of the motivation circuitry (Novejarque et al., 2011) is associated with LP females not avoiding pups after detecting and sniffing them. By contrast, in virgin females the more approach-and-retreat responses to pups, the higher the activity in the AcbC (Rho = 0.801; p = 0.017). This finding further reveals important changes in the activity of the motivation circuitry of the brain in response to pups during pregnancy. A similar situation is found in the MePD and the PAG, where approach-and-retreat responses to pups are positively correlated with cFos-ir cell density in virgins (Rho = 0.801; p = 0.017 for the MePD and Rho = 0.726; p = 0.041 for the PAG) but not in LP females (Table S3, Figures S2D and S2H).

Finally, nest building and retrieval are quite common behaviors in virgin and LP females exposed to pups (Table S1). Our data indicate that occurrence of nest building behavior is significantly and positively correlated with activation of the VTAs only in LP females (Rho = 0.756; p = 0.049; see Table S2 and Figure S1E), whereas pup retrieval is negatively correlated with activation of the PaA in LP females (Rho = −0.826; p = 0.022; see Table S3 and and Figure S1F). As couples of same-condition females were tested together, we also analyzed inter-female interaction (affiliative behaviors), which negatively correlates with the activity of the VMHVL in LP females (Rho = −0.954, p = 0.001) (see Table S3 and Figure S1G).

Principal component analysis supports the SBN/motivation circuitry identity

In the previous sections, we have analyzed independently the data of cFos expression of each nucleus and their possible correlation with pup-induced behaviors. To further understand how the whole brain network...
We performed a principal component analysis (PCA) after standardizing each variable to have mean equal to zero and SD (standard deviation) equal to 1. From the 13 principal components obtained, we focus on the two main principal components that together explain almost 65% of the variance (Figure 4A). The contribution of the remaining principal components (PCs) to data variability is much lower (<9%).

For the first principal component, the loading factor vector $\phi_1 = (\phi_{11}, \cdots, \phi_{1n};$ see Star Methods) only shows positive factors (see first coordinate of vectors in Figure 4B), so that PC1 is a weighted average of the degree of activity of the whole system. As all the variables have been standardized (transformed to mean equal to zero and SD equal to 1), positive and negative PC1-scores are obtained. PC1 scores are high for females exposed to pups (either virgins or LP), thus indicating a strong brain activation induced by exposure to pups.
by pups in both kinds of females. However, buttons activate brain centers of virgins (high values of PC1), but not those of LP females, thus fitting the results of group comparison analysis (standard statistics).

By contrast, PC2 has positive and negative loading factors for the different nuclei. Positive loading factors correspond to the nuclei belonging to the motivation brain circuitry (with the only exception of AcbSh, whose loading factor is nearly null; 0.00928) and the PAG, whereas the remaining nuclei of the SBN show negative loading factors (see the second coordinate of the vectors in Figure 4B); this indicates that the studied nuclei actually belong to two different functional systems that respond differently to pups and buttons in the two groups of females. The PAG is an exception to that rule, as it belongs to the SBN but presents a positive loading factor, similar to most motivational nuclei; this is probably reflecting a role of the PAG not just in the expression of social behavior (as part of the SBN) but also in motivation (e.g., expression of motivated attacks to pups, see Discussion), as suggested by direct neuroanatomical connections between the PAG and VTA and other functional and neurochemical data (Ntamati et al., 2018; Vázquez-León et al., 2021).

The vectors representing the loading factors of PC1 and PC2 within a given functional system are all of them oriented similarly as the angles they form with the axes are similar, thus indicating that the activities of these nuclei are positively correlated; this is true for both the SBN and the motivation circuitry (with the exceptions of the AcbSh and PAG). By contrast, the vectors corresponding to the loading factors of the SBN centers are nearly perpendicular to those of the motivation circuitry, revealing a lack of correlation between the activities of both systems.

Notably, PC2 gives a quantitative measure of the balance between both systems under the different situations, e.g. female status and stimulus presented. High, positive PC2 scores indicate a large activation of the motivational circuit with minor activation of the SBN. As shown in Figure 4B, this is the case of virgin females exposed to buttons. On the other hand, virgins exposed to pups showed negative scores for PC2, fitting a higher activation of the SBN than the motivation circuit.

In addition, PC2 scores for LP females exposed to buttons are virtually zero in most cases, probably reflecting a low, similar activation of socio-sexual and motivational nuclei in these conditions. Finally, LP females exposed to pups display more variability in PC2 scores than LP-B females, meaning a variable, high activation of the SBN and the motivational system. But altogether, LP-P females showed more neutral scores of their PC2 scores than both virgin groups.

**Discriminant analysis: Activity pattern of the SBN/motivational circuitry predicts the condition of the female and stimulus**

Finally, we decided to study whether the pattern of activity of the brain of the females of each condition (group/stimulus) was able to predict the animal condition. There are different classification techniques, such as multinomial regression or linear discriminant analysis, which can be used in this context. We have carried out a linear discriminant analysis (LDA) to predict a response variable, Y (with 4 categories: Class 1 = LP-P; Class 2 = LP-B; Class 3 = V-P; Class 4 = V-B) from:

a) The cFos-ir cell density values of the individuals in the p nuclei analyzed (p = 13 in our case).

b) In addition, as the medial preoptic area is claimed to be the main nucleus controlling expression of maternal behaviors (see Kohl and Dulac, 2018), we also performed a linear discriminant analysis using as predictor just the data of the MPO, in order to clarify whether its activity can explain by itself the response of the females to the stimuli.

**Linear discriminant analysis using the original data (cFos-ir cell density)**

The coefficients of the three linear discriminant functions built from the cFos-ir cell density in the 13 nuclei analyzed are shown in Figure S3A. The proportions of trace of the two first LD functions explain more than 86% of the between-class variance (Figure S3A). As shown in Figure 5A, the first discriminant function (LD1) gives positive scores for females exposed to pups and negative values for females exposed to buttons, allowing to distinguish between both groups (Figures 5, S3B and S3C). The scores of the individuals in the second discriminant function (LD2) show negative values for LP-P and positive values for most V-P, allowing to distinguish between both groups, although the distinction is not so clear between LP-B and V-B.
Last, the third discriminant function (LD3) gives positive scores to LP-B and negative scores for V-B, whereas in females exposed to pups the majority of scores are closer to zero (Figures S3B and S3C).

Classification methods are applied with these data based on the iterative leave-one-out cross-validation procedure. The results indicate that, when applied to cFos-ir cell density in the 13 nuclei of interest (see Figure 5), classification based on linear discriminant analysis assigns correctly all the females to their actual group with the exception of only two individuals, i.e. a V-B is classified as an LP-B, and a V-P is classified as an LP-P. No mistakes relative to the stimulus occur, therefore. This classification renders an accuracy of 93.33% with an associated p value of $2.028 \times 10^{-14}$, and the sensitivity is 1.000 for LP-B, but zero for LP-P and 0.8750 for V-B and V-P (Figure 5B).

Overall, statistical analysis reveals that data of brain activity (cFos-ir cell density) in the 13 nuclei of the SBN and motivation brain centers of our experimental females are sufficient to predict with high accuracy the status of a female (either virgin or LP) and whether the animal was exposed to a social stimulus (pups) or a nonsocial one (buttons).

Linear discriminant analysis using only cFos-ir cell density in the MPO

Because previous literature suggests that the MPO is the key center for the expression of maternal behaviors, which would change by the end of pregnancy, we next checked whether data on the MPO (density of cFos-ir cells in the MPO) might allow appropriately classifying the animals in their experimental group. When linear discriminant analysis was applied to the MPO, dataset rendered a single linear discrimination function (LD1) with a coefficient $<0.001$ (Figure 6A). Once again, we checked the performance of the discriminant functions by leave-one-out cross-validation, and the comparison between the real and the predicted classes is shown in Figure 6B. In this case, there are multiple mistakes in the V-B, V-P, and LP-P groups. This classification results in an accuracy of 66.67%, with an associated p value of $5.374 \times 10^{-6}$, with a sensitivity of 1.000 for LP-B, but zero for LP-P and 0.6250 for V-B and V-P.

Figure 5. Linear discriminant analysis using the original data of cFos-ir cell density in the socio-sexual and motivational circuits

(A) 3D representation of the individual scores (dots) of each subject, for the three linear discriminant functions (LD1–LD3) obtained using the full set of data (cFos cell density in all the 12 nuclei analyzed). The color code represents the groups of females: virgins exposed to buttons (V-B in green), virgins exposed (V-P in blue), late-pregnant exposed to buttons (LP-B in yellow), and late-pregnant exposed to pups (LP-P in red). Open dots correspond to females having attacked pups. In order to help visualization, colored regions are drawn to approximately representing the term of the partition obtained after the LDA to which the individuals of each category belong (LP-P, LP-B, V-P, V-B). By clicking on the graph, the reader can get access to Figure S3 showing an accurate interactive 3D graph that plots the actual limits of the LDA partition and the classification of individuals according to that partition, together with 2D graphs with two-by-two projections on the three LDA functions.

(B) Confusion Matrix table indicating the number of animals of each group (True group) assigned to each group by means of the prediction procedure (Prediction). The results of the statistical analysis are summarized below.

(Figures S3B and S3C). Last, the third discriminant function (LD3) gives positive scores to LP-B and negative scores for V-B, whereas in females exposed to pups the majority of scores are closer to zero (Figures S3B and S3C).
As a conclusion, using the data of the MPO alone renders a very low performance in the classification as compared with using data from the whole set of nuclei.

**DISCUSSION**

Using cFos expression assessment, we have shown that pups (a source of relevant novel social stimuli) and buttons (novel nonsocial objects) activate differently the brains of female mice, and these responses change by the end of pregnancy. We have focused on relevant nuclei known to participate in the expression of different social behaviors (the SBN) and in motivational aspects of behavior (the motivation circuitry of the brain). Neither late-pregnant nor virgin females had previous experience with pups during their adult life, so that novelty cannot contribute to the variability of the results and does not explain differences between groups and stimuli.

**Pup care and maternal motivation: Changes induced by pregnancy**

Our results confirm, firstly, that the SBN is more activated by a social stimulus (pups) than by a nonsocial one (buttons), thus validating our experimental design, as we are comparing two relevant stimuli that differ in their capacity to activate the social brain. The second conclusion is that this activation of the SBN seems largely independent of the status of the female, e.g. virgin and LP females show not differential response to the stimuli, no significant FEMALExSTIMULUS interaction. The exceptions to this are the AC/ADP, the MePD, and the PAG. In all three cases, FEMALExSTIMULUS interaction is mainly due to a decrease in the activity elicited by buttons in LP females, a fact that fits the decreased investigation of buttons observed in pregnant females (Figure S1).

By contrast, in the reward brain circuitry, there are clear differences in the activity induced by social (pups) and nonsocial stimuli in both groups of females. Although the conditions of our experiment are not appropriate to reveal changes in motivation toward pups (e.g. interaction with the pups requires low effort; see discussion in Abellán-Alvaro et al., 2021; Salais-López et al., 2021), changes of this somewhat hidden aspect of behavior can be inferred from the expression of cFos in the brain motivation circuit. In fact, among virgin females, buttons activate the motivation circuitry of the brain as much as pups or even more (as in the AcbC, BLA, and VTAp); this contrasts with the situation in LP females, in which pups are a more powerful, activating stimulus of the motivation brain circuit than buttons. In other words, pregnancy changes the salience of nonsocial stimuli, leading to less exploration to buttons (Figure S1) and decreased activation of the reward brain circuitry. In addition, during pregnancy there is an enhanced activation by pups of some
nuclei, such as the AcbC where the activity in LP females exposed to pups is significantly higher than in virgins.

Our results on this point partially agree with those obtained by Matsushita et al. (2015) that, using a cFos approach, also observed that pup exposure is a powerful stimulus to activate the motivational brain regions in lactating females. However, in that study, lactating females of 3–5 days postpartum were used as experimental animals, so that pups constituted novel stimuli only for virgins. Moreover, they did not expose control females to a nonsocial stimulus but use just nonexposed females as controls. Therefore, our results are not completely comparable, although both studies suggest an impact of motherhood on the activation of the motivational circuitry by pups that, according to our results, starts before parturition, namely due to the action of pregnancy hormones on the brain.

The PCA analysis gives interesting information on the differential response of the SBN and brain reward circuitry during pregnancy. First, the second principal component, PC2, shows positive loading factors for the centers of the reward brain circuitry (with the exception of the AcbSh) and negative loading factors for the nodes of the SBN (with the exception of the PAG, as discussed later); this indicates neat differences in the activity of both brain circuits between animals.

The fact that loading factors’ vectors of nuclei of a functional system (SBN or motivation circuitry) form a small angle between them reinforces the view that these nuclei act cooperatively (showing intra-system correlation). In addition, the fact that vectors of one system form large angles with those of the other system (see Figure 4B) reveals that both systems are largely independent (their activity is not correlated), thus demonstrating that motivation is a somewhat independent component of different behaviors (e.g., caring for pups or sniffing/gnawing buttons).

The distribution of individuals in the PCA biplot (PC1 vs PC2; Figure 4B) elegantly summarizes the differences between groups. Because PC1 somewhat reflects the general activation of the considered brain centers, the results of the PCA fit those of the standard statistics in showing that LP females exposed to buttons show less activation than the rest of the females (yellow dots are grouped at left), reflecting the curiosity of virgins toward buttons and the power of pups as stimulus for both types of females. Regarding PC2, the inverse sign of its loading factors for the SBN and the motivation brain circuit allow considering those individuals with positive scores as “more motivated than social” and those with negative scores as “more social than motivated.” Accordingly, virgins exposed to buttons are “more motivated than social,” although virgins exposed to pups are “more social than motivated.” Late-pregnant females show both positive or negative PC2 scores, but globally the scores are closer to zero, thus suggesting that LP are both “social and motivated” while interacting with pups. Considering that both groups exposed to pups (LP-P and V-P) show similar maternal behavior, these results fit previous work of the group describing how motherhood-associated hormones specifically enhance the motivational aspects of maternal behavior (Salais-López et al., 2021).

**Pregnancy hormones indirectly influence maternal motivation**

Because the only difference between virgin and LP females is pregnancy, our data reveal a strong effect of pregnancy hormones onto the brain of females, in the activity of the motivation circuitry. LP females seem to lose interest in nonsocial stimuli (there is a reduction of button-induced activity as compared with virgin females due, at least in part, to a reduced investigation of buttons) and focus on pups, thus resulting in a pup-elicited higher activation of, at least, the AcbC. It is interesting to note that, apparently, this higher activation of the AcbC is not due to increased interaction with pups in LP females, as there are not significant behavioral differences on that point (see Table S1), but probably due to enhanced motivational valence of pup-derived stimuli, paralleled by a decrease in motivational valence of buttons during pregnancy.

These findings raise the question of which are the mechanisms that allow pregnancy hormones to modify the functioning of the motivation circuitry of the brain of females. There is strong evidence indicating that sexual steroids (late-pregnancy progesterone withdrawal and estradiol surge) plus placental lactogens (late pregnancy) and/or hypophysial prolactin (postpartum) (see Bridges, 2020) are needed to induce fully motivated maternal behaviors. Recent work has shown that during pregnancy and postpartum, lactogens are able to influence the SBN nodes (Salais-López et al., 2017, 2021) that also concentrate estradiol-sensitive cells (Mitra...
et al., 2003; Simerly et al., 1990), whereas the nuclei composing the motivational brain circuitry do not express prolactin receptors (Kokay et al., 2018; Salais-López et al., 2018) or few estrogen receptors (Mitra et al., 2003; Simerly et al., 1990). Therefore, the influence of pregnancy hormones on motivation must be indirect, e.g. they likely act onto neurons projecting to the motivation circuitry rather than on the motivation circuitry itself. The only exception to this rule is the VTA and the reticular division of the substantia nigra, where according to the study of Mitra et al. (2003) in the mouse, the beta-receptor of estrogens is expressed at high levels, thus suggesting a locus for the action of estrogens on motivation during motherhood.

In this respect, the medial amygdala may also play an important role. Previous studies with juvenile individuals as a social, rewarding stimulus, have shown that a medial amygdala-to-hypothalamus pathway is critical for social motivation (Hu et al., 2021). Thus, optogenetic stimulation of GABAergic MePD neurons projecting to the MPO (or simply with their axons terminating in the MPO) induces (a) dopamine release in the Acb; (b) place preference acquisition; and (c) auto-stimulation. By contrast, optogenetically blocking this pathway interferes with social, but not other natural rewards. In fact, using fiber photometry of calcium signals, these authors report that nonsocial rewarding items (chocolate, sugar) seem not to activate MePD-to-MPO projecting neurons, whereas social stimuli do it, thus fitting our cFos-expression data (pups > buttons induced cFos expression in the MePD and the MPO).

These data also fit a previous study of our group in which, using a motivated pup retrieval test, a clear correlation was observed between prolactin signaling and maternal motivation in several nuclei of the SBN, including the MePD and the MPO (as well as the PaA), plus other nuclei not belonging to the SBN (e.g. central amygdala and posterior intralaminar thalamus) (Salais-López et al., 2021). Thus, lactogens, acting onto several nuclei of the SBN (e.g. MePD and MPO), may change specifically the response to pup stimuli of some cell populations, enhancing the rewarding valence of pups by the end of pregnancy. In our current experiment, this is revealed by a differential activation by pups/buttons in the nuclei of the reward/motivation system of the brain during late pregnancy, together with a similar effect in the MePD (and the PAG, see below).

The lack of a similar differential response to pups/buttons in the MPO between LP and virgin females suggests that concurrent changes must occur in other targets of the MePD also involved in reward/motivation of social stimuli. In this respect, direct amygdalo-striatal pathways (Novejarque et al., 2011) have been involved in some forms of social reward, e.g. female attraction for male pheromones (Agustín-Pavón et al., 2014; Dibenedictis et al., 2015). The possibility exists, therefore, that amygdalo-striatal projections sensitive to sexual steroids and lactogens, such as pathways from the medial amygdala to the ventral striatum—either direct (Pardo-Bellver et al., 2012) or indirect—using intra-amygdaloid connections (basolateral/basomedial amygdala, cortical amygdala [Novejarque et al., 2011; Pardo-Bellver et al., 2012]), might also contribute to changes in maternal motivation during late pregnancy. In fact, Numan et al. (2010) demonstrated the importance of basolateral/basomedial amygdala, projecting to the Acb (Novejarque et al., 2011), in pup-directed maternal responses in postpartum rats (inhibited by muscimol injected in the BLA/basomedial amygdala), thus fitting our data on cFos expression in the BLA and the AcbC induced by pups and buttons in LP females (see Figure 3). The lack of effect of muscimol inhibition of the medial amygdala on maternal behavior by Numan et al. (2010) suggests that the MePD-BLA-Acb pathway may have an important role on maternal motivation, whereas the MePD-MPO projection might be involved in other forms of social reward (Hu et al., 2021).

**Females possess specific circuitry for decision-making of whether to care, ignore/avoid, or attack pups**

In females exposed to pups, we monitored the behavior during the first 8 min of exposure and, with a single exception, LP and V females displayed no significant differences; this might seem surprising because previous work of ours (Martín-Sánchez et al., 2015b) and other groups (Alsina-Llanes et al., 2015; Stolzenberg and Rissman, 2011) indicate that lactating dams exhibit more and quicker maternal care when pups are (re) introduced in their cages than age-matched virgin females. However, in those previous studies dams differed from virgins not only in the influence of motherhood-associated hormones (during pregnancy, parturition, and postpartum) but also in their previous intensive experience with pups (from parturition to the day of the experiment), which virgins lacked. Our experiment suggests, therefore, that experience with pups has a pivotal role in the increase in maternal care observed in postpartum dams, and the lack of such experience in LP females minimizes differences with pup-naïve virgin females.
The only pup-directed behavior that significantly differs between LP and virgins is pup-directed attacks (see Table S1), displayed only by some LP females but not virgins. Although maternal behavior is usually equated to pup care (maternal care), in some conditions attacking and eating pups is quite frequent and should be considered an adaptive maternal behavior (Hrdy, 2000). In other words, contrary to what is usually thought, a truly adaptive maternal behavior does not consist of taking care of every possible pup in every possible moment but choosing when it is convenient (given the conditions) to do so, or to eat the pups instead, postponing motherhood until a better occasion. When confronted to alien pups, LP females apparently have the two drives, caring and eating. A control of when to attack and eat pups is therefore important for maternal behavior to be fully adaptive.

Our previous study on this subject (Navarro-Moreno et al., 2020) revealed a specific pathway within the vomeronasal system, apparently related to pup attack, as cFos expression in the accessory olfactory bulb (AOB) and posteromedial part of the medial bed nucleus of the stria terminalis (BSTMPM) is positively correlated with pup-aggression score. Here we have found that in LP females there is a highly significant, strong and negative correlation between pup-aggression score and activity in the VTAp (Table S2) and a strong positive correlation in the PAG (Table S3). These data lead us to hypothesize that during pregnancy, pup-induced activity of the mesolimbic dopaminergic system is part of the decision-making mechanisms for attacking/caring pups. Because the increased activity of the VTAp (mainly containing dopamine neurons) triggers a higher activity of the mesolimbic dopaminergic pathway (see Fields et al., 2007; Ikemoto, 2010), it seems that elevated activity of this pathway may be associated with pup caring, whereas reduced activity would be associated with pup aggression. The lack of pup attacks in virgin females, in which the VTAp shows relatively low cFos-ir cell density after exposure to pups, indicates that high VTAp activity in LP refrains a drive to attack pups that might depend on another brain region (in males, a region of the post-eromedial BST seems involved; Tsuneoka et al., 2015; in our females BSTMPM activity is correlated with pup attacks; Navarro-Moreno et al., 2020). This brain region is likely not activated by pups in virgins. Therefore, only in LP females an attack-or-care decision is necessary, and the VTAp seems to be related to this decision-making system.

In addition, also in LP females the activity (cFos-ir cell density) of the PAG shows a positive and significant correlation with pup aggression score, suggesting a role of this brain region in pup-directed aggression. Different studies (summarized by Canteras, 2012) relate the PAG with the medial hypothalamic column involved in aggressive/defensive reactions to predators (dorsolateral column) and to conspecifics (lateral column), e.g. agonistic behaviors. In this context, our findings suggest that pup-directed attack is an additional reaction to conspecifics (a social behavior) that occurs in virgin males but also in late-pregnant or postpartum females under specific conditions (cold-induced stress, Zafar et al., 2018; caloric restriction during pregnancy, Bronson and Marsteller, 1985). Pup attack seems to involve part of the social behavior network (e.g. portions of the bed nucleus of the stria terminals; Navarro-Moreno et al., 2020; Tsuneoka et al., 2015) and, according to our results, also the PAG. Therefore, in LP females, pup-directed attacks are under the control of two neural systems acting antagonistically: activity in the VTAp is negatively correlated with pup attacks, whereas activation of the BSTMPM/PAG correlates positively with this behavior.

Another behavior displayed by females in our context is what we call "approach-and-retreat," a reaction similar to risk assessment: the female approaches a pup, sniffs at it for a while, and then retreats without retrieving it, what can be interpreted as a "not-to-retrieve/care" decision. This behavior is displayed similarly by virgin and LP females (see Table S1) but only in LP females it is negatively correlated with cFos-ir cell density in the BLA and the AcbC (Table S2). Although causal relationships cannot be established with this experiment, activation of these two interconnected (Novejarque et al., 2011) centers of the reward circuitry is associated with LP females not retrieving after detecting and sniffing pups. By contrast, in virgin females approach-and-retreat responses to pups positively correlate with activation of the MePD, the AcbC, and the PAG (Tables S2 and S3). This finding further reveals important changes in the activity of the motivation-sociosexual circuitry of the brain in response to pups during pregnancy, leading to not avoiding or ignoring pups, but caring or attacking them instead.

Concerning virgin females, although in mice they approach pups with a short delay (see Alsina-Llanes et al., 2015; Martín-Sánchez et al., 2015b; Stolzenberg and Rissman, 2011), it seems that pup-sniffing-related activation of the AcbC and the MePD facilitates or is facilitated by not retrieving the pups. This recalls the situation in the rat, where lesions of the MePD in virgin females dramatically shorten their
sensitization period (Sheehan et al., 2001). By contrast, the strong activation of the BLA and the AcbC related to pup sniffing in LP females seems to be associated with not avoiding pups after detecting and sniffing at them, fitting the results of lesion/inactivation experiments of the BLA obtained in rats (Lee et al., 2000; Numan et al., 2010). In fact, although inhibition with 100 ng of muscimol infused in the BLA of female rats did not alter pup sniffing, it significantly delayed pup retrieval.

Other social and maternal behaviors are also correlated with activation of specific nuclei. Thus, pup retrieval shows a strong, negative correlation with activation of the PaA in LP females (but not virgins; see Table S3 and Figure S1F). We tentatively interpret this as related to the parental style of the females. According to Bosch (2011), the basal level of anxiety is reduced during motherhood, with the paraventricular hypothalamus being involved in the regulation of this basal anxiety. Nonetheless, high-anxiety mothers display a more protective maternal style than low-anxiety ones: they retrieve pups more quickly and display more frequently arched-back posture and pup licking/grooming. Our results suggest that PaA activity has an inhibitory role of anxiety, may be mediated by somato-dendritic or axonal oxytocin release (Neumann, 2007), so that the higher the activity, the lower the anxiety and, consequently, the less frequent is pup retrieval. The anxiolytic activity of the PaA would already be present in LP females.

Moreover, nest building is a quite common behavior in virgin or LP females exposed to pups, but our data indicate that occurrence of this behavior is significantly and positively correlated with activation of the VTAa only in LP females (see Table S2 and Figure S1E). Similarly, affiliative behaviors between adult females (inter-female interactions), which are similarly expressed by virgin and LP females (Table S1), show a negative, highly significant correlation with activity in the VMHVL in LP but not virgin females. Because LP females already show aggression to unfamiliar intruders (prepartum or pregnancy-induced aggression; Mann and Svare, 1982), and the VMHVL has been involved in intermale (Lin et al., 2011) and maternal aggression (Hashikawa et al., 2017), our results can be interpreted as a tonic inhibition of aggression allowing affiliative contact with the familiar female, with which the experimental LP female is living in the same home cage.

A neural network view of social behaviors: SBN versus hierarchical labeled-line circuits

Most of the studies on the neural or neuroendocrine basis of social behaviors try to identify a key nucleus for the expression of a given social behavior. For maternal behaviors, the classic studies by Michael Numan and his group (Numan et al., 1990; Numan and Numan, 1996) revealed that lesions restricted to, or isolating the medial preoptic area/ventral BST, resulted in a reduction of maternal care. Similar lesion experiments in mice led Tsuneoka et al. (2013) to identify a region in the MPO, what they called the central MPO, the lesion of which in females (using fiber-sparing neurotoxic drugs) resulted largely in pup killing instead of pup care. This analysis was further detailed by Wu et al. (2014), who identified a specific galanin-expressing population of neurons in the MPO that project to the VTA that seems involved in motivational aspects of maternal behavior. Ablation of these cells abolishes pup care in virgin and lactating females and promotes pup killing in virgin females and male mice. Otherwise, their activation promotes pup care in males and is correlated with parental behaviors in males, virgin females, and mothers.

However, the detailed analysis carried out by Kohl et al. (2018) has shown that the cell population of galanin-expressing cells in the MPO is interconnected with some 20 nuclei, and this network seems to encode together the complex interactions with pups during parenting and other aspects of non-pup-directed parental behaviors. The analysis of the effects of optogenetic activation and inhibition of MPO-galaninergic cells suggests the involvement of some of the pathways arising from the MPO in motivation for pups (MPO to VTA), pup-directed grooming (MPO to PAG), and reduction of social interactions with adults (MPO to medial amygdala). However, the complex, reciprocal connections of the MPO with these centers (e.g. the medial amygdala) makes difficult to accept a hierarchical organization within the social brain. For instance, MPO galanin cells projecting to the medial amygdala are active during different components of parenting (sniffing, grooming, retrieving, and entering the empty nest), but inhibiting or activating these cells seems to have no clear effects on interaction with pups (see Kohl et al., 2018).

Overall, although not specifically focused on the galaninergic population of the MPO, our data suggest that the functioning of the whole brain network, instead of just the MPO, encodes maternal behaviors. First, although the MPO is activated by pups more than by buttons, this is also the case for every center of the
SBN. Second, late-pregnant and virgin females without previous experience with pups show similar activation by pups in the MPO, as it happens in most of the remaining nuclei of the SBN.

In this respect, the discriminant analysis performed indicates that the data of activity of the MPO are not enough to predict the nature of the stimulus the animal has been exposed to (pups or buttons) and the physiological status of the female (virgin or late-pregnant). By contrast, considering for the discriminant analysis all 13 nuclei belonging to the SBN and motivation brain circuit allows a nearly perfect classification of the animals into their respective groups, with an accuracy of 93.33% and no mistakes on the stimulus the animals are exposed to (buttons or pups); this suggests that, although the MPO would be an important node of the network, the whole system composed of the SBN and the circuit for motivated behaviors encodes together the nature of the stimulus the animal is perceiving and the changes in the response to it associated with pregnancy.

This gives direct support to the view, already proposed by Sarah Winnans Newman more than 20 years ago (Newman, 1999), that social behaviors, including maternal ones (pup care and non-pup-directed behaviors), are encoded by the activity of a network of brain centers. The opposite view, namely that there are specific circuits and centers controlling the expression of different social behaviors (e.g. male and female sexual behaviors, aggressive/agonistic behaviors; affiliative relationships), considers a series of linear pathways (labeled line model) in which there is a hypothalamic nucleus responsible for the execution of a given social behavior (male sexual behavior; female sexual behavior; agonistic/territorial behavior, including aggression; affiliative interactions; pup care/attack) that would be triggered by a key social stimulus under a given hormonal status. This hypothalamic key nucleus (e.g. the hypothalamic aggression locus; Lin et al., 2011) would activate the behavior through descending projections to the midbrain/brainstem (Falkner et al., 2020) but would work under a certain control of telencephalic centers (e.g. the lateral septum; Wong et al., 2016). In some cases, this scheme has been applied to the control of maternal behaviors, with the MPO as the hypothalamic key center projecting to the ventrolateral periaqueductal gray and the medial amygdala as the telencephalic center for control.

Our data indicate, instead, that the social brain network (SBN) encodes not only interactions between adult individuals, as proposed by Newman (1999), but also pup-directed behaviors, another special form of social behavior. According to the hypothesis by Winnans Newman, the resulting behavior would not depend on the activity of one of the nodes of the SBN, but on the pattern of activity on the whole network (see Figure 7A). The whole SBN responds strongly to the presence of pups (as compared with buttons), with a different pattern in virgin and LP females, with higher activity in the MePD and the PAG, and low activity in the MPO and the LSV of LP females as compared with virgins. The results relative to the centers controlling motivational aspects of behavior in our study reveal that they play an important role in the response of the females to social and nonsocial stimuli (Figure 7B). Here, the status of the female (virgin or late-pregnant) determines their response to the stimuli, with pregnancy inducing a high activity in the whole system in response to pups, whereas virgins react strongly to buttons.

As discussed earlier, the change in the response of both systems is with all likelihood due to the action of pregnancy hormones that, according to the distribution and action of estrogens and prolactin, are concentrated in the SBN nodes; this would indirectly modulate the activity of the motivation brain circuit, thus enhancing the incentive properties of pup-derived stimuli and reducing those of nonsocial stimuli. Altogether, these changes would result in motivated, pup-directed behaviors once the litter is born, ensuring their survival and well-being (Kohl et al., 2018; Salais-López et al., 2021).

Limitations of the study

A possible limitation of our study is that we have not recorded and measured the interactions with the control stimulus, buttons, in the same females in which cFos has been analyzed. We considered buttons as a nonsocial novel object, appropriate as a simple control object (pups were also a novel object for the females). However, after the experiments we realized that many of the buttons had been intensely gnawed, thus indicating that they are not just novel but salient enough as to induce goal-directed behaviors (gnawing). In fact, our results of cFos immunoreactivity indicate that button-induced activation is observed in most nuclei of the motivation circuit. There, virgins show generally more activation by buttons than by pups (opposite to what happens in LP females), and button-elicited cFos immunoreactivity is denser in virgin than LP females (see Figure 2). In a different set of females (LP and virgins), we measured button-induced
behaviors, and this confirms that increased button-elicited brain activity in virgins relates to more prolonged interaction with buttons, enhanced rewarding properties of buttons, or both. Therefore, the pattern of activity in the SBN and the motivation circuitry and direct analysis of behavior indicate a reduction of salience of buttons during late pregnancy. Because females were exposed to either pups or buttons, this is not due to a competing effect of the presence of pups, but it reflects an actual decrease in salience of this control, nonsocial stimulus during pregnancy.

Another limitation of our study is related to the use of c-Fos immunoreactivity to evaluate brain activity. A more dynamic procedure (calcium imaging, electrophysiological procedures) would allow analyzing temporal aspects of brain activity, related to specific conducts, thus helping to clarify causal relationship. However, using these techniques, it is technically difficult to analyze simultaneously the activity of a network of 13 nuclei distributed in distant regions of the brain. Our data may help to focus future research on specific groups of these nuclei, to evaluate how their activity relates to maternal care in postpartum and virgin females. For instance, the possible role of amygdalo-striatal pathway systems in maternal motivation can be further explored using these techniques.

**STAR+METHODS**

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ACKNOWLEDGMENTS
This work has been funded by Spanish Ministry of Science and Innovation (PID2019-107322GB-C21 to F. M-G. and C22 to C. A-P.; PID2019-108562GB-I00, to E.L.), Generalitat Valenciana (GV-2020-173; to M.J. S-C.) and Universitat Jaume I de Castelló (UJI-A2019-14 to M. J. S-C.; UJI-B2020-22 to M.V. I-G.). C. N-M. was awarded a predoctoral grant by UJI; this work is part of her Doctorate Thesis. The authors are indebted to Rafael Goterris-Cerisuelo and Sandra Sanahuja-Irene for their invaluable help.

AUTHOR CONTRIBUTIONS
Conceptualization, F. M-G., M. J. S-C., and C. A-P.; Methodology, C. N-M. and M. B-M.; Investigation, C. N-M.; Formal Analysis, M. V. I-G., C. N-M., and F. M-G.; Writing—Original Draft, C. N-M., M. V. I-G., and F. M-G.; Writing—Review & Editing, M. J. S-C., E. L., and C. A-P.; Visualization, C. N-M. and M. V. I-G.; Funding Acquisition, F. M-G. M. J. S-C., M. V. I-G., E. L., and C. A-P.; Supervision, F. M-G., M. J. S-C., C. A-P. and E. L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
Being this a work on the neural basis of maternal behavior, we have used females as our experimental subjects. We consider that analyzing the biological basis of parental behaviors may be helpful to promote social gender equality.

Received: January 27, 2022
Revised: April 13, 2022
Accepted: May 30, 2022
Published: July 15, 2022

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Animals             | Janvier Labs | RRID:MGI:5652638 |
| Rabbit anti-cFos    | Synaptic Systems, Ref. 226003 | RRID:AB_2231974 |
| Goat anti-rabbit IgG, biotinylated | Vector labs, Ref. BA1000 | RRID:AB_2313606 |
| Avidin-Biotin Peroxidase complex, Vectastain-Elite | Vector labs, Ref. PK6100 | RRID:AB_2336819 |
| ImageJ              | NIH | RRID:SCR_003070 |
| SPSS version 27     | IBM | RRID:SCR_016479 |
| R                   | The R Project for Statistical Computing, https://www.r-project.org/ | RRID:SCR_001905 |
| Original data       | Mendeley Data | RRID:SCR_015671 |
| Data on cFos cell density and behaviour | Mendeley Data | https://doi.org/10.17632/spfmdng5dz.1 |

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fernando Martinez-Garcia (femartin@uji.es).

Materials availability

This study did not generate new unique reagents or animal lines.

Data and code availability

- The original data reported in this paper are deposited in a public repository Mendeley Data https://doi.org/10.17632/spfmdng5dz.1
- All microscopic images and videos derived from the experiments reported in this paper will be shared by the lead contact upon request, as well as any additional information required to reanalyse data reported in this paper.
- This paper does not report original codes.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In the present work we have used adult female mice (Mus musculus) of the strain CD1 (n = 52), as well as adult stud males (n = 24). All the females were 10 weeks old at the beginning of the procedure and stud males were 3–6 months old. Animals were treated throughout following the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1) and, accordingly, experimental procedures were approved by the Committee of Ethics and Animal Experimentation of the Universitat Jaume I, and a license was issued by the Direcció General de Producció Agrària i Ramaderia de la Generalitat Valenciana (code 2015/VSC/PEA100055 type 2).

To avoid isolation stress, animals were housed in homologous pairs at least twenty days before the experiment (exposure to pups or buttons) in polypropylene cages with a controlled temperature of approximately 24°C and under a 12-h light/dark hour cycle (lights on at 08:00h) with ad libitum water and food supply.
Females were randomly assigned to the experimental groups. Those females assigned to the late-pregnant (LP) groups, were stimulated by introducing fresh male-soiled bedding in their cages for two days and introduced then in the cage of an adult stud male for a single night in a quiet room to ensure that mating occurred. After mating all the females were weighted daily for ten days to check that they showed a weight gain corresponding to pregnancy (as compared to virgins). Fourteen pregnant females were used as experimental LP females, and six additional females were allowed to mate four days before (using the same procedure) and employed as donors of pups for our experiments.

Females assigned to the groups of virgins (V) were simply housed in pairs and weighted daily for 10 days and left undisturbed until the experiment began. Five days prior to the experiments, shredded filter paper was introduced in the home cage of the females (both virgins and LP) as material for the nest.

**METHOD DETAILS**

**Experimental design**

When the pregnant females were calculated to be in their postconception day 17 (two days before parturition) both late pregnant (LP) and virgin (V) females were exposed to 3–4 day old pups from donor dams or, alternatively, to control non-social objects, e.g. plastic buttons of a similar size as pups. Again, LP and V females were randomly assigned to pups- or button-exposed groups. Thus, our experimental design includes 4 groups of females: 1) LP exposed to pups (LP-P; n = 7), 2) virgins exposed to pups (V-P; n = 8), 3) LP exposed to buttons (LP-B; n = 7), and 4) virgins exposed to buttons (V-B; n = 8).

Two days prior to stimulus exposure and behavioural testing, females were habituated to the experimental setup and the experimenter by introducing eight glass marbles in their home cage daily at the time in which experiments were scheduled. The next day (test), pairs of virgins or LP female mice were exposed to eight buttons or eight pups that were introduced in the opposite side of the cage relative to the nest. The behaviour of the females exposed to pups was video recorded for 90 min for subsequent analysis.

Ninety minutes after introduction of the stimulus, females were deeply anaesthetised with an intraperitoneal (i.p.) injection of sodium pentobarbital (Vetoquinol, Madrid, Spain; i.p. injection of 0.02 mg/g of body weight, (Shipley and Adamek, 1984)) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4. Brains were carefully extracted from the skull.

**Observation and quantification of behaviour of the females during exposure to pups**

An observer that was blind to the experimental conditions and design observed the videos of the females exposed to pups (see Table S1). Observation was restricted to the first 8 min following pup introduction (Martín-Sánchez et al., 2015b), during which the observer analysed thirty-two 5-s periods (four 5-s periods per minute, separated by 10-s intervals). For each 5-s period, he/she registered the most maternal behaviour exhibited by the female, according to the following hierarchy: in nest, females stayed inside the nest in close contact with pups; retrieval, females carried the pups to the nest; nest building, females gathered pieces of nest material; on nest, females were located on the nest, near the pups but not in contact with them; approach-and-retreat, sniffing at a pup out of the nest followed by retreat without retrieval; and off nest, females were out of the nest without interacting with pups. For each animal, thus, 32 behavioural events were registered distributed among the categories described above.

Occasionally, females attacked and ate pups. An observer blind to the experimental conditions re-analysed the video/audio-recordings and identified those moments in which pup-directed attacks occurred, which were easy to recognise as they always occurred while the female was out of the nest, licking-grooming a pup, which suddenly started emitting strong distress vocalisations which stopped after a few seconds. We measured the latency to each attack to a pup and assigned it to the female that displayed pup-directed aggression. For each female we calculated a pup aggression score:

\[
Pup\ Aggression\ Score = \sum_{i=1}^{8} (25 - \text{latency to attack pup } i)
\]

Those females not attacking any pup were assigned a latency of 25, as all pup attacks occurred during the first 24 min. This way, pup aggression score was zero for the females not expressing pup-directed aggression, and it was higher for those females attacking more pups and/or attacking pups with a lower latency.
Finally, the interaction between females in the same cage was also measured for each of these thirty-two 5-s periods as present (1) or not present (0), considering an interaction when a female sniffed the other.

Since we were interested only in pup-directed behaviours, we did not record behavioural responses of the females exposed to buttons.

The behavioural items described above were used to calculate maternal score for each animal. The maternal score is a weighted sum of those episodes in which female’s behaviour reflects a maternal state (pup retrieval, nest building, in nest and on nest):

\[
\text{Maternal Score} = 5 \times \text{Retrieval} + 5 \times \text{In Nest} + 4 \times \text{Nest Building} + 2 \times \text{On Nest}
\]

**Observation and quantification of behaviour of the females during exposure to buttons**

A second set of females, both virgins (n = 8, 4 pairs) and late pregnant (n = 8, 4 pairs), were treated exactly as those in the previous experiment, e.g. they were habituated for two days to introducing 8 objects (glass marbles) in their home cage at the same time of the day. The next day, when pregnant females were in post-conception day 17, 8 buttons were deposited in their home cage and their behaviour was videorecorded for 20 min.

Then, using BORIS (Behavioral Observation Research Interactive Software, Friard and Gamba, 2016), a person blind to the experimental conditions, observed the videos and recorded interaction of the females with buttons (sniffing at them) and inter-female interaction for the first 5 min after introduction of the buttons (Figure S1).

**Tissue processing and immunohistochemistry**

After perfusion of the females with fixative solution, brains were post-fixed overnight in 4% PFA at 4° C and cryoprotected in 30% sucrose in 0.01M PB at 4° C until they sank. Using a freezing microtome (Microm HM-450, Walldorf, Germany), 40 µm-thick coronal sections were obtained collected in five parallel series in 30% sucrose in PB and stored at −20°C for subsequent cFos immunohistochemistry, an immediate early gene considered a good indicator of neural activity during exposure to the stimuli (Chaudhuri, 1997; Sagar et al., 1988). Sections of animals of the different groups (LP and virgin females exposed to buttons and pups) were processed simultaneously using the same batches of reagents and antibodies, to minimize inter-individual variability and to avoid inter-group bias. To do so, a series of sections of each animal was allowed to thaw and free-floating immunohistochemistry was performed. In brief, sections were treated, under mild rocking at room temperature (except otherwise specified), as follows: (a) 3 × 10 min rinses in TRIS buffered (0.01M, pH 7.6) saline (TBS); (b) immersion in 1% H2O2 in TBS for 30 min for endogenous peroxidase inhibition; (c) 3 × 10 min rinses in TBS; (d) 1-h immersion in a blocking solution consisting of 3% normal goat serum, 3% bovine serum and 0.3% Triton X-100 in TBS, pH 8; (e) overnight incubation at 4° C with the primary antibody (rabbit anti-cFos n°. 226003; Synaptic Systems) diluted 1:5000 in the blocking solution; (f) 3 × 10 min rinses in TBS; (g) 2-h incubation in 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (Vector BA1000) in the blocking solution; (h) 3 × 10 min rinses in TBS; (i) 90-min incubation in 1:50 avidin-biotin-peroxidase complex (Vectastain-Elite, Vector Laboratories) in TBS; (j) 2 × 10 min rinses in TBS and 2 × 10 min additional rinses in TBS (Tris Buffer 0.05M pH 7.6); finally, (k) the resulting peroxidase activity was revealed with diaminobenzidine tetrahydrochloride (DAB) reaction (0.025% DAB and 0.01% H2O2 in TB). The reaction was stopped by rinsing sections repeatedly in TB and, finally, in 0.2% gelatine in TB. Sections were then mounted from warm (37° C) 0.2% gelatine in TB on slides, dehydrated, cleared with xylene and coverslipped in DPX (Scharlab Laboratory).

**Image capture**

Expression of cFos was quantified in a selection of 6 brain nuclei involved in the control of socio-sexual behaviours (see Figures 1 and 2): lateral septum ventral portion (LSV), medial amygdala (posterodorsal division, MePD), medial preoptic nucleus (MPO), ventromedial hypothalamic nucleus (VMHVL), paraventricular hypothalamic nucleus (anterior part, PaA), anterior commissure nucleus/anterodorsal preoptic area (AC/ADP) and lateral column of the periaqueductal grey (PAG).

In addition, we also sampled 6 nuclei/cortical regions belonging to the motivation brain circuitry (see Figure 3), namely anterior and posterior ventral tegmental (VTAa and VTAp), accumbens shell and core (AcbSh...
and AcbC), basolateral amygdala (BLA) and medial prefrontal cortex (including prelimbic and infralimbic cortex, PrL-IL).

To reduce variability, for each one of these brain centres a specific anteroposterior, dorsoventral and medio-lateral region of interest was selected using the mouse brain atlas (Paxinos and Franklin, 2004; see Figures 1, 2 and 3). Pictures of both hemispheres were systematically taken using a Leica DFC450 digital camera attached to a Leica DM750 microscope. For each region an appropriate magnification was used to cover the region of interest and, when needed, a specific portion of the picture was manually selected as a region of interest (ROI) and the density of cFos-ir cells measured using image analysis techniques.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis
To quantify the density of cFos-immunoreactive cells in each region, image processing and analysis were conducted on ImageJ software (NIH). In brief: a) the green channel of each RGB image was selected; b) images were then binarized setting the threshold at 75% of the mode of the grey histogram, so that every pixel below this threshold was considered labelled; c) the resulting binary images were further filtered using commands fill holes, open (3 iterations) and watershed; d) particles were automatically counted, discarding those smaller than half the average size of the cells from that specific nucleus (calculated by measuring the average area of six randomly selected intensely labelled cells in the nucleus); e) the density of cFos-ir cells (cells/mm²) was calculated by dividing the total number of particles detected in the images of the right and left hemispheres, by the sum of the areas of the ROI (see Figures 1, 2 and 3).

Statistical analysis
Statistical analysis was performed using SPSS software package (IBM) and R (R Core Team, 2020). The significance level was set at p < 0.05.

Behaviour
Data on behaviour of pup- or button-exposed females were first used to compare behaviour elicited by pups between LP and V females, by means of a Student’s t test. When behavioural data did not display normal distribution, even after logarithmic transformation (Kolmogorov Smirnov test), we used a non-parametric Mann-Whitney test.

Intra-nucleus comparison of cFos-ir cell density
Concerning cFos expression, when data accomplished normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test), what sometimes required logarithmic transformation of data (Log10 [n+1]), a two-way ANOVA was performed, with FEMALE (V or LP) and STIMULUS (buttons or pups) as factors. Significant FEMALExSTIMULUS interactions were explored by post-hoc pairwise comparison with Bonferroni corrections.

Otherwise, in those nuclei in which one or more of the 4 groups (V-B, V-P, LP-B, LP-P) failed to accomplish normality or homoscedasticity, we assessed the differences in the main factors separately; FEMALE (all virgins vs all LP) and STIMULUS (all females exposed to buttons vs all females exposed to pups). A two-sample t-test (for samples showing normality), or a Mann-Whitney test for those displaying no normal distribution, was performed with non-transformed data.

Brain-behaviour correlation analysis
Since we had measured pup-induced behaviour and brain activity (density of cFos-ir cells in the nuclei of the sociosexual and motivation brain networks) in the same animals, e.g. LP and V females exposed to pups, we explored the relationship between activity in these brain nuclei with the expression of pup-elicited behaviours, by means of a Spearman correlation analysis (most behaviours did not follow normal distribution). We did so separately in LP and virgin females exposed to pups. This allows exploring changes in brain-behaviour relationship during pregnancy.

Principal components analysis (PCA)
Principal component analysis provides a way to achieving a global analysis of the data, which may shed light of the response of the whole system (the whole set or nuclei analysed) to the two stimuli in the two
kinds of females. This statistical approach reduces a set of intercorrelated variables (cFos-ir cell density in the different nuclei of the SBN and motivation brain circuitry) into a few dimensions that gather a big amount of the variability (e.g. information) of the original variables. These dimensions are called principal components (PCs) and have the properties of collecting highly correlated variables within each component while being uncorrelated with each other. Therefore, PCA was performed after standardizing each variable to have mean zero and standard deviation one. The results reveal 12 principal components, and we focused on the two main principal components (PC1 and PC2) that, together, explain about 65% of the variance.

Each principal component is obtained using a linear combination of the original data \( \mathbf{X} \) to \( \mathbf{X}_p \), where \( X_1, X_2, \ldots, X_p \), are the cFos-ir cell density in the \( p \) nuclei analyzed; \( p = 12 \) in our case, with a specific loading factor \( \phi;1 > \phi > -1 \) for each nucleus. For instance, for the \( j \)-th principal component (PC):

\[
PC_j = \phi_1X_1 + \phi_2X_2 + \cdots + \phi_pX_p, \quad \text{with } \sum_{i=1}^{p} \phi_i^2 = 1
\]

Using this equation, we calculate the scores of each component for each female. We show the scores of each animal in the two first principal components as a biplot (PC1 vs PC2), in which each animal is represented as a dot with a colour code indicating the group it belongs to (LP-P, LP-B, V-P, V-B). In addition, the loading factors for PC1 and PC2 for each nucleus, \( p \), are indicated as vectors \( \langle \phi_1, \phi_2 \rangle \) in Figure 3B. The orientation (direction/angle) of the vector indicates how much the variable contributes to the principal component: the more parallel to a principal component axis, the more it contributes only to that PC. The length of the vector indicates how well the two principal components explain the variability of the density of cFos expression in this nucleus. The angles between vectors corresponding to different nuclei show their correlation: small angles represent high positive correlation, right angles represent lack of correlation, opposite angles represent high negative correlation.

**Linear discriminant analysis**

Assuming that \( \mathbf{X} = (X_1, \ldots, X_p) \) is the vector with the covariates/predictors measured for each individual, and that these observations in each group, (LP-P, LP-B, V-P, V-B), follow a multivariate gaussian distribution, Bayes’ theorem is used to flip these around into estimates for \( \Pr(Y = \text{LP-P} | \mathbf{X}) \), \( \Pr(Y = \text{LP-B} | \mathbf{X}) \), \( \Pr(Y = \text{V-P} | \mathbf{X}) \) and \( \Pr(Y = \text{V-B} | \mathbf{X}) \). Mathematically, the problem turns into the problem of finding the borders of the zones defined by the covariates, which maximize each probability. The linear discriminant analysis looks for linear borders, linear combinations of \( X_1, \ldots, X_p \) (discriminant functions) that allow to characterize or separate the zones that maximize the probability of each category. Once these discriminant functions are estimated, they can be used as classifiers for new individuals. Then, given a new individual with measurements \( \mathbf{X}_{\text{new}} = (X_{1\text{new}}, \ldots, X_{p\text{new}}) \), it will be labelled as a LP-P if \( \Pr(Y = \text{LP-P} | \mathbf{X}_{\text{new}}) \) is greater than the probabilities of the other groups. The number of discriminant functions in this technique is either \( N_g - 1 \) where \( N_g \) is the number of groups (4 in our case), or \( p \), the number of predictors, whichever is smaller. This renders three LD functions for the analysis using the full set of data and a single LD function for the analysis using MPO cFos density.

So, we used our data to first calculate the linear discrimination functions, each one characterised again by a set of coefficients applied to the original dataset. With this, the proportion of trace is calculated, e.g. the proportion of between-class variance that is explained by successive discriminant functions and, if considered enough (>75%) the two first discriminant functions are used for classification.

Usually, the classification methods split the dataset into a subset of observations used to get the discriminant functions, the so-called training data, and another subset of observations called test data, which is used to check the performance of these discriminant functions. In our case, due to the limited size of our dataset we are checking the performance of the discriminant functions by leave-one out Cross Validation. It is an iterative procedure where in each step we use as a training set the whole data set except one observation that is used as a test set. Then we compare the classification obtained by the iterative procedure with the real classification of the individuals to determine the global accuracy (proportion of individuals correctly classified), sensitivity of the classification for each class (number of individuals of a given class that are correctly classified), specificity of the classification for each group (number of individuals assigned to a group that actually belong to that group, not shown). We also calculate the No Information Rate (NIR; maximum accuracy if all individuals were assigned to the same group).
These data allow assessing the correctness of the classification based on the dataset employed, using Kappa ($K = 1$ perfect classification; $K = 0$ null correctness):

$$K = \frac{N \sum_{i=1}^{n} m_{ii} - \sum_{i=1}^{n} (G_i C_i)}{N^2 - \sum_{i=1}^{n} (G_i C_i)}$$

Where:

- $i$ is the class number
- $N$ is the total number of classified values compared to truth values
- $m_{ii}$ is the total number of values belonging to the truth class $i$ that have also been classified as class $i$ (e.g. values found along the diagonal of the confusion matrix)
- $C_i$ is the total number of predicted values belonging to class $i$
- $G_i$ is the total number of truth values belonging to class $i$

An associated $p$-value contrasts whether classification is significantly better ($p < 0.05$) than NIR.

To illustrate the results, instead of the traditional graphics showing the classification on the basis of the 13 original variables, which would render a high number of graphics, we use a single 3D graphic on the scores in the three linear discriminant functions (Figure S3). For the case of the MPO, we represent the classification using the original data of the cFos density.