Doublecortin-immunoreactive immature neurons in the olfactory system across pregnancy and lactation in female mice

Michele Prina[1*], Rafael Esteve-Pérez[1*], Cinta Navarro-Moreno[1§], María Abellán-Álvaro[1], Enrique Lanuza[1], MªJosé Sánchez-Catalán[2], Fernando Martínez-García[2], Carmen Agustín-Pavón[1#+], Jose Vicente Torres-Pérez[1#+]

[1]Departament de Biologia Cel·lular, Funcional i Antropologia Física, Universitat de València

[2]Departament de Medicina, Universitat Jaume I de Castelló

§ Current address: CIBERER - IIS La Fe

*These authors contributed equally to the work

[+]These authors contributed equally to the work

[#]Corresponding authors: CAP (pavon@uv.es), JVTP (jose.vicente.torres@uv.es). Departament de Biologia Cel·lular, Biologia Funcional i Antropologia Física, Universitat de València; C/Dr Moliner, 50. 46100 Burjassot, València, Spain

Abstract

In mammalian species, motherhood is a critical period of neural plasticity, allowing the dam to adapt its behaviour to the demanding task of taking care of the pups. For example, in mice, pregnancy and lactation promote proliferation of neuroblasts in the subventricular zone and adult neurogenesis in the olfactory bulb. Thus, we hypothesised that the non-canonical neurogenesis described in other areas of the olfactory system would be similarly affected by either pregnancy or lactation. To test this hypothesis, we analysed the expression of doublecortin (DCX) and Ki67, markers of immature neurons and proliferation respectively, across the reproductive cycle in adult CD1 female mice. In a group of late pregnant females, there was a trend towards increase of DCX cells in the olfactory bulb. In the piriform cortex, lactating females had significantly fewer immature neurons, and of lower complexity and diameter, than pup-sensitised females. This suggests that both lactation, and/or exposure to pups, and the preceding pregnancy are require to promote maturation of this population of embryonically generated DCX-ir neurons. By contrast, the number of DCX-immunoreactive cells in the olfactory tubercle was not significantly affected by motherhood. Finally, we found Ki67 nuclei located in close apposition to clusters of DCX-ir cells in this region, with some of DCX-ir neurons co-expressing both markers. Taken together, our results expand our current knowledge on how motherhood remodels the brain and reveal the olfactory tubercle as an unexplored niche for adult neurogenesis.

Key words: piriform cortex; olfactory tubercle; neurogenesis; matrescence; lactation

Introduction

Throughout the lifespan of a female mammal, a myriad of psychobiological changes can determine their behavioural and neural responses. Motherhood constitutes a period of special importance due to its adaptive nature (Pawluski et al. 2022; Hoekzema et al. 2022). Not surprisingly, inadequate maternal behaviours are highly correlated with developmental and health deficits in the offspring (Fitzgerald, Hor, and Drake 2020). Additionally, motherhood, particularly the first time, represents a challenging period for the mothers themselves. Despite its importance, many studies have focused on the long-lasting consequences to the offspring while neglecting the neurobehavioral changes in the mother (Pawluski et al. 2022; Navarro-Moreno et al. 2022; Kundakovic and Champagne 2015; Hoekzema et al. 2022; Hillerer et al. 2014).

Like the transition from adolescence to adulthood during puberty, the maternal brain goes through hormonal and psychological adaptations during motherhood. The period of 'matrescence' will prepare the individual for the stage of motherhood, characterised by a change in behaviour (Orchard et al. 2023). For instance, female rodents start building nests and showing signs of aggression towards conspecifics by the end of their pregnancy (Mayer and Rosenblatt 1984; Mann and Svare 1982; Navarro-Moreno et al. 2022). Similarly, the postpartum period is characterised by an increased load of caring-related tasks (e.g., nurturing and grooming the offspring) and limited or shifted interactions with other adult conspecifics (Leuner and Sabihi 2016; Navarro-Moreno et al. 2022; Pawluski et al. 2022). These behaviours are mediated by neuroplastic adaptations in the brain of the dams during both pregnancy and postpartum, which are dynamically adjusted to the needs of the offspring (Salais-López et al. 2017; Fleming et al. 1994). Thus, motherhood represents a stage of long-lasting neuroplastic adaptation in the female brain with potential recurrence during their adult life.

An evolutionary conserved architectural framework seems to orchestrate this maternal brain functioning in all mammals studied, the so called 'maternal behaviour neurocircuitry', included in the socio-sexual brain network (Swain and Ho 2019; Newman 1999). This circuitry includes cortical areas, hypothalamus, amygdala, olfactory bulbs (OB; both main -MOB- and accessory -AOB), and hippocampus (Leuner and Sabihi 2016), among others. Adult neurogenesis, the production of new neurons from adult neural precursors, has emerged as key contributor to parental neuroplasticity in this circuitry. Adult neurogenesis is sensitive to hormonal input (e.g., prolactin) and has been involved in odour-mediated, stress and socio-sexual behaviours (Leuner and Sabihi 2016; Medina and Workman 2020; Shingo et al. 2003; Pawluski et al. 2022; Mak and Weiss 2010; Furuta and Bridges 2005). In this regard, doublecortin (DCX), a microtubule-associated protein expressed by emerging neurons, can be used as a proxy to assess immature neurons.

DCX is a protein exclusively expressed by neuronal precursors during both embryonic and adult stages. Although its function(s) has not been fully elucidated yet, DCX seems involved in neurite development and/or migration (Francis et al. 1999; Gleeson et al. 1999; Friocourt et al. 2003). Its expression localises with the outermost part of the immature soma and diminishes upon maturation concurring with the expression of NeuN, an archetypical marker of mature neurons (Brown et al. 2003; Nacher, Crespo,

and McEwen 2001). DCX immunoreactivity (DCX-ir) within the adult brain is sparse. Nonetheless, it includes niches of adult neurogenesis: a) the sub-granular zone (SGZ) of the hippocampal dental gyrus (DG) where some of them mature into glutamatergic granular neurons; and b) the sub-ventricular zone (SVZ), which gives rise to the neuroblast that migrate via the rostral migratory stream (RMS) onto the OB, where they integrate as inhibitory interneurons (Brown et al. 2003; Nacher, Crespo, and McEwen 2001; Leuner and Sabihi 2016).

Studies in different species indicate that DCX-ir can also be observed in non-proliferative areas of the adult brain, including the layer II of the piriform cortex (Pir) and the layer I of the olfactory tubercle (OT) (Bédard et al. 2002; Patzke et al. 2013; la Rosa et al. 2020; Brown et al. 2003; Nacher, Crespo, and McEwen 2001). Pir's population of DCX-ir cells are considered of embryonic origin (Shapiroet al. 2007). Although their number declines with age, these cells remain in a posed neuroblast state (arrested maturation) throughout adulthood and get integrated, probably as glutamatergic or GABAergic neurons (unsettled in the literature), in response to olfactory-mediated plasticity events (Gómez-Climent et al. 2008; la Rosa et al. 2020; Benedetti et al. 2020; Rotheneichner et al. 2018; Martínez-Rodríguez et al. 2019). According to their morphology, this population can be classified as tangled, precursors with small diameter and none-to-few dendrites, and complex, with bigger soma and more composed arborisations.

The population of DCX-ir cells at the OT is quite small and has not been fully characterised yet (Martínez-Rodríguez et al. 2019; Torres-Pérez et al. 2022). These cells are of uncertain origin, possibly embryonic arriving via RMS (Bédard et al. 2002; Patzke et al. 2013; de Marchis, Fasolo, and Puche 2004). Nevertheless, like Pir, OT is related to the motivational nature of chemosensory intraspecific communication (Gómez-Climent et al. 2008; Agustín-Pavón, Martínez-García, and Lanuza 2014; Gadziola and Wesson 2016), which has a prominent role in the behavioural changes associated with motherhood, particularly in rodents (Navarro-Moreno et al. 2022).

In the present investigation, we sought to assess potential variations on the population of immature neurons within olfactory areas (OB, Pir and OT) of female mice based on their reproductive phases. Thus, brains from wild-type female mice from distinct reproductive stages where isolated, sectioned and double-immunoreacted against DCX and Ki67, a marker of cellular proliferation (Kee et al. 2002). We designed two experiments to investigate, first, the contribution of pregnancy hormones by comparing DCX cells in virgin vs. pregnant female mice, and second, the possible additive effect of pup contact, (pup-sensitised virgins vs. lactating dams). We hypothesised that pregnancy and/or lactation would alter the density of immature neurons and/or their maturation stages within the olfactory areas.

Material and Methods

Animals

Samples from a total of 29 female mice (*Mus musculus*) from the wild-type strain CD1 were used for this study. Their age at the time of sacrifice was 13-14 weeks old. Subjects were housed in age-matched homologous pairs to avoid isolation stress and

kept in polypropylene cages at ~24°C under a 12-h light/dark hour cycle (lights on at 8:00 am) with *ad libitum* access to water and food. Samples corresponding to some groups (see 'Experimental design') were those spared from previous published work (Navarro-Moreno et al. 2022; 2020), allowing for a reduction in the number of culled animals (from 44 to 15) and the need to use additional male mice for mating in the present investigation.

Animal procedures were approved by the Committee of Ethics and Animal Experimentation of the Universitat Jaume I, performed in accordance with the European Union Council Directive of June 3rd, 2010 (6106/1/10 REV1), and under an animal-usage license issued by the Direcció General de Producció Agrària i Ramaderia de la Generalitat Valenciana (code 2015/VSC/PEAI00055 type 2).

Experimental design

Subjects were randomly assigned to the different experimental groups. Our experimental design included 4 groups, which were divided in two different experiments: Experiment 1) virgin (n = 8) vs. pregnant (n = 7) adult females; and Experiment 2) pup-sensitised virgin (n = 7) vs. lactating animals (n = 7).

Samples collected from previous research (Navarro-Moreno et al. 2022; 2020) were repurposed to entirely constitute Experiment Design. Briefly, females from Experiments 1 and 2 were 10 weeks old at the beginning of the procedure and samples collected between around 13 weeks-of-age. Pregnant females, and age-matched virgins, were culled at gestational days 17-19, while lactating dams and corresponding pup-sensitised females were sacrificed at postpartum day 4 (PPD4; for more details see Navarro-Moreno et al.).

In all cases, at corresponding age, females were deeply anaesthetised with an intraperitoneal (i.p.) injection of sodium pentobarbital (Vetoquinol, Madrid, Spain) at 0.02 mg per g of body weight, and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4.

Tissue processing

After perfusion, brains were carefully extracted from the skull, post-fixed overnight at 4°C with similar fixative solution and kept in a cryopreserving solution of 30% sucrose in 0.01M PB at 4°C until sinking. Five parallel series of 40 μm-thick free-floating coronal sections were obtained from each brain using a freezing microtome (Microm HM-450, Walldorf, Germany). Sections were kept in 30% sucrose in 0.1M, pH 7.6, at −20°C until use.

Double immunohistochemistry against DCX and Ki67

From the present investigation, one out of the five series from each subject were used. Sections from all four experimental groups were simultaneously processed with similar batches of reagents and antibodies to ensure minimal procedural biases.

Immunohistochemistry was performed in free-floating conditions. First, endogenous fluorescence was blocked by incubating the sections in 1% NaBH in 0.05M tris-buffered saline (TBS) for 30 minutes at room temperature (RT). Next, to block

nonspecific binding, sections were incubated in a 3% mixture of normal donkey serum (NDS) and normal gout serum (NGS) in 0.3% Tx100 in 0.05M TBS, pH 7.6, for one hour at RT. Then, sections were incubated with a combination of primary antibodies against DCX (1:4000; EMD Millipore Corp., AB2253) and Ki67 (1:5000; Abcam, ab15580) diluted in 0.05M TBS, pH 7.6, with 2% NDS/NGS in 0.3% Tx100 for an overnight at 4°C in agitation. Following day, sections were incubated with a mix of secondary antibodies (1:200 each; Alexa Fluor 488-conjugated goat anti-guinea pig, Molecular Probes; Rhodamine Red™-X-conjugated donkey anti-rabbit, Jackson ImmunoResearch) diluted in 0.05M TBS, pH 7.6, with 2% NDS in 0.3% Tx100 for 90 minutes at RT. When required, samples were thoroughly rinsed between steps in TBS.

To fluorescently label nuclei, sections were also incubated with 600nM DAPI (4',6'-diamino-2-feniindol) in TBS for 45 seconds at RT. Finally, sections were mounted in glass slides using 0.2% gelatine in TB and cover-slipped with fluorescent mounting medium (FluorSave Tm Reagent; Dako, Glostrup, Denmark).

Image acquisition and quantification

DCX/Ki67 immunoreactivity was quantified throughout representative levels according to the stereotaxic atlas (Paxinos and Franklin 2004) for OB (Bregma 4/3.6 mm), Pir (Bregma 1/-2 mm) and OT (Bregma 1/0.6 mm). Within Pir and OT (sparse immunoreactivity) pictures were only taken whenever signal was clearly identified by the examiner, who was always blind to experimental conditions. All pictures were taken using digital DFC495 camera attached to a Leitz DMRB microscope (Leica, Wezlar, Germany). Unless stated otherwise, pictures were taken at 20x magnification. When possible, pictures were obtained from both hemispheres. Quantification was performed using ImageJ analysis software (Schneider, Rasband, and Eliceiri 2012).

To perform OB analysis, two sets of images (one representative sampling image per area) per animal were taken at three different levels: granular layer (GrO) of the MOB, glomerular layer (Gl) of the MOB and accessory bulb (AOB). At GL, low density of DCX- and Ki67-ir allowed to manually count the number of immunoreactive cells using the multi-point tool, with DAPI signalling used to recognize individual nucleus. Ki67 signalling at GrO and GLA was similarly done. However, for DCX immunopositivity at GrO and GLA, the channel corresponding to DCX was isolated, converted to greyscale and signalling threshold defined as a value 140% above the mode. The resulting images were used to calculate % of positive area (with a region of interest -ROI- of 12,000 μm^2).

Positive signalling at Pir (layer II, occasionally migrating towards layer III) and OT (layer I and, occasionally migrating towards layer II) was also done using the multi-point tool. Additionally, DCX-ir cells were classified into tangled or complex based on two criteria: morphology and size (Rotheneichner et al. 2018; Benedetti et al. 2020; Rubio et al. 2016). According to morphology, a cell was determined to be tangled if it had none to one branching process while complex if it had two or more. To determine size, main diameter of the DCX-ir cells (when nuclei staining was fully apparent) was calculated. If diameter was smaller or equal than 10 μm cells were considered tangled, while those with larger diameters than 10 μm were determined as complex. In both criteria studied, data was normalised to the number of images analysed per animal.

Additionally, a new category of ‘clustered’ (grouped) DCX-ir cells at OT (layer I) are reported.

Statistical analysis

Data were analysed using the software IBM SPSS Statistics 22.0. Normalization was determined using the Kolmogrov-Smirnov and the Shapiro-Wilk tests. After removing outliers, a student’s t-test analysis for independent samples with significance set al $p < 0.05$. In a comparison was statistically significant, its effect size was calculated with Cohen’s D. Graphs were created using GraphPad Prism 9.5.4 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Motherhood did not change DCX- and Ki67-ir at the OB

Within the OB (Figure 1), we assessed DCX- and Ki67-ir at three different areas: at both glomerular layer (Gl) and granule layer (GrO) within the main olfactory bulb (MOB), and at the accessory bulb (AOB).

At Gl, DCX-ir was sparsely distributed (Figure 1A). There was no co-localization between both DCX and Ki67 except a single occurrence within one ‘lactating’ animal (image not included). Although no statistical differences were reported, a tendency towards increased number of DCX-ir cells was reported at Experiment 1 (virgin < pregnant; $t = 2.260$, $p = 0.052$; Cohen’s D = -1.145). Similarly, no statistical differences were observed at Experiment 2 (pup-sensitised virgin vs. lactating animals; DCX: $t = 0.330$, $p = 0.747$; Ki67: $t = 2.065$, $p = 0.061$; Figure 1C).

At GrO the pattern of DCX-ir expression was more densely distributed than at Gl while Ki67-ir was similarly scattered (Figure 1D). Therefore, DCX immunoreactivity was measured as proportion of area with positive signal within designated ROI while Ki67-ir was manually counted. Statistical analysis did not reveal any significant difference on either DCX-ir (Experiment 1: $t = 0.617$, $p = 0.551$, Figure 1E -left panel; Experiment 1: $t = 1.571$, $p = 0.142$, Figure 1F-left panel) or Ki67-ir (Experiment 1: $t = 0.548$, $p = 0.595$, Figure 1E -right panel; Experiment 2: $t = 1.573$, $p = 0.144$, Figure 1F -right panel).

At the AOB, the distribution pattern was similar to GrO (Figure 1G) and thus analysed likewise. As with the MOB, no significant differences were found (Experiment 1: DCX Area: $t = 0.724$, $p = 0.485$, Ki67 count: $t = 0.662$, $p = 0.522$, Figure 1H; Experiment 2: DCX Area: $t = 0.934$, $p = 0.084$, Ki67 count: $t = 1.053$, $p = 0.320$, Figure 1I).

DCX immunoreactivity was affected by lactation but not pregnancy alone in Pir

Both complex and tangled DCX-ir cells (Figure 2A-B) were identified within the piriform cortex (Pir) with similar characteristics as previously described (Rubio et al. 2016; Rotheneichner et al. 2018). Briefly, complex cells had one or more processes and presented larger soma than tangled cells (Figure 2C, yellow and green respectively). Thus, DCX-ir cells were classified twice based in either their morphological complexity or their soma dimensions (ranging from 5 to 22 µm). Additionally, there was no Ki67-ir

within the layer II of the Piriform Cortex (Pir), which agrees with previous studies on the non-proliferative nature of this neurogenic niche (Rubio et al. 2016).

Statistical analysis failed to show any significant difference within Experiment 1 (virgin vs. pregnant) based on either morphology (Total DCX: $t = 0.397$, $p = 0.698$; DCX complex: $t = 0.224$, $p = 0.826$; DCX tangled: $t = 0.494$, $p = 0.630$; Figure 2D) or diameter (DCX average: $t = 0.216$, $p = 0.832$; DCX complex: $t = 0.023$, $p = 0.982$; DCX tangled: $t = 0.298$, $p = 0.771$; Figure 2E).

On the contrary, there was a statistical difference in Experiment 2 by which lactating females had significantly less complex DCX-ir cells than pup-sensitised females (Fig 6F: t-test=2.432, p-value=0.035, Cohen's D=1.509). Additionally, the average diameter of DCX-ir cells ($t = 3.015$, $p = 0.013$, Cohen's $D = 1.702$), and that of the complex population ($t = 3.311$, $p = 0.008$, Cohen's $D = 0.129$), were significantly reduced in lactating females (Figure 2G) while tangled DCX-ir cells remained unaffected in both numbers and diameter ($p > 0.05$).

The OT was not affected by motherhood but showed signs of continuous proliferation in adulthood

We have identified a small population of DCX-ir cell at the olfactory tubercle (OT) which, in opposition to the Pir, co-labelled with Ki67. Upon careful assessment of this DCX-ir cells, we identified two types: clustered (Figures 3A-C), aggregates of tangled-like cells found within layer I of the OT; or complex (Figure 3D-G), single cells (or few cells) with multiple processes and found throughout layers I (Figure 3D-E) and layer II (Figure 3F-G) of the OT. Interestingly, only the clustered type harboured those that were also Ki67-ir.

No significant differences on DCX-ir were found in either experiment (Experiment 1: Total DCX: $t = 0.316$, $p = 0.757$, DCX complex: $t = 1.911$, $p = 0.080$, DCX clustered: $t = 0.008$, $p = 0.993$, Figure 3H; Experiment 2: Total DCX: $t = 0.128$, $p = 0.901$, DCX complex: $t = 1.112$, $p = 0.289$; DCX clustered: $t = 0.329$, $p = 0.748$, Figure 5I). In similar fashion, Ki-67-ir remained unchanged (Experiment 1: Ki67: $t = 0.769$, $p = 0.457$; DCX/Ki67: $t = 1.929$, $p = 0.078$, Figure 5H; Experiment 2: Ki67: $t = 0.288$, $p = 0.779$; DCX/Ki67: $t = 0.261$, $p = 0.799$, Figure 3I).

Discussion

We have assessed the population of DCX-ir and Ki67-ir cells at the olfactory bulbs (OB), piriform cortex (Pir) and olfactory tubercle (OT) of female mice throughout different reproductive stages. Strikingly, despite previous reports, motherhood and/or exposure to pups did not result in significant differences at the OB. Interestingly, we observed a decrease in the density of embryonically generated immature neurons in the Pir, likely due to an increase on neuronal maturation, when pregnancy was followed by lactation. This result suggests that both matrescence and the posterior exposure interaction with the pups are required to induce this phenomenon. Finally, we show that the population of DCX-ir cells at the OT is likely proliferative in young adult female mice, since DCX-ir cell clusters are in close apposition to Ki67-ir nuclei, and even these markers co-localize in a subset of small, clustered cells. Although we did not find

reproductive stage-mediated differences, our results expand previous observations suggesting that the OT is a niche of postnatal neurogenesis.

Motherhood resulted in increased neuronal maturation at the piriform cortex

Motherhood has been previously associated with neuroplastic changes including adult neurogenesis at SVZ which, via the canonical pathway, migrate to the OB and integrate as mature neurons (Leuner and Sabihi 2016; Medina and Workman 2020; Shingo et al. 2003). These plastic changes are adaptive since they are associated with novel odour learning, maternal care and hence an increased offspring survival (Hillerer et al. 2014). Nonetheless, we did not find a significant effect induced by motherhood at OB on either DCX-ir or Ki67-ir cells. Although unexpected, this lack of changes did not affect odour learning as our previous research using these same females did not show impaired maternal behaviours (Navarro-Moreno et al. 2022; 2020). In agreement, experiments reducing adult neurogenesis in mice via irradiation at the SVZ did not result in a significantly altered maternal behaviour (Feierstein et al. 2010). Nonetheless, our lack of differences can suggest that migrating cells from the RMS might take longer to reach the OB. Others have reported an increase in new neurons, cells co-labelled with BrdU/NeuN, within different areas of the main OB (MOB) at gestational day 7 and PPD7 (Shingo et al. 2003). In our research, however, pregnant and lactating dams were sacrificed at gestational days 17-19 and PPD4 respectively. In support of this hypothesis, a trend towards increased number of DCX-ir cells at the glomerular layer of the main olfactory bulb (Gl) can be seen in the group of pregnant animals.

The population of DCX-ir cells at the Pir has been previously described and regarded as a non-canonical non-proliferative neurogenic niche (Rubio et al. 2016; Gómez-Climent et al. 2008; la Rosa et al. 2020; Ghibaudi et al. 2023; Rotheneichner et al. 2018). In accordance with the non-proliferative nature, we did not find Ki67-labelling within the DCX-ir cells at the Pir. Additionally, we observed a significant reduction in the number and size of the DCX-ir complex cells, the ones in a more advanced maturation stage towards functional neurons, within the lactating group. This difference was not reported in the group of pup-sensitized virgin animals. Similarly, there were no differences between the virgin and pregnant animals. Therefore, our results indicate that both pregnancy and exposure to pups are required for the motherhood-associated maturation to take place at Pir. Thus, the physiological changes accompanying pregnancy seem to engage the DCX-expressing cells at the Pir into a state of predisposition towards maturation which takes place in response to exposure to pups, probably associated with olfactory plasticity (Benedetti et al. 2020; Rotheneichner et al. 2018).

The differential maturation at the Pir of lactating mice was restricted to the complex cells. The population of tangled, the DCX-ir cells regarded as more immature, was unchanged. In addition, the ones that remained as complex showed a smaller diameter, although above-threshold to still be considered complex. This pattern suggests that the cells that were closer to maturation engaged in neuronal differentiation upon pregnancy and postpartum signals. Thus, the almost-mature population of cells lost their DCX expression in the maturation process while the less close to maturity continued progression throughout a similar route. In this complex scenario, lack of change in the number of tangled cells indicates that this population of neuroblast-like cells remains

relatively steady, in a state of arrested maturation, awaiting potential further signals to engage in plasticity events, like those reported elsewhere (Gómez-Climent et al. 2008; la Rosa et al. 2020; Benedetti et al. 2020; Rotheneichner et al. 2018; Martínez-Rodríguez et al. 2019). For instance, if a new pregnancy and posterior lactation period take place, the associated signals would lead this pool of tangled DCX-ir cells to their progression towards maturation and integration as means to adjust their behaviour to the new event. Further studies looking at consecutive periods of motherhood might help to entangle this dynamic system.

The olfactory tubercle as a novel niche of proliferation in adulthood

An analogous situation to that of the OB was observed at the OT where the population of DCX-ir cells was not significantly altered in either group. Noteworthy, despite lack of differences by motherhood, we found clusters of DCX-ir cells in which some were Ki67-positive at layer I of the OT. Similar cluster of DCX-positive cells have been identified at the human paralaminar nuclei between gestational week 38 and birth and regarded as proliferating (Sorrells et al. 2019). Thus, the clustering distribution together with the Ki67 co-labelling suggests that layer I of the OT retains some proliferative activity during adulthood in mice, possibly serving as a reservoir of immature cells to be integrated at other layers of the OT upon relevant inductive events. However, considering the importance of OT to rodents' motivational nature towards chemosensory intraspecific communication (Gadziola and Wesson 2016; Agustín-Pavón, Martínez-García, and Lanuza 2014; Gómez-Climent et al. 2008), the lack of differences in this population by motherhood is noticeable. Nonetheless, the overall small number of DCX-ir cells within all the animals studied could be masking such an effect.

Even if the population of DCX-ir cells at the OT were not altered by motherhood, the question of whether this location retain some proliferative activity and/or their origin remains uncertain. Previous research pointed at migrations during embryogenesis via the RMS (Bédard et al. 2002; Patzke et al. 2013; de Marchis, Fasolo, and Puche 2004), which could be feasible since DCX is a microtubule-associated protein involved in cellular migration (Francis et al. 1999; Gleeson et al. 1999; Friocourt et al. 2003). However, results from other have suggested that recruitment from afar locations via long-range neuronal migrations are unlikely (Sorrells et al. 2019). Therefore, sources within the neighbourhood of the OT such as meninges, which bear neuronal precursor-like cells even during adulthood (Bifari et al. 2015), should be considered.

Conclusion

Many studies have focused on the effect of motherhood to the offspring while failing to look after the consequences to the mothers themselves (Pawluski et al. 2022; Navarro-Moreno et al. 2022; Kundakovic and Champagne 2015). Different imaging techniques have allowed identification of structural and functional neuroplastic adaptations associated with motherhood in humans (Hoekzema et al. 2022). However, these studies can only provide limited data and do not allow for cellular-type identification. Here, we have used a rodent model to identify a decrease in the number of complex DCX-ir at the Pir of animals that had undergone pregnancy and were in the active process of lactating their offspring. This suggests that both processes, matrescence and postpartum care of

the offspring, are required to induce a motherhood-induced change in neuronal maturation as assessed by DCX immunoreactivity. The other areas studied, OB and OT, were unaffected by motherhood in terms of DCX-ir density. Nonetheless, OT has revealed as a potential novel niche of adult neurogenesis which, despite the lack of differences by motherhood, makes it of interest for future investigations.

Limitations

Although this study has produced some compelling data, we have identified two potential caveats. Firstly, despite being considered a microtubule-associated protein with functions on differentiation and migration of neurons (Francis et al. 1999; Gleeson et al. 1999; Friocourt et al. 2003), DCX could be involved in other processes which might be related to neuronal development (Yap et al. 2012) or not (Dhaliwal et al. 2016). Thus, interpretations on neuronal maturation based on DCX-ir alone should be done with caution. Secondly, different commercial antibodies against both DCX and Ki67 might yield variations on data depending on the species and cerebral area studied (Ghibaudi et al. 2023), a source of inconsistency that should also be accounted for.

Data availability

Raw data generated within this manuscript can be requested to the corresponding authors.

Author contributions

MP acquired images, analysed data, figure preparation and contributed to writing. RE-P performed the immunohistochemistry procedures and contributed to writing. CN-M performed animal experiments and tissue processing. MA-A contributed to animal experiments and tissue processing. MJS-C, FM-G and EL participated in the study design and contributed to writing and funding acquisition. CA-P designed the study, supervised research, data analysis and manuscript writing. JVT-P participated in image acquisition, data analysis, prepared figures, and wrote the manuscript. Final version of this manuscript was discussed and approved by all authors.

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Conflict of interest

Authors have no conflicts of interest to declare.

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Figures and figure legends

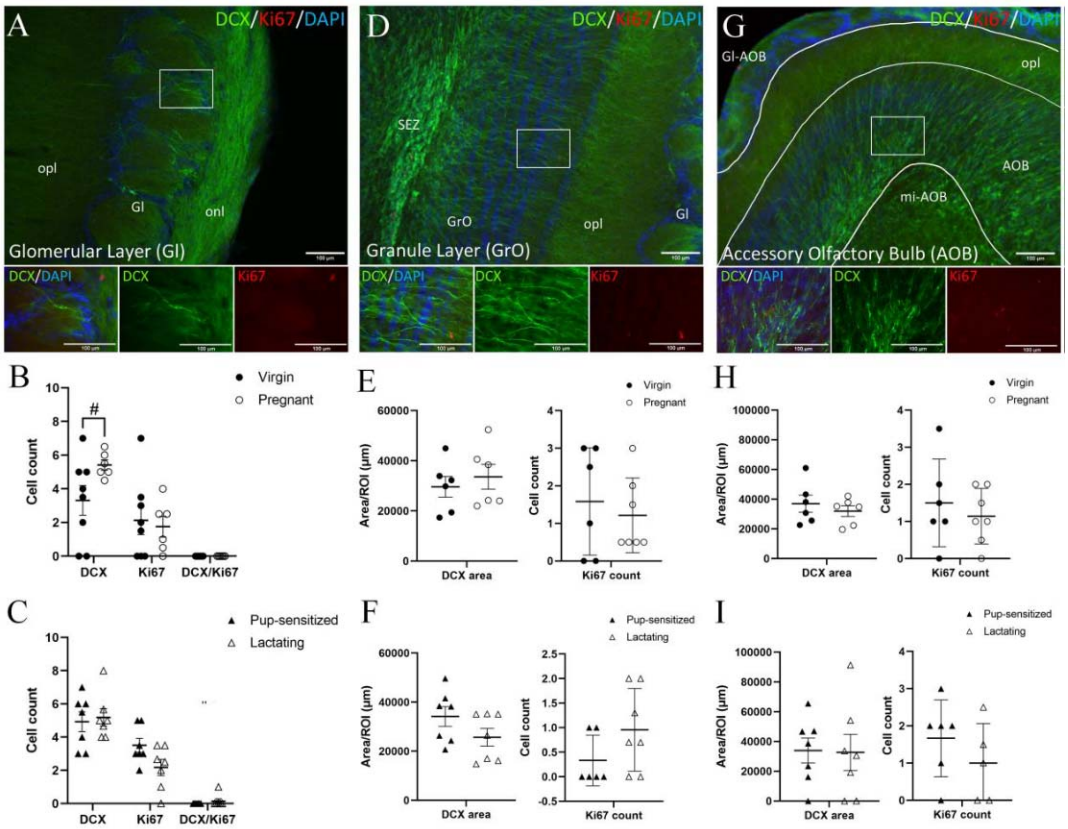

Figure 1. DCX- and Ki67-immunoreactivity was not significantly affected by motherhood in the OB. **A)** Representative microphotographs from the glomerular layer (Gl) of a lactating animal stained for DCX (green), Ki67 (red) and DAPI (blue). The outer plexiform layer (opl) and the olfactory nerve layer (onl) are also visible. Below, zoom in on a single DCX-positive cell. **B)** Interleaved scatter plot for DCX-ir cell count, Ki67-ir cell count and DCX/Ki67-ir co-labelling for Experiment 1 (virgin vs. pregnant). **C)** Interleaved scatter plot showing DCX-ir cell count, Ki67-ir cell count and DCX/Ki67-ir co-labelling for Experiment 2 (pup-sensitized vs. lactating). **D)** Representative microphotograph for granular layer (GrO) stained as in A). The subependymal zone (SEZ), the outer plexiform layer (opl) and a portion of the glomerular layer (Gl) are also visible. Below, zoom in on a DCX-ir cell with a Ki67-ir cell in close proximity. **E)** and **F)** interleaved scattered plots with DCX-ir measured as proportion of positive area and Ki67-ir as cell counts for Experiment 1 and Experiment 2 respectively at the GrO. **G)** Representative microphotograph for accessory olfactory bulb (AOB) similarly stained. The outer plexiform layer (opl) and the glomerular layer

and mitral layer of AOB are also visible. Below, a zoom in on a group of DCX-ir cells together with Ki67-ir cells. **H)** and **I)** interleaved scattered plots similar to E) and F) and corresponding to the AOB. In all cases, scale bars represent 100 µm. Graphs show individual values and mean ± standard error mean (SEM). # p = 0.05

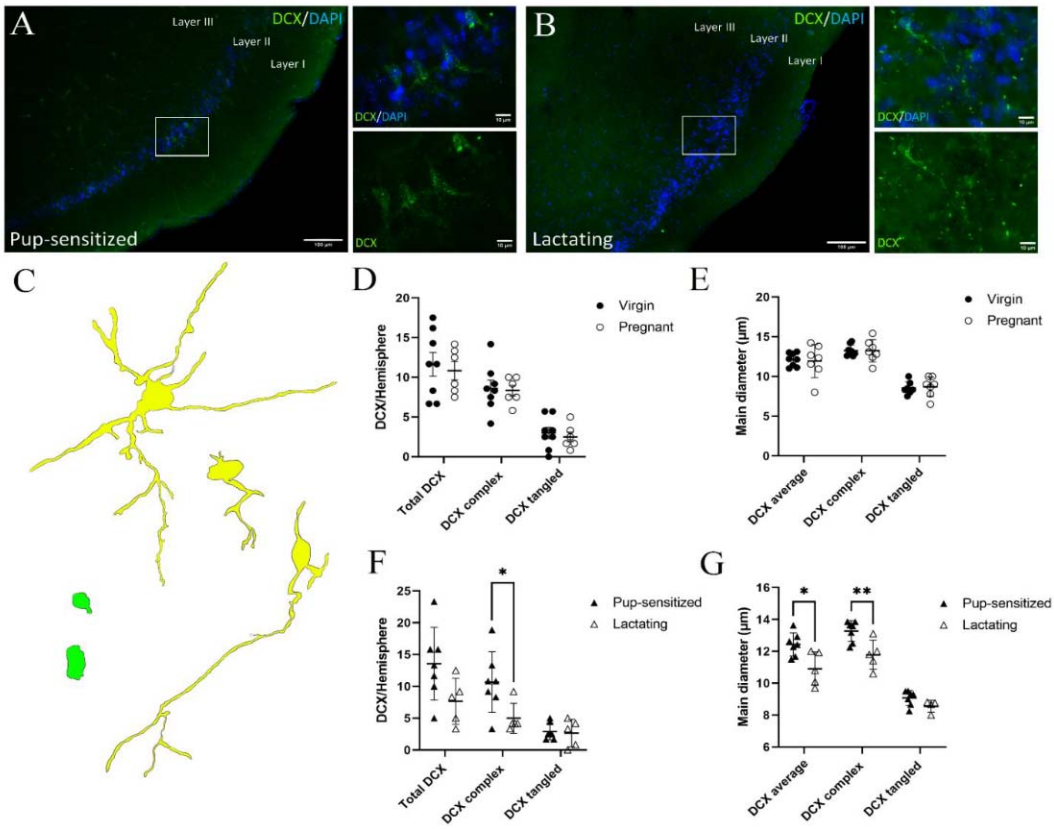

Figure 2. DCX-ir cell density and diameter were reduced in the piriform cortex (Pir) of lactating dams. Representative microphotograph from a **A)** pup-sensitized and **B)** lactating animal showing DCX (green) and DAPI (blue). Layer II of Pir is characterised by high nuclei density. Note that there is no Ki67 staining (red). Right panel shows a zoom in to a group of tangled and complex cells (just a single complex cell in B). **C)** Schematic representation on the morphological differences between tangled (green) and complex (yellow) DCX-ir cells found at Pir. Interleaved scatter plots for Experiment 1 based on **D)** morphology and **E)** main diameter measurement. **F)** and **G)** similar interleaved scatter plot than D) and E) but for Experiment 2. In all cases, scale bars represent 100 µm. Graphs show individual values and mean ± SEM. * p < 0.05; ** p <0.01.

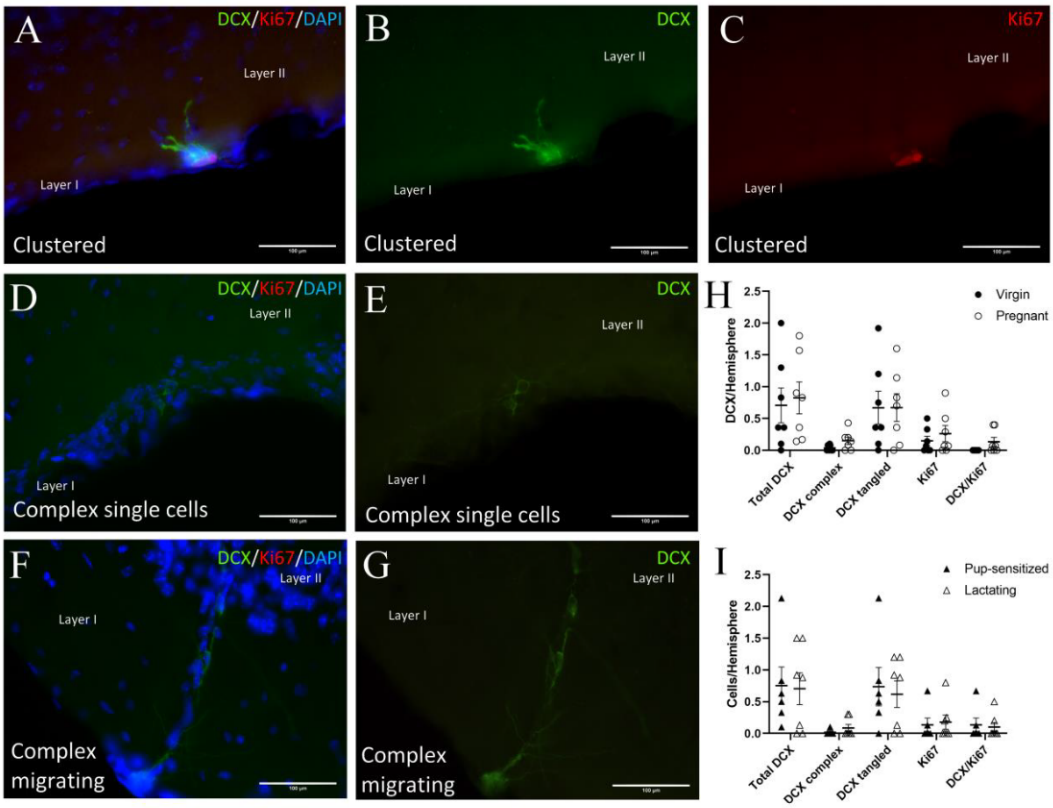

Figure 3. Motherhood does not significantly affect DCX-ir cell population in the olfactory tubercle (OT) but proliferation occurs continuously in all conditions studied. Representative microphotographs stained against DCX (green), Ki67 (red) and DAPI (blue) for **A-C)** clustered, and **D-E)** complex cells within the OT. Note that co-labelling Ki67/DCX only occurs at the clustered cells. Also note that DCX-ir in F-G seems distributed throughout layers I-III of the OT. Interleaved scattered plots for total DCX, DCX complex, DCX tangled, Ki67 and DCX/Ki67 co-labelling for H) Experiment 1 (virgin vs. pregnant) and I) Experiment II (pup-sensitized vs. lactating). In all cases, scale bars represent 100 µm. Graphs show individual values and mean ± SEM.