Puberty and motherhood promote region-specific maturation of doublecortin cells in olfactory circuits of mice

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Abstract

In mammalian females, both adolescence and motherhood are critical periods modulating behavioural changes to favour evolutionary survival. In mice, olfaction is a key driver of social behaviours, and adult neurogenesis in the olfactory bulb is an important form of neural plasticity underlying adequate behavioural responses towards conspecifics. In this study, we sought to investigate whether transition to adulthood and motherhood would affect the populations of immature neurons described in other areas of the olfactory system. To do so, we analysed the expression of doublecortin (DCX), a marker of immature neurons, and Ki67, a marker of proliferation, in the olfactory tubercle and piriform cortex of pre-pubertal and adult CD1 female mice. Our results revealed sustained proliferation of neuroblasts in the olfactory tubercle in both pre-pubertal and adult females, with Ki67 nuclei located in close apposition to clusters of DCX-immunoreactive cells. However, the density of DCX-immunoreactive cells in this area was not affected by either puberty, pregnancy, or lactation. In contrast, lactating females had significantly less DCX- immunoreactive cells than pup-sensitised virgin females at the piriform cortex, with a similar non-significant tendency observed in the group of pregnancy alone. This suggests that lactation can act on top of pregnancy to promote the maturation of these embryonically generated DCX- immunoreactive neurons at the piriform cortex. 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: doublecortin; Ki67; neurogenesis; matrescence; lactation

Introduction

Throughout the lifespan of a female mammal, a myriad of psychobiological changes can determine their behavioural and neural responses. Both the transition to adulthood and motherhood constitute a period of special importance due to its adaptive nature (Carmona et al. 2019; Pawluski et al. 2022; Hoekzema et al. 2022; Piekarski, Boivin, and Wilbrecht 2017). Not surprisingly, inadequate social and maternal behaviours are highly correlated with developmental and health deficits in the females and their 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 females during both pregnancy and postpartum, which are dynamically adjusted to the needs of the offspring (Martínez-García et al. 2021; 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 the social brain functioning in all mammals studied, the so called "socio-sexual behaviour 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 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; Doetsch and Alvarez-Buylla 1996). 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 dentate gyrus (DG) where some of these neurons mature into glutamatergic granular neurons; and b) the ventricular-subventricular zone (V-SVZ), which gives rise to the neuroblasts that migrate via the rostral migratory stream (RMS) towards the OB, where they disperse and 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 in 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 (Shapiro et al. 2007; Klempin et al. 2011; Nacher, Crespo, and McEwen 2001). Although their number declines with age, these cells remain in a posed neuroblast state (arrested maturation) throughout adulthood and get integrated, probably as glutamatergic neurons, 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). 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 (Benedetti et al. 2020).

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. 2020).

In the present investigation, we sought to assess the proliferative potential of DCX-ir cells within the OT by co-labelling against DCX and Ki67, a marker of cellular proliferation (Kee et al. 2002), and the potential influence of both adolescence and motherhood at the OT and Pir in terms of DCX-ir. For this purpose, we designed three experiments. First, to assess the effect of sexual steroids and transition to adulthood by comparing pre-pubertal vs. adult female mice. Secondly, the effect of pregnancy hormones by comparing in virgin vs. pregnant female mice. And, thirdly, a potential additive effect of lactation by comparing pup-sensitised virgins vs. lactating dams. We hypothesised that both transition to adulthood and pregnancy and/or lactation would promote maturation which could result in a decrease on the number of immature embryonically-generated DCX-ir neurons at Pir. Additionally, we also hypothesised that these critical periods might alter the population of DCX-ir cells at the OT.

Materials and Methods

Animals

Samples from a total of 42 female mice (*Mus musculus*) from the wild-type strain CD1 were used for this study. Their age at the time of sacrifice was 4 weeks old for the pre-

pubertal animals, and 12-14 weeks old for the virgins, pregnant females and dams. 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 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 8 groups, which were divided in three different experiments: Experiment 1) pre-puber (n = 6) vs. young adult (n = 7); Experiment 2) virgin (n = 8) vs. pregnant females (n = 7); and Experiment 3) pup-sensitised virgin (n = 7) vs. lactating dams (n = 7).

Samples collected from previous research (Navarro-Moreno et al. 2022; 2020) were repurposed to constitute Experiment 2. Briefly, females from Experiments 2 and 3 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 (for more details see Navarro-Moreno et al. 2020), while lactating dams and corresponding pup-sensitised females were sacrificed at postpartum day 4 (PD4). Pre-pubertal animals were sacrificed at 4 weeks of age at the same time as young adult virgins, age-matched with controls of Experiments 2 and 3.

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 saline (0.9%) 4% paraformaldehyde (PFA) in 0.1 M 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 0.01 M 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 0.1 M, pH 7.6, at −20°C until use.

Co-immunohistochemistry against DCX and Ki67

We used one out of the five brain series from each subject. Sections corresponding to each Experiment were simultaneously processed using same 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 0.05 M 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 0.05 M TBS, pH 7.6, for one hour at RT. Then, sections were incubated overnight with a combination of primary antibodies against DCX (1:4000; EMD Millipore Corp., AB2253) and Ki67 (1:5000; Abcam, ab15580) diluted in 0.05 M TBS, pH 7.6, with 2% NDS/NGS in 0.3% Tx100 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 0.05 M 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 600 nM 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 OT, all sections of the slides between Bregma 1 and 0.6 mm were inspected to quantify all positive cells within this structure. For Pir, we selected 4 sections representative of four Bregma levels: 1, 0, -1 and -2 mm. Images were acquired whenever signal was clearly identified by the examiner, who was always blind to experimental conditions. All images were taken using digital DFC495 camera attached to a Leitz DMRB microscope (Leica, Wezlar, Germany). Unless stated otherwise, pictures were taken at 20x magnification. Pictures were obtained from both hemispheres at selected levels. For the OT, we obtained pictures whenever we found DCX-ir, whereas for the Pir we took a single picture centred at layer II in selected levels.

Positive signalling at OT (layer I and, occasionally migrating towards layer II) and Pir (layer II, occasionally migrating towards layer III) was counted by using the multi-point tool of ImageJ analysis software (Schneider, Rasband, and Eliceiri 2012). Results were expressed as number of positive cells per hemisphere in the OT, and number of positive cells per mm^2 in the Pir.

We categorized DCX-ir cells in the OT as 'clustered' (grouped, generally with little or no neurites, Figure 1A) and complex-like (isolated or not in close apposition to neighbours, and with well-developed and visible neurites of diverse length (Figure 1B). In the Pir, DCX-ir cells were classified into tangled or complex based on 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 and 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. In general, diameter was smaller or equal than 10 µm cells in tangled cells, while those with larger diameters than 10 µm were complex. Data was normalised to the number of sections or pictures analysed

per animal, and animals lacking a complete representation of any Bregma level because of damaged tissue were discarded for further analysis.

Representative images for figure preparation were taken with a confocal microscope (Leica, TCS-SPE) using 20x and 63x objectives. Figures were processed using Fiji software (Schneider, Rasband, and Eliceiri 2012) and Adobe Photoshop CS6 (Adobe Systems, MountainView, CA, USA). Animals used for figure preparation included a pregnant female (internal code: M18130), a young adult female (internal code: M1933), pre-puber female (internal code: M1902) and a virgin female (internal code: M18134).

Statistical analysis

Data were analysed using the software IBM SPSS Statistics 26.0. Normality was determined using the Kolmogrov-Smirnov and the Shapiro-Wilk tests, and outliers were removed following Tukey's hinges method. We applied a student's t-test analysis for independent samples when possible, and if data violated normality, we used a Mann-Whitney test. Significance was set al $p < 0.05$. Graphs were created using GraphPad Prism 9.5.4 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Ki67 co-labelling with DCX-ir cells in the OT suggest continuous proliferation.

The small population of DCX-ir cell at the olfactory tubercle (OT), in opposition to the Pir (see below), was co-labelled with Ki67 (Figure 1A, B). Upon careful assessment of this DCX-ir cells, we identified two types. Clustered DCX-ir cells are found as aggregates of round cells with little or no neurites located at the external edge of layer I of the OT (Figure 1A). Complex-like DCX-ir cells are either grouped but not in close contact (Figure 1B,C), or isolated, describing multiple processes and mainly located within the outermost region of layer I (Figure 1B). Infrequently, this isolated DCX-ir cells were found closer to layer II (Figure 1C) and, on some occasions, in close apposition to the Islands of Calleja (Figure 1D).

Interestingly, a small minority of the clustered-type DCX-ir cells were co-labelled with Ki67-ir (Fig 1A) and, more frequently, single-labelled Ki67 nuclei were found in intimate contact with the cluster (Fig 1A). This suggests that, unlike DCX-ir cells in the Pir, which are generated during embryonic development and are never co-labelled with Ki67 (nor Ki67-ir is found in the Pir, see below), OT DCX-ir neurons might retain certain proliferative capacity.

Statistical analysis did not find significant differences on DCX-ir density in any of our Experiments (Experiment 1: Total DCX: $U = 15$, $p = 0.445$, DCX clustered: $U = 10$, $p = 0.138$, DCX complex-like: $U = 15.5$, $p = 0.445$, Figure 2A; Experiment 2: Total DCX: $U = 20.5$, $p = 0.620$, DCX clustered: $U = 24.5$, $p = 1$; DCX complex-like: $U = 12$, $p = 0.128$, Figure 2B; Experiment 3: Total DCX: $U = 21$, $p = 1$, DCX clustered: $U = 20$, $p = 0.945$; DCX complex-like: $U = 17.5$, $p = 0.706$, Figure 2C).

Ki67-ir nuclei were found throughout layer I of OT, sometimes in close apposition or near DCX-ir clusters. General expression of Ki67-ir was strongly affected by age in Experiment 1, with a highly significant density of labelled nuclei in the OT of pre-pubertal

females when compared to post-pubertal animals ($t = 8.098$, $p < 0.001$, Figure 2A'). Nonetheless, the expression of Ki67-ir was not significantly affected by motherhood and or posterior care/exposure to the offspring (Experiment 2, $t = 1.397$, $p = 0.188$, Figure 2B'; Experiment 3, $t = -0.834$, $p = 0.422$, Figure 2C'). Double labelling of DCX cells with Ki67 was not found in either pre-pubertal mice or adults in Experiment 1 or virgins in Experiment 2. By contrast, double labelling was found in the OT of females of the pregnant group (percentage of DCX-ir cells co-labelled with Ki67, 13.03%±6.36), pup-sensitized (17.5%±12.2) and lactating (6.7%±4.8).

The population of DCX-ir at Pir decreases upon transitioning to adulthood.

Both complex and tangled DCX-ir cells (Figure 3A) were identified within layer II of 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 3B). Importantly, and in contrast with what we found in the OT, there was no Ki67-ir within layer II of the Piriform Cortex (Pir), which agrees with previous studies on the non-proliferative nature of this DCX-ir population (Rubio et al. 2016). As expected, we did not find Ki67-ir nuclei co-labelling with DCX-ir cells, nor Ki67-ir single labelled nuclei in layer II of the Pir.

In agreement with the previously described maturation process in this population of immature neurons with age, we found a strong difference in Experiment 1 between groups, with pre-pubertal females harbouring much higher number of total DCX-ir neurons (Total DCX: $t = 4.608$, $p < 0.001$; DCX tangled: $t = 2.660$, $p = 0.055$; DCX complex: $t = 4.624$, $p < 0.001$; Figure 4A). Average diameter of DCX-ir cells, by contrast, was not significantly affected by age ($t = 0.527$, $p = 0.609$, Figure 4B).

Number of DCX-ir cells and their average diameter was decreased by lactation at Pir

Pregnancy alone failed to significantly affect any of the measures on Pir DCX-ir cells, except for a slight trend towards reduction of complex DCX-ir cells (Total DCX: $t = 1.415$, $p = 0.188$; DCX tangled: $t = 0.455$, $p = 0.329$; DCX complex: $t = 1.544$, $p = 0.077$; DCX average diameter, $t = 0.105$, $p = 0.459$, Figure 4C, D). By contrast, lactating animals had significantly lower density of DCX-ir cells, due to lower number of the complex subtype (Total DCX-ir, $t = 2.410$, $p = 0.035$; DCX tangled, $t = 0.654$, $p = 0.532$; DCX complex, $t = 0\ 2.748$, $p = 0.019$, Figure 4E). Accordingly, the average diameter of DCX-ir cells was significantly lower in lactating females ($t = 2.412$, $p = 0.03$, Figure 4F). This result suggests that lactation and/or contact with pups might act on top of pregnancy to promote maturation of DCX-ir cells in the Pir.

Discussion

We have assessed the population of DCX-ir immature cells at the olfactory tubercle (OT) and piriform cortex (Pir) of female mice throughout different reproductive stages. Our results suggest that the population of DCX-ir cells at the OT is likely proliferative in adult female mice. Indeed, DCX-ir cell clusters are located in close apposition to Ki67-ir nuclei, and these markers co-localize in a small subset of clustered cells in the subjects exposed to either pregnancy or contact with pups. Although we did not find reproductive stage-mediated differences in the number of DCX-ir cells, our results suggest that the OT is a niche of postnatal neurogenesis. Further, according to previous reports, we did not find

proliferative activity in the Pir, and the population of embryonically generated immature neurons decreased after puberty. Interestingly, we observed a trend towards decrease on the number of complex DCX-ir cells in pregnant females, which reaches significance in lactating dams. This decrease in DCX-ir cells is likely due to an acceleration of maturation rate of these cells when pregnancy was followed by lactation. This result suggests that both matrescence and the posterior exposure and interaction with the pups are required to induce this phenomenon.

The olfactory tubercle as a novel niche of proliferation in adulthood

One of our main findings stems in the sparse clusters of DCX-ir cells in which some were Ki67-positive at layer I of the OT. Similar clusters 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. This population of DCX-ir cells could be serving as a reservoir of immature cells to be integrated into other layers of the OT upon relevant inductive programming. Interestingly, we have previously shown that DCX-ir cells in the OT are significantly increased in a mouse model of Rett syndrome (Martínez-Rodríguez et al. 2019; Torres-Pérez et al. 2022).

Considering the importance of OT to rodents' motivational nature towards chemosensory intraspecific communication (Gadziola and Wesson 2016; Agustín-Pavón, Martínez-García, and Lanuza 2014), the lack of differences in this population by puberty and motherhood is noticeable. Nonetheless, the overall small number of DCX-ir cells within all the animals studied could be masking such an effect. In fact, we found double labelling in DCX-ir cells only in animals exposed to pups and in pregnant females. Future studies should be carried out to better characterize this process.

Even if the density of DCX-ir cells at the OT was not altered across the life of animals, at least at the ages studied in this report, the question of whether this area retains some proliferative activity and/or their origin deserves future studies. Previous research pointed at migrations during embryogenesis via the RMS (Bédard et al. 2002; Patzke et al. 2013; Sanai et al. 2011; 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 others have suggested that recruitment from afar locations via long-range neuronal migrations are unlikely (Sorrells et al. 2019; Paredes et al. 2016). 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.

Motherhood resulted in a decrease on immature neurons at the piriform cortex

Motherhood has been previously associated with neuroplastic changes including adult neurogenesis at the V-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; Doetsch and Alvarez-Buylla 1996). 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).

The population of DCX-ir cells at the Pir has been previously described and regarded as a non-canonical non-proliferative neurogenic niche, where DCX-ir cells decrease with age as they mature and integrate into the circuit (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 and found a strong decrease of DCX-ir in young adult virgins as compared to pre-pubertal females. Albeit we found no significant differences between virgin and pregnant animals, we observed a trend towards decrease of the more complex DCX-ir in the latter group. Interestingly, there was a significant reduction in the number of total DCX-ir cells in lactating animals, mainly due to a decrease of DCX-ir complex cells, the ones in a more advanced maturation stage. Accordingly, the average main diameter of the DCX-ir cells was reduced in this group of females. 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).

By contrast, the population of tangled, the DCX-ir cells regarded as more immature, was unchanged. 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 seem to have 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). It is tempting to speculate that, if a new pregnancy and posterior lactation period took 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.

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. In addition, OT has revealed as a potential novel niche of adult neurogenesis which, despite the lack of differences by transition to adulthood and 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

RE-P and MP performed the immunohistochemistry procedures, acquired images, analysed data, figure preparation and contributed to writing. CN-M and MB-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.

References

Agustín-Pavón, Carmen, Fernando Martínez-García, and Enrique Lanuza. 2014. "Focal Lesions within the Ventral Striato-Pallidum Abolish Attraction for Male

Chemosignals in Female Mice." *Behavioural Brain Research* 259. https://doi.org/10.1016/j.bbr.2013.11.020.

Bédard, Andréanne, Martin Lévesque, Patrick J. Bernier, and André Parent. 2002. "The Rostral Migratory Stream in Adult Squirrel Monkeys: Contribution of New Neurons to the Olfactory Tubercle and Involvement of the Antiapoptotic Protein Bcl-2." *European Journal of Neuroscience* 16 (10). https://doi.org/10.1046/j.1460-9568.2002.02263.x.

Benedetti, Bruno, Dominik Dannehl, Richard König, Simona Coviello, Christina Kreutzer, Pia Zaunmair, Dominika Jakubecova, et al. 2020. "Functional Integration of Neuronal Precursors in the Adult Murine Piriform Cortex." *Cerebral Cortex* 30 (3). https://doi.org/10.1093/cercor/bhz181.

Bifari, Francesco, Valeria Berton, Annachiara Pino, Marijana Kusalo, Giorgio Malpeli, Marzia Di Chio, Emanuela Bersan, et al. 2015. "Meninges Harbor Cells Expressing Neural Precursor Markers during Development and Adulthood." *Frontiers in Cellular Neuroscience* 9 (OCT). https://doi.org/10.3389/fncel.2015.00383.

Brown, Jason P., Sébastien Couillard-Després, Christiana M. Cooper-Kuhn, Jürgen Winkler, Ludwig Aigner, and H. Georg Kuhn. 2003. "Transient Expression of Doublecortin during Adult Neurogenesis." *Journal of Comparative Neurology* 467 (1). https://doi.org/10.1002/cne.10874.

Carmona, Susanna, Magdalena Martínez-García, María Paternina-Die, Erika Barba-Müller, Lara M. Wierenga, Yasser Alemán-Gómez, Clara Pretus, et al. 2019. "Pregnancy and Adolescence Entail Similar Neuroanatomical Adaptations: A Comparative Analysis of Cerebral Morphometric Changes." *Human Brain Mapping* 40 (7). https://doi.org/10.1002/hbm.24513.

Dhaliwal, Jagroop, Yanwei Xi, Elodie Bruel-Jungerman, Johanne Germain, Fiona Francis, and Diane C. Lagace. 2016. "Doublecortin (DCX) Is Not Essential for Survival and Differentiation of Newborn Neurons in the Adult Mouse Dentate Gyrus." *Frontiers in Neuroscience* 9 (JAN). https://doi.org/10.3389/fnins.2015.00494.

Doetsch, Fiona, and Arturo Alvarez-Buylla. 1996. "Network of Tangential Pathways for Neuronal Migration in Adult Mammalian Brain." *Proceedings of the National Academy of Sciences of the United States of America* 93 (25). https://doi.org/10.1073/pnas.93.25.14895.

Fitzgerald, Eamon, Kahyee Hor, and Amanda J. Drake. 2020. "Maternal Influences on Fetal Brain Development: The Role of Nutrition, Infection and Stress, and the Potential for Intergenerational Consequences." *Early Human Development* 150. https://doi.org/10.1016/j.earlhumdev.2020.105190.

Fleming, A. S., C. Kuchera, A. Lee, and G. Winocur. 1994. "Olfactory-Based Social Learning Varies as a Function of Parity in Female Rats." *Psychobiology* 22 (1). https://doi.org/10.3758/BF03327078.

Francis, Fiona, Annette Koulakoff, Dominique Boucher, Philippe Chafey, Bruce Schaar, Marie Claude Vinet, Gaëlle Friocourt, et al. 1999. “Doublecortin Is a Developmentally Regulated, Microtubule-Associated Protein Expressed in Migrating and Differentiating Neurons.” *Neuron* 23 (2). https://doi.org/10.1016/S0896-6273(00)80777-1.

Friocourt, Gaëlle, Annette Koulakoff, Philippe Chafey, Dominique Boucher, Fabien Fauchereau, Jamel Chelly, and Fiona Francis. 2003. “Doublecortin Functions at the Extremities of Growing Neuronal Processes.” *Cerebral Cortex* 13 (6). https://doi.org/10.1093/cercor/13.6.620.

Furuta, Miyako, and Robert S. Bridges. 2005. “Gestation-Induced Cell Proliferation in the Rat Brain.” *Developmental Brain Research* 156 (1). https://doi.org/10.1016/j.devbrainres.2005.01.008.

Gadziola, Marie A., and Daniel W. Wesson. 2016. “The Neural Representation of Goal-Directed Actions and Outcomes in the Ventral Striatum’s Olfactory Tubercle.” *Journal of Neuroscience* 36 (2). https://doi.org/10.1523/JNEUROSCI.3328-15.2016.

Ghibaudi, Marco, Alessia Amenta, Miriam Agosti, Marco Riva, Jean-Marie Graïc, Francesco Bifari, and Luca Bonfanti. 2023. “Consistency and Variation in Doublecortin and Ki67 Antigen Detection in the Brain Tissue of Different Mammals, Including Humans.” *International Journal of Molecular Sciences* 24 (3): 2514. https://doi.org/10.3390/ijms24032514.

Gleeson, Joseph G., Lin Peter T, Lisa A. Flanagan, and Christopher A. Walsh. 1999. “Doublecortin Is a Microtubule-Associated Protein and Is Expressed Widely by Migrating Neurons.” *Neuron* 23 (2). https://doi.org/10.1016/S0896-6273(00)80778-3.

Gómez-Climent, María Ángeles, Esther Castillo-Gómez, Emilio Varea, Ramón Guirado, José Miguel Blasco-Ibáñez, Carlos Crespo, Francisco José Martínez-Guijarro, and Juan Nácher. 2008. “A Population of Prenatally Generated Cells in the Rat Paleocortex Maintains an Immature Neuronal Phenotype into Adulthood.” *Cerebral Cortex* 18 (10). https://doi.org/10.1093/cercor/bhm255.

Hillerer, Katharina Maria, Volker Rudolf Jacobs, Thorsten Fischer, and Ludwig Aigner. 2014. “The Maternal Brain: An Organ with Peripartal Plasticity.” *Neural Plasticity*. https://doi.org/10.1155/2014/574159.

Hoekzema, Elseline, Henk van Steenbergen, Milou Straathof, Arlette Beekmans, Inga Marie Freund, Petra J.W. Pouwels, and Eveline A. Crone. 2022. “Mapping the Effects of Pregnancy on Resting State Brain Activity, White Matter Microstructure, Neural Metabolite Concentrations and Grey Matter Architecture.” *Nature Communications* 13 (1). https://doi.org/10.1038/s41467-022-33884-8.

Kee, N., S. Sivalingam, R. Boonstra, and J. M. Wojtowicz. 2002. “The Utility of Ki-67 and BrdU as Proliferative Markers of Adult Neurogenesis.” *Journal of Neuroscience Methods* 115 (1). https://doi.org/10.1016/S0165-0270(02)00007-9.

Klempin, Friederike, Golo Kronenberg, Giselle Cheung, Helmut Kettenmann, and Gerd Kempermann. 2011. “Properties of Doublecortin-(DCX)-Expressing Cells in the Piriform Cortex Compared to the Neurogenic Dentate Gyrus of Adult Mice.” *PLoS ONE* 6 (10). https://doi.org/10.1371/journal.pone.0025760.

Kundakovic, Marija, and Frances A. Champagne. 2015. “Early-Life Experience, Epigenetics, and the Developing Brain.” *Neuropsychopharmacology*. https://doi.org/10.1038/npp.2014.140.

Leuner, Benedetta, and Sara Sabihi. 2016. “The Birth of New Neurons in the Maternal Brain: Hormonal Regulation and Functional Implications.” *Frontiers in Neuroendocrinology* 41. https://doi.org/10.1016/j.yfrne.2016.02.004.

Mak, Gloria K., and Samuel Weiss. 2010. “Paternal Recognition of Adult Offspring Mediated by Newly Generated CNS Neurons.” *Nature Neuroscience* 13 (6). https://doi.org/10.1038/nn.2550.

Mann, Martha A., and Bruce Svare. 1982. “Factors Influencing Pregnancy-Induced Aggression in Mice.” *Behavioral and Neural Biology* 36 (3). https://doi.org/10.1016/S0163-1047(82)90867-6.

Marchis, Silvia de, Aldo Fasolo, and Adam C. Puche. 2004. “Subventricular Zone-Derived Neuronal Progenitors Migrate into the Subcortical Forebrain to Postnatal Mice.” *Journal of Comparative Neurology* 476 (3). https://doi.org/10.1002/cne.20217.

Martínez-García, Magdalena, María Paternina-Die, Erika Barba-Müller, Daniel Martín De Blas, Laura Beumala, Romina Cortizo, Cristina Pozzobon, et al. 2021. “Do Pregnancy-Induced Brain Changes Reverse? The Brain of a Mother Six Years after Parturition.” *Brain Sci* 2021: 168. https://doi.org/10.3390/brainsci.

Martínez-Rodríguez, Elena, Ana Martín-Sánchez, Simona Coviello, Cristina Foiani, Emre Kul, Oliver Stork, Fernando Martínez-García, et al. 2019. “Lack of MeCP2 Leads to Region-Specific Increase of Doublecortin in the Olfactory System.” *Brain Structure and Function*. https://doi.org/10.1007/s00429-019-01860-6.

Mayer, A. D., and J. S. Rosenblatt. 1984. “Prepartum Changes in Maternal Responsiveness and Nest Defense in Rattus Norvegicus.” *Journal of Comparative Psychology (Washington, D.C. : 1983)* 98 (2). https://doi.org/10.1037/0735-7036.98.2.177.

Medina, Joanna, and Joanna L. Workman. 2020. “Maternal Experience and Adult Neurogenesis in Mammals: Implications for Maternal Care, Cognition, and Mental Health.” *Journal of Neuroscience Research*. https://doi.org/10.1002/jnr.24311.

Nacher, Juan, Carlos Crespo, and Bruce S. McEwen. 2001. “Doublecortin Expression in the Adult Rat Telencephalon.” *European Journal of Neuroscience* 14 (4). https://doi.org/10.1046/j.0953-816X.2001.01683.x.

Navarro-Moreno, Cinta, Manuela Barneo-Muñoz, María Victoria Ibáñez-Gual, Enrique Lanuza, Carmen Agustín-Pavón, María José Sánchez-Catalán, and Fernando Martínez-García. 2022. “Becoming a Mother. Pregnancy Shifts the Activity of the

Social and Motivation Brain Networks in Female Mice." *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.4020518.

Navarro-Moreno, Cinta, Maria Jose Sanchez-Catalan, Manuela Barneo-Muñoz, Rafael Goterris-Cerisuelo, Maria Belles, Enrique Lanuza, Carmen Agustin-Pavon, and Fernando Martinez-Garcia. 2020. "Pregnancy Changes the Response of the Vomeronasal and Olfactory Systems to Pups in Mice." *Frontiers in Cellular Neuroscience* 14. https://doi.org/10.3389/fncel.2020.593309.

Newman, Sarah Winans. 1999. "The Medial Extended Amygdala in Male Reproductive Behavior. A Node in the Mammalian Social Behavior Network." In *Annals of the New York Academy of Sciences*. Vol. 877. https://doi.org/10.1111/j.1749-6632.1999.tb09271.x.

Orchard, Edwina R., Helena J.V. Rutherford, Avram J. Holmes, and Sharna D. Jamadar. 2023. "Matrescence: Lifetime Impact of Motherhood on Cognition and the Brain." *Trends in Cognitive Sciences* xx (xx). https://doi.org/10.1016/j.tics.2022.12.002.

Paredes, Mercedes F., Shawn F. Sorrells, Jose M. Garcia-Verdugo, and Arturo Alvarez-Buylla. 2016. "Brain Size and Limits to Adult Neurogenesis." *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.23896.

Patzke, N., C. Kaswera, E. Gilissen, A. O. Ihunwo, and P. R. Manger. 2013. "Adult Neurogenesis in a Giant Otter Shrew (Potamogale Velox)." *Neuroscience* 238. https://doi.org/10.1016/j.neuroscience.2013.02.025.

Pawluski, Jodi L., Elseline Hoekzema, Benedetta Leuner, and Joseph S. Lonstein. 2022. "Less Can Be More: Fine Tuning the Maternal Brain." *Neuroscience and Biobehavioral Reviews*. https://doi.org/10.1016/j.neubiorev.2021.11.045.

Paxinos, George, and Keith B.J. Franklin. 2004. "The Mouse Brain in Stereotaxic Coordinates, 2nd Edition." *Academic Press*.

Piekarski, David J., Josiah R. Boivin, and Linda Wilbrecht. 2017. "Ovarian Hormones Organize the Maturation of Inhibitory Neurotransmission in the Frontal Cortex at Puberty Onset in Female Mice." *Current Biology* 27 (12). https://doi.org/10.1016/j.cub.2017.05.027.

Rosa, Chiara la, Francesca Cavallo, Alessandra Pecora, Matteo Chincarini, Ugo Ala, Chris G. Faulkes, Juan Nacher, et al. 2020. "Phylogenetic Variation in Cortical Layer II Immature Neuron Reservoir of Mammals." *ELife* 9. https://doi.org/10.7554/eLife.55456.

Rotheneichner, Peter, Maria Belles, Bruno Benedetti, Richard König, Dominik Dannehl, Christina Kreutzer, Pia Zaunmair, et al. 2018. "Cellular Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors into Excitatory Neurons." *Cerebral Cortex* 28 (7). https://doi.org/10.1093/cercor/bhy087.

Rubio, A., M. Belles, G. Belenguer, S. Vidueira, I. Fariñas, and J. Nacher. 2016. "Characterization and Isolation of Immature Neurons of the Adult Mouse Piriform Cortex." *Developmental Neurobiology* 76 (7). https://doi.org/10.1002/dneu.22357.

Salais-López, Hugo, Enrique Lanuza, Carmen Agustín-Pavón, and Fernando Martínez-García. 2017. "Tuning the Brain for Motherhood: Prolactin-like Central Signalling in Virgin, Pregnant, and Lactating Female Mice." *Brain Structure and Function* 222 (2). https://doi.org/10.1007/s00429-016-1254-5.

Sanai, Nader, Thuhien Nguyen, Rebecca A. Ihrie, Zaman Mirzadeh, Hui Hsin Tsai, Michael Wong, Nalin Gupta, et al. 2011. "Corridors of Migrating Neurons in the Human Brain and Their Decline during Infancy." *Nature* 478 (7369). https://doi.org/10.1038/nature10487.

Schneider, Caroline A., Wayne S. Rasband, and Kevin W. Eliceiri. 2012. "NIH Image to ImageJ: 25 Years of Image Analysis." *Nature Methods*. https://doi.org/10.1038/nmeth.2089.

Shapiro, Lee A., Kwan L. Ng, Richard Kinyamu, Patricia Whitaker-Azmitia, Eldon E. Geisert, Mathew Blurton-Jones, Qun Yong Zhou, and Charles E. Ribak. 2007. "Origin, Migration and Fate of Newly Generated Neurons in the Adult Rodent Piriform Cortex." *Brain Structure and Function* 212 (2). https://doi.org/10.1007/s00429-007-0151-3.

Shingo, Tetsuro, Christopher Gregg, Emeka Enwere, Hirokazu Fujikawa, Rozina Hassam, Colleen Geary, James C. Cross, and Samuel Weiss. 2003. "Pregnancy-Stimulated Neurogenesis in the Adult Female Forebrain Mediated by Prolactin." *Science* 299 (5603). https://doi.org/10.1126/science.1076647.

Sorrells, Shawn F., Mercedes F. Paredes, Dmitry Velmeshev, Vicente Herranz-Pérez, Kadellyn Sandoval, Simone Mayer, Edward F. Chang, et al. 2019. "Immature Excitatory Neurons Develop during Adolescence in the Human Amygdala." *Nature Communications* 10 (1). https://doi.org/10.1038/s41467-019-10765-1.

Swain, James E., and S. Shaun Ho. 2019. "Early Postpartum Resting-State Functional Connectivity for Mothers Receiving Buprenorphine Treatment for Opioid Use Disorder: A Pilot Study." *Journal of Neuroendocrinology* 31 (9). https://doi.org/10.1111/jne.12770.

Torres-Pérez, Jose Vicente, Elena Martínez-Rodríguez, Anabel Forte, Carlos Blanco-Gómez, Oliver Stork, Enrique Lanuza, Mónica Santos, and Carmen Agustín-Pavón. 2022. "Early Life Stress Exacerbates Behavioural and Neuronal Alterations in Adolescent Male Mice Lacking Methyl-CpG Binding Protein 2 (Mecp2)." *Frontiers in Behavioral Neuroscience* 16 (August): 1–16. https://doi.org/10.3389/fnbeh.2022.974692.

Yap, Chan Choo, Max Vakulenko, Kamil Kruczek, Bashir Motamedi, Laura Digilio, Judy S. Liu, and Bettina Winckler. 2012. "Doublecortin (DCX) Mediates Endocytosis of Neurofascin Independently of Microtubule Binding." *Journal of Neuroscience* 32 (22). https://doi.org/10.1523/JNEUROSCI.5318-11.2012.

Figure legends

Figure 1. Representative pictures of DCX-ir (green) and Ki67-ir nuclei (red) immunofluorescence in the olfactory tubercle. Clustered round (little or no neurites) DCX-ir cells are found at the external edge of layer I in intimate contact with Ki67-ir cells (A) while complex-like DCX-ir cells, with well-developed and visible neurites, can be found either grouped but not in close contact or isolated within the outermost region of layer I (B). On some occasions, DCX-ir cells were located closer to layer II (C; arrowhead) and/or in proximity to the Islands of Calleja (D; DCX-ir cell in inset, border of Island of Calleja marked in a dashed line). Scale bars represent 10 μm (figures A'-D': green channel; and A''-D'': red channel) and 50 μm (figures A-D: merged).

Figure 2. Bar charts showing the density of immature neurons and proliferating nuclei in the olfactory tubercle. The density of immature DCX-ir is not affected by either puberty (A), pregnancy (B) or lactation (C). By contrast, Ki67-ir proliferative nuclei are significantly decreased by transition to adulthood (A'), but not altered by motherhood (B', C'). *** p value < 0.001.

Figure 3. Representative pictures of DCX-ir (green) immunofluorescence in the piriform cortex. Sections were stained for both DCX-ir (green) and Ki67-ir (red). Note that, contrary to OT, there was no positive labelling against Ki67 (A). Both tangled (A'; arrowhead) and complex, with larger soma and more processes (A''; arrowpoint), can be found within layer II of the piriform cortex. Scale bars represent 10 μm (figures A' and A'': green channel only) and 50 μm (figures A: merged).

Figure 4. Bar charts showing the density and diameter of immature neurons in the piriform cortex. Number of total, tangled and complex DCX-ir cells decrease after puberty (A), whereas their diameter average diameter remains stable (A'). Pregnancy does not affect DCX-ir (B) or their diameter (B'). Lactation significantly decreased the density of DCX-ir cells, an effect due to a decrease in the number of DCX-ir complex neurons (C). This decrease in complex neurons leads to a significant decrease in the average diameter of DCX-ir cells (C'). * p value < 0.05; ** p value < 0.01; *** p value < 0.001.

Figure 1

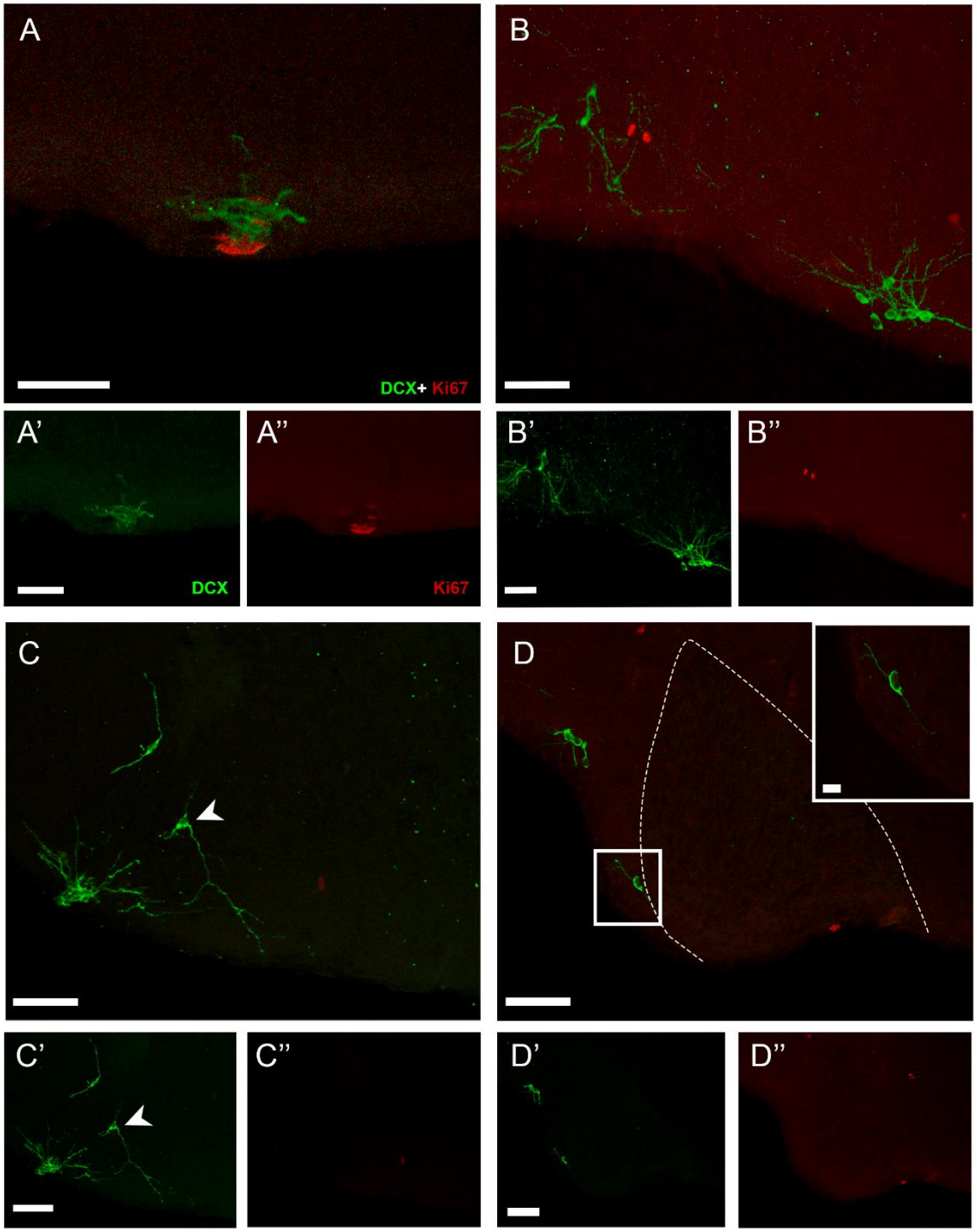
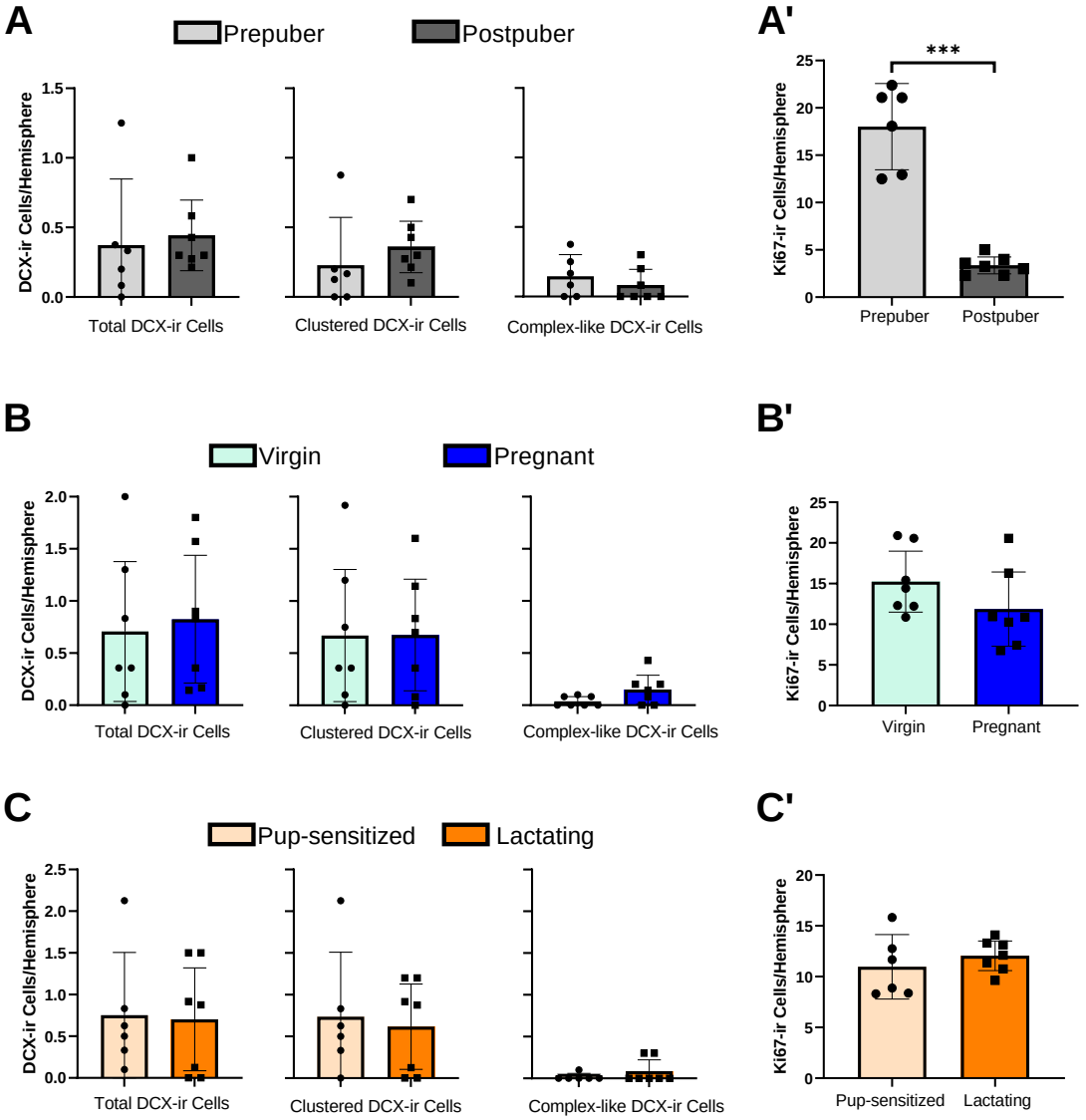


Figure 2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Ki67-ir Cells/Hemisphere
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DCX-ir Cells/Hemisphere
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Figure 3

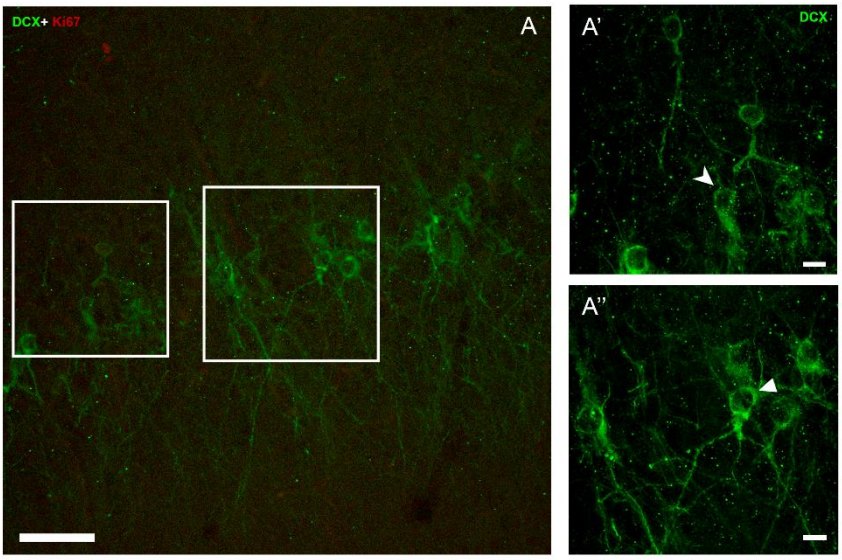
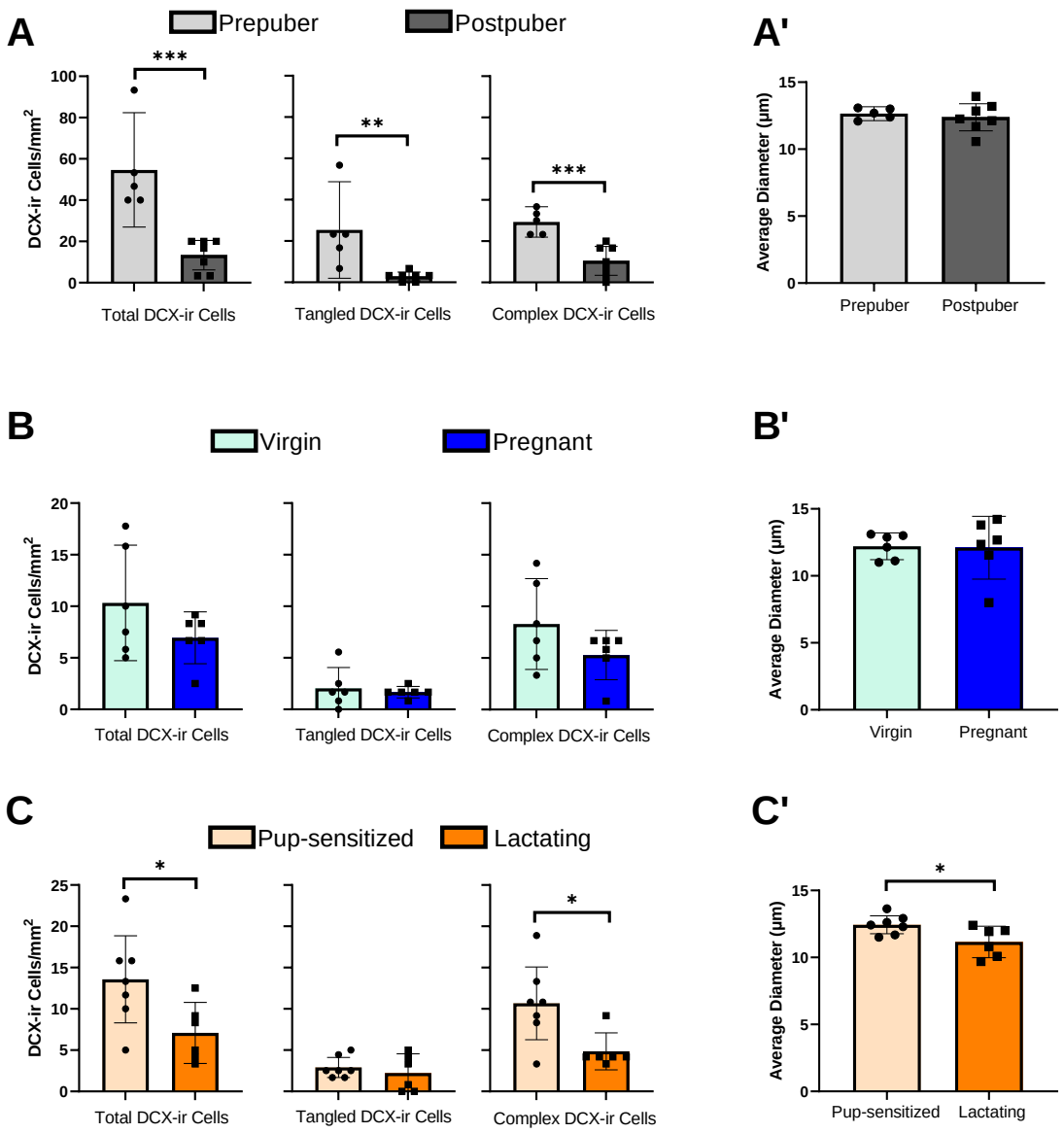


Figure 4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