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Hemaswetha Rajagopalan April 9, 2019

Expression of MeCP2 in a Social Maternal Learning Paradigm in Mice

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An abstract of
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Abstract

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Learning and the formation of memories of salient sensory stimuli is an essential aspect of motherhood that facilitates maternal responsiveness. Two factors that influence this period of learning include the direct interaction and experience the mother has with her infants and the contribution of circulating hormones that may affect the neural circuitry resulting in a behavioral response. In our study, we examine the gene, Methyl-CpG-binding protein 2 (*Mecp2)*, which is known to be associated with experience-dependent learning and acts through epigenetic regulation to modulate activity of other genes and facilitate synaptic changes in the brain. We performed immunohistochemical staining to obtain the expression of MeCP2 in the auditory cortex of intact CBA/CaJ female mice. Our cohorts consisted of animals manipulated by their level of pup experience (naïve vs. 5 days) and physiological state (virgin vs. pregnant). We found that MeCP2 levels in these adult animals did not differ significantly across groups, indicating that neither the maternal physiological state nor the experience caring for pups are associated with changes in this developmentally-implicated mediator of experience-dependent plasticity. Our findings suggest changes in MeCP2 expression levels does not appear to be associated with plasticity in the auditory cortex during infant cue learning,

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Introduction

Learning and memory formation are fundamental for species survival and motivate important questions in neuroscience. There is a tremendous growth in our knowledge of the different ways species detect information and learn from their surroundings as well as their behavioral response in social situations. Neuroscience research can explore behaviors in social situations at the molecular level of protein interactions or the modified neural circuitry during a learning experience.

The acquisition of new information is broadly categorized into short-term or long-term memory. Short-term memory spans from milli-seconds to several minutes, obtaining information seen or heard in the immediate past or present. There is however, a limited capacity, such that information that does not receive attention and repeated activation will be lost. Sufficiently rehearsed information is transferred into long-term memory, which can be retained from days to years. Long-term memory is classified as explicit (declarative) and implicit (nondeclarative). Implicit memory includes learning of procedural skills and conditioning while not requiring conscious awareness to use this memory. Brain regions involved include the cerebellum, amygdala, and basal ganglia (Kandel et al., 2014). Explicit memory involves the learning of concrete events, objects, and places and greatly relies on the hippocampus and cortex.

These two types of memories recruit different mechanisms in the brain and vary in processing. Learning and memory is divided into three major processing stages: 1) Encoding, 2) Storage, and 3) Retrieval. Encoding creates memory traces to store incoming information. Storage is the result of information consolidation and retrieval involves accessing stored information to perform a behavior (Gazzaniga et al., 2014). The neural mechanism of learning includes structural alterations in neurons, modifications of synapses, and changes in molecular protein expression within neurons (Thompson, 1986). Dysfunction in the neural circuitry via improper development, lack of sufficient neural stimulation at critical periods of learning, and genetic mutations greatly affect the capacity of our brains to learn and store new information, resulting in a vast and complex range of disorders. Researchers are continuously discovering new interactions of molecules and dysfunctions in activation or inhibition of pathways, filling gaps in our knowledge of the mechanisms underlying these disorders.

The Methyl-CpG-binding protein 2 (*Mecp2*) gene is associated with epigenetic regulation during experience-dependent learning. It is X-linked and therefore mutations of *Mecp2* predominantly affect females. *Mecp2* contains two main domains: (1) a methyl-CpG binding domain which binds CpG dinucleotides and (2) a transcriptional repression domain that represses transcription of target genes (Pelka et al., 2006). The methyl-CpG binding domain methylates DNA by attaching a methyl group on sites where a cytosine nucleotide is followed by a guanine nucleotide. The transcriptional repression domain acts on downstream other transcriptional repressors resulting in activation of genes, such as cAMP response element binding (CREB) protein and brain derived neurotrophic factor (BDNF), both of which are associated with synaptic plasticity (Chahrour et al., 2008). By these actions, MeCP2 epigenetically modulates the activity of other genes and facilitates synaptic changes in different parts of the brain (Pelka et al., 2006). Although the literature discusses particular targets specific to synaptic plasticity or Rett, a comprehensive list of exact targets of MeCP2 and how they are regulated is currently unknown (Chahrour et al., 2008; Peddada et al., 2006).

MeCP2 is expressed throughout the brain (Mullaney et al., 2004), and its expression affects other gene's expression as well as behavioral phenotypes. In the hypothalamus, Chahrour et al. (2008) found that MeCP2 concentrations induced changes in thousands of genes expression levels. As MeCP2 is an activity-dependent regulator of gene expression, they predicted that MeCP2 played a key role in responding to new physiological states (Chahrour et al., 2008). Partial or complete loss of function of *Mecp2* through gene mutation or knockout in mice (Amir et al., 1999) results in a range of phenotypes including progressive ataxia, diminished acoustic startle response (Ito-Ishida et al., 2015), impaired hippocampal learning and memo ry (Chao et al., 2010), and reduced contextual fear conditioning (Pelka et al., 2006).

The most prominent disorder associated with this gene is Rett Syndrome; a severe and progressive neurodevelopmental brain disorder (Amir et al., 1999) that's symptoms include mental retardation, language and learning disabilities and other compromised brain functions (Moog et al., 2003). Rett pathogenesis manifests itself within the first ten years of life, calling attention to the importance of *Mecp2* in early development. From birth to two years of life, there is evident loss of language and behavioral skills, reduced growth rate, and microcephaly leading to the early onset of developmental stagnation. As a result, individuals with Rett have difficulties in communication, learning and coordination (Liyanage and Rastegar, 2014). Due to early onset

of Rett Syndrome, much of our knowledge on the function and role of *Mecp2* is limited to this time period of development (Amir et al., 1999; Liyanage and Rastegar., 2014).

In the cerebral cortex and hippocampus of normal rats, the intensity and expression of *MeCP2* increases significantly during early postnatal development. These changes in expression coincide with periods of synaptogenesis in rats (Mullaney et al., 2004), supporting data that shows *Mecp2*'s role in regulating genes involved in the formation or maintenance of synaptic connections (Chahrour et al., 2008) and highlighting *Mecp2*'s association with plasticity at times of learning. It is apparent that MeCP2 protein levels increase through development and are high in adulthood (Mullaney et al., 2014); however, is it unclear whether or how MeCP2 expression may change in adulthood during learning experiences. There is a lack of studies demonstrating the role MeCP2 may play to regulate gene expression during learning and memory formation of other key periods of adulthood, such as motherhood.

Motherhood is an important time period for changes in the brain in order for maximum infant cue recognition and maternal responsiveness. When a female transitions from a virgin to a mother, she undertakes a multitude of new responsibilities and demands to ensure the survival of her infants including infant cue recognition and maternal responsiveness (Kinsley et al., 1999). Parenting in females involves the learning of offspring cues, regulation of maternal motivation, and manifestations of species-specific parental behaviors (Kohl et al., 2017), of which sensory learning of infant cues is important in facilitating maternal responsiveness. In mice, motherhood involves the integration of sensory stimuli including olfactory, tactile, and auditory information, after which, signals are sent to motivational and motor centers to elicit a maternal behavioral response (Kohl et al., 2017). During motherhood, learning of certain infant cues is also important to being able to drive appropriate maternal responses. Infant sounds, such as cries or other vocalizations, provide valuable information to their caretaker. Learning their meaning is key for proper communication in many species, and experience with caretaking leads to plasticity within the auditory cortex, the site of sound processing (Galindo-Leon et al., 2009). We can use a mouse maternal model to investigate the mechanisms of social auditory learning with the ultrasonic vocalizations (USV's) emitted by infant mice. The auditory cortex is a key site of integration of auditory learning and memory (Weinberger, 2004), where neural plasticity at a molecular level can be induced through sound stimulus presentation, including complex social sounds (Ivanova et al., 2011; Ivanova et al., 2017).

The USVs produced by mouse pups include pup isolation calls and wriggling calls and have a salient communicative value, inducing responses such as retrieval or suckling (Sewell, 1970; Ehret and Koch 1989). A typical parental behavior in mother mice is to retrieve pups when they are outside the nesting area. The performance of retrievals is a measure of maternal responsiveness. The playback of pup-calls is sufficient to elicit an approach behavior by mothers; however, this preference is not exhibited in non-mother virgin female mice without pup experience (Ehret, 1987). Lin et al. (2013) demonstrated that a behavioral preference to approach pup-calls over a neutral sound was correlated with the strength of USV-evoked inhibition in the AC. This inhibitory plasticity is thought to improve pup call detection and learning (Galindo-Leon et al., 2009; Lin et al., 2013). The authors also demonstrated that early co-carers, prior to weaning of pups, produce call-evoked inhibition similar to that of mothers. After weaning, the pup experience fades and, as a result, the inhibition decays to levels of virgin females. This decay was not seen in mothers after weaning pups, hinting at the importance of a maternal physiological state in retention of pup-call recognition and learning (Lin et al., 2013).

To further understand this plasticity caused by learning about pup vocalizations, it is important to consider the contribution of hormones on maternal experience-dependent sensitization and the learning of infant cues. Using a maternal mouse model uniquely allows one to test auditory plasticity with the manipulation of maternal hormones and pup exposure in order to explore how experience and internal state influence social sound processing (Banerjee and Liu, 2013; Moreno et al., 2018). Although the molecular changes leading to experience-induced auditory cortex plasticity are still poorly understood, we can hypothesize that genes, such as *Mecp2*, associated with learning and plasticity, may play a role in facilitating social cue learning and recognition during a maternally sensitive period.

Krishnan et al. (2016) demonstrated a connection between Mecp2 and maternal response. They used a maternal mouse model to study *Mecp2's* effect on maternal behavior by creating a heterozygous genetic ($Mecp2^{het}$) female mouse. Maternal pup retrieval behavior was assayed between the heterozygous and wild type mice. Heterozygous surrogate females performed significantly worse than mothers and wild type surrogates, with longer latencies in retrieving the pups and a greater number of pup retrieval errors. Additionally, they observed decreases adult inhibitory plasticity in the AC of $Mecp2^{het}$ surrogates displaying poor pup retrieval behavior. To assess the role of the AC specifically, they performed a conditional knock-down of *Mecp2* in

surrogates, again finding impaired pup retrieval behavior. The authors demonstrated that *Mecp2* expression within the AC is important in maternal retrieval behavior. Perhaps just as *Mecp2* plays a role during early development inducing synaptogenesis and the regulation of neural connections (Mullaney et al., 2004), it plays a similar role during the drastic changes in neural circuitry experienced during parturition (Kohl et al., 2017). However, it is unknown whether *Mecp2* expression changes during motherhood or through the process of learning about infants.

Parturition and maternal care lead to changes in synaptogenesis and the molecular make-up of the brain, particularly through increases in N-methyl-D-aspartate (NMDA) and brain-derived neurotrophic factor (BDNF) (Liu et al., 2000). Motherhood is an important period of learning, where the high concentration of hormones is related to maternal sensitization (Kohl et al., 2017; Marlin et al., 2015; Zilkha et al., 2017). The rise in estrogen and progesterone during mid to late pregnancy may facilitate the onset of maternal behavior in mammalian species (Pederson, 1997). In a study by Ehret and Koch (1989), the authors reported that gonadectomized male and female mice do not retrieve spontaneously, but with an injection of sex steroids, in particular estrogen and progesterone, the mice demonstrated retrieval behavior of the pups.

The literature suggests that an interaction between hormones and *Mecp2* may play a role in critical periods of learning (Auger et al., 2011; Muller et al., 2003; Westberry et al. 2010; Wilson et al. 2011). Westberry et al. (2010) indicated an association between the pattern of MeCP2 and estrogen receptor α (ERα) expression during postnatal development. Typically, ERα mRNA expression is high early in postnatal development and begins to decline by postnatal day 10. A mutant form of MeCP2 resulting in a defective protein, resulted in an altered ERα expression pattern, which demonstrated MeCP2 playing a direct role in the regulation of ERα gene expression via epigenetic *Mecp2* methylation (Westberry et al. 2010; Wilson et al. 2011).

Kurian et al. (2007) identified a sexually dimorphic effect of *Mecp2* mRNA in the amygdala and ventromedial hypothalamus in early postnatal days one to ten in rats. Importantly, this dimorphism is regional and marks the time points of a sensitive period of sexual dimorphism in the rat brain (Kurian et al., 2007), where there are drastic changes in hormone concentrations. This helps to indicating that differences in hormone exposure may influence the expression of this gene. Auger et al. (2011) demonstrated that methylation at CpG sites, a function of MeCP2, on promoter regions of hormone receptors was affected by changes in the concentration of

hormones. Furthermore, the increase in estrogen increases methylation, hinting that hormone changes must be engaging in methylators like MeCP2. Only a few studies have directly examined the relationship between MeCP2 and hormones, resulting in a lack of clarity on how hormone presence may influence the expression of *Mecp2* (Auger et al., 2011; Muller et al., 2003; Westberry et al. 2010; Wilson et al. 2011), but we can consider the potential of this interaction.

The aim of our study is to identify the differential effects of gaining pup experience on *Mecp2* expression in the AC in the presence of maternal hormones. Due to motherhood being an important period of learning similar to that of early development, we expect *Mecp2* expression to be modulated by the physiological state of motherhood during an experience-dependent learning event in regions of the brain associated with maternal behavior and infant cue learning. *Our predictions follow that recent mothers, during a crucial period of experience-dependent learning, will display significantly different expression in the auditory cortex when compared to co-carers or naïve animals.* This study may highlight a modulatory effect of the critical period of motherhood in experience-dependent social learning.

Materials and Methods

Behavioral Procedures

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. A total of 16 female mice of CBA/CaJ strain (11-15 weeks old) were used in this study (Figure 4). Animals were on a 12-hour light/12-hour dark cycle and provided with food and water ad libitum. The four different experiment groups (Figure 3) included virgin females naïve to pups during adulthood (n = 4), mated and as a result pregnant naïve females (n = 4), mothers with five days of pup experience (p5 mothers, n = 4), and virgin females with five days of caring with birth to five day old pups (p5 virgin co-carers, n = 4). By these experimental groups, we are able to test the effects of undergoing pregnancy and the hormones associated with it as well as the effects of obtaining experience with pups. In all cases, these hormone manipulations were natural and not through hormone implants. On the respective day of testing for each group, mother mice and co-carers were separated from pups and individually

experimented. On the experimental day, subjects were placed in an anechoic chamber for 4 hours, because the experience of being in a silent chamber may potentially affect the activity of immediate early genes interacting with *Mecp2*, which typically change in expression during the first hour of a new experience before returning to baseline (Cullinan et al., 1995). Animals were sacrificed using carbon dioxide and perfused with KPBS and 4% paraformaldehyde over 10 minutes. Brains were removed, post-fixed overnight in 4% paraformaldehyde overnight at 4C° and transferred to 30% sucrose until no longer floating at 4C°. All habituation and testing occurred during the dark phase.

Pregnancy Stage Determination

Mothers at p5 were selected between Theiler stages 23 and 26, which correspond to days post coitum (dpc) 15 through 18. Theiler stage was determined visually via post perfusion necropsy of fetal pups from the mouse atlas (https://www.emouseatlas.org/emap/ema/theiler_stages/downloads/theiler2.pdf).

Immunohistochemistry

Brains were coronally sectioned at 45 microns (μm), and sections containing the auditory cortex and other regions of interest were stored in cryoprotectant at -20C°. Immunohistochemistry labeling of MeCP2 antibody was conducted on every other or every third section. After washing sections in Tris, sections were incubated for 30 min in 0.3% Triton-X in Tris followed by blocking with 5% normal goat serum (NGS) in 0.3% Triton-X in Tris for 1 hour. Sections were transferred to MeCP2 primary antibody (Cell Signaling Technology, #3456S) at a dilution of 1:500 in Tris (0.05% Triton-X, 1.5% NGS) and stored at 4C° for 2 days with gentle agitation. After 2 days of incubation, sections were rinsed in Tris and incubated for 1 hour in biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, BA-1000) at a dilution of 1:500 in 1.5% NGS and Tris. Sections were then washed in Tris and incubated in an ABC solution for 1 hour. After rinsing in Tris, sections were placed in DAB (2% DAB, 1.5% Nickel Solution, and 1.6% H2O2) for protein visualization. After staining, brain sections were washed with Tris, mounted, dehydrated and cover-slipped using Permount.

Cell quantification

Quantification of protein expression was performed on sufficient tissue representing the full range of the primary auditory cortex (7 sections +/- 1 per animal). Images were obtained on an Aperio ScanScope whole slide scanner at 40x magnification and analyzed on the QuPath open-source software (Bankhead et al., 2017). Training of cell quantification on QuPath was adapted from previous studies performing digital image analysis (Bankhead et al., 2017; Loughrey et al., 2018). Before beginning the analysis, preprocessing steps were performed for proper image reading. Image type was set to Brightfield-DAB on each whole slide image. The *Estimate stain vectors* tool was applied to a region large enough containing both background and stained cells of a single 'typical' image of a stained batch (Figure 1). These stain vectors were then applied to the remaining slides of the same staining batch. QuPath utilizes a color-deconvolution algorithm that allows better classification (Ruifrok et al., 2003) and should be applied to images with chromogenic stains such as hematoxylin or DAB for initial processing of the stain vectors to identify the range of staining. This prevents the software from measuring negative stain amounts or other unusual values. QuPath was set to analyze the images based on nuclear DAB optical density. Cells were then quantified using the *positive cell detection* tool. Using a representative section from each condition, parameters were set to train QuPath to identify cell bodies and categorize them with the appropriate staining intensity. Using the distribution of the DAB mean intensity of nuclear staining, cutoff values of 0.25, 0.53, and 0.81 were then set to classify weak, moderate, and strong immunohistochemistry thresholds respectively (Figure 2).

Statistical Analysis

In order to test the effects of the different conditions on the intensity of MeCP2 expression in the auditory cortex, the H-score was calculated. The H-score is an intensity measurement value calculated for each tissue section on a scale of 0 (all nuclei negative) to 300 (all nuclei strongly positive). As described in Goulding et al., (1995), the H-score is calculated by adding 3 x % strongly stained nuclei, 2 x % moderately stained nuclei, and 1 x % weakly stained nuclei. The average H-score across the four animal groups (Virgin naïve females, pregnant naive females, mothers with 5 days of pups, virgin co-carers with 5 days of pup experience), was analyzed using both a one-way and two-way ANOVA. The proportion of positively detected cell bodies in either weak, moderate, or strong were each compared cross the four animal groups using one-way ANOVAs.

Qupath Script

```
setImageType('BRIGHTFIELD_H_DAB');
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"smoothBoundaries": true,  "makeMeasurements": true,  "thresholdCompartment": "Nucleus:
DAB OD mean",  "thresholdPositive1": 0.25,  "thresholdPositive2": 0.53,  "thresholdPositive3":
0.81,  "singleThreshold": false}');
```

Results

MeCP2 expression in the auditory cortex

For the initial analysis of the number of cells expressing MeCP2, animals were assigned one of four groups where they had 5 days of pup experience or no experience and undergone pregnancy or were virgins (Figure 2). There was no significant main effect in the one-way ANOVA test ($F_{3,11} = 1.182$, $p = 0.361$), which demonstrated that MeCP2 is a constitutively expressed protein in adulthood (Table 1, Figure 5).

Effects of pup experience and maternal hormonal state on MeCP2 expression intensity in the auditory cortex

We then assessed the expression of MeCP2 in the auditory cortex using a 2 x 2 ANOVA test (Table 1, Figure 6). There was no significant main effect of either pup experience ($F_{1,11} = 0.093$, $p = 0.766$) or maternal hormonal state ($F_{1,11} = 1.187$, $p = 0.299$) on the H-score intensity value. Additionally, there was no significant interaction between the two factors ($F_{1,11} = 0.005$, $p = 0.944$).

Due to the possibility that exposure to pups may modulate the hormonal state of virgin females (Ziegler, 2000) similar to that of mothers, we may not be able to distinguish the

hormonal state between the virgins and mothers. Since we did not directly control the concentration of hormones in the animals, we do not know if they were significantly greater than baseline. For this reason, we also conducted a one-way ANOVA (Table 1, Figure 6) on the calculated H-scores, which also showed no significant main effect ($F_{3,11} = 0.428$, $p = 0.737$). Therefore, we were unable to identify an effect of either pup experience or maternal state on the H-score intensity measurement in the AC.

Effects of pup experience and maternal hormonal state on MeCP2 expression on proportion of nuclei in each intensity category of the auditory cortex

In addition to comparing H-scores, we wanted to determine if the proportion of cells within each intensity category differed. A 2 x 2 ANOVA revealed no significant main effect on either pup experience or maternal state on the expression of low, medium or high intensity expressing cells (Table 1). A one-way ANOVA (Table 2, Figure 7) revealed no significant differences when comparing the proportion of positively detected nuclei in weak ($F_{3,11} = 0.623$, $p = 0.615$), moderate ($F_{3,11} = 0.328$, $p = 0.805$), or strong intensity ($F_{3,11} = 0.277$, $p = 0.841$). Therefore, our methods were unable to detect an effect of pup experience and maternal state on the intensity of MeCP2 expression in the AC.

Distribution of staining intensities across conditions in the auditory cortex.

Although the previous results were not significant, the variability within each condition was a detail that we further investigated. Looking at the intensity distribution within each experimental condition, we identified a unique pattern in which the virgin conditions exhibited a similar distribution and the non-virgin conditions exhibited a similar distribution (Figure 8). The virgin conditions showed a left skewed intensity distribution, whereas the non-virgin conditions presented a more bimodal distribution of staining intensities, both of which appeared to be distinct.

Discussion

In this study we show pup experience and maternal physiological state does not affect *MeCP2* expression in the AC Contradictory to our original hypothesis, the presence of maternal

hormones does not appear to modulate, or increase the expression of MeCP2 relative to naïve, virgin females. We did not find a significant difference in the H-score or the proportion of cells in each intensity.

One difficulty with the analysis of our study is controlling for a pure experience-only condition. Exposing the adult female mice to pups and maternal experiences may lead to fluctuations in hormone concentrations similar to that of mothers (Ziegler, 2000). There is contradictory information in the literature of whether hormone production is altered in response to infant interactions, so we must regard this factor when looking at our results. Studies in humans have shown that a playback of crying can affect cortisol, prolactin and testosterone in mothers and fathers (Berg et al., 2001; Fleming et al., 2002). On the other hand, Stallings et al. (2001) identify that nonpostpartum women did not present the same physiological changes in salivary cortisol levels and heart rate that multiparous mothers experience. This leaves the possibility that our conditions may not be completely independent from one another; therefore, we may not be able to make a completely separate experience and hormonal state in our experiment and analysis.

Our results suggest that changes in MeCP2 expression levels do not appear to be associated with plasticity in the auditory cortex during infant cue learning. MeCP2 may not act via changes in expression during adult learning and motherhood as it appears to do in early development. Due to the constitutive characteristic of this gene, it may be more important to maintain expression at some physiological range for proper functioning. The reduced expression caused by mutations is associated with Rett-like symptoms (Amir et al., 1999) and we also see that the overexpression of MeCP2 can result in other motor dysfunctions, such as swaying, tremors, and gait ataxia in mice (Luikenhuis et al., 2004). Although our data suggests that MeCP2 expression levels are not significantly different across conditions, the distinct intensity distributions suggests that the variability of MeCP2 expression may be correlated to being in a maternal physiological state. Our next step would be to analyze the type of distribution physiological mothers and non-mothers have.

Additional Analysis

Although we did not find any significant differences in MeCP2 expression in the AC, there may be an interaction in other sex-hormone sensitive brain regions that are associated with

maternal responsiveness. The maternal context can influence drastic changes in the neural circuitry for learning and recognition that is distinct to motherhood. In the growing body of research of this epigenetic modulator, *MeCP2* has being implicated in influencing many regions of the brain including the primary somatosensory cortex (Orefice et al., 2016). As this study was meant to be exploratory, we collected tissue spanning the medial preoptic area (MPOA), medial amygdala (MeA), and ventral tegmental area (VTA) to process and analyze these trends. The MPOA is a well-studied region in the context of sexual behavior and maternal responsiveness. Hormonal changes are known to modify receptors and the neural circuitry in this region (Numan, 2015; Numan and Insel, 2003) and may be more sensitive to subtle changes in the expression of *Mecp2*. The MeA appears to have inhibitory effects on maternal responsiveness, such that lesioning this area would lead to virgin females to respond maternally more quickly due to a reduced presentation of fearfulness (Fleming et al., 1980). The VTA is also associated with parental responsiveness (Kohl et al., 2017), such that inactivation of neurons via muscimol injections disrupted retrieval of pups and nursing behavior (Numan et al., 2009). While processing these regions, we can perform double-staining to visualize *Mecp2* as well as either excitatory or inhibitory neurons to get more specific expression information.

Future Studies

In order to isolate an effect of the hormones in this physiological state of motherhood, we would suggest the following future experiments. This experiment could be replicated using ovariectomized females with either an estradiol or vehicle-implant to control for the hormonal state as the experience of pups may lead to changes in the physiological state. We would also suggest looking at a longer, more comprehensive timescale during the period of motherhood. This would enable us to also test pup-call recognition at a later time point, providing evidence of maternal state enabling a long-term retention of auditory and social memory. Additionally, it would be useful to also collect behavioral data on maternal behaviors presented towards the pups such as grooming, nest building, and retrieving after inducing a reduced or increased expression of *MeCP2*. The results of these future studies would be important in understanding the how MeCP2's presence affects neural circuitry and the display of maternal behavior.

Table 1.

Main effect of pup experience and maternal state as well as their interaction on the H-score and proportion of three levels of expression intensity of MeCP2 protein in the auditory cortex using a 2 X 2 ANOVA test.

	Factor	F Statistic	P-value
H-Score	Pup Experience	$F_{1,11} = 0.093$	0.766
	Maternal State	$F_{1,11} = 1.87$	0.299
	Experience * State	$F_{1,11} = 0.005$	0.944
Proportion 1+ (weakly stained)	Pup Experience	$F_{1,11} = 0.229$	0.642
	Maternal State	$F_{1,11} = 1.579$	0.235
	Experience * State	$F_{1,11} = 0.062$	0.808
Proportion 2+ (moderately stained)	Pup Experience	$F_{1,11} = 0.432$	0.525
	Maternal State	$F_{1,11} = 0.031$	0.864
	Experience * State	$F_{1,11} = 0.521$	0.485
Proportion 3+ (strongly stained)	Pup Experience	$F_{1,11} = 0.021$	0.642
	Maternal State	$F_{1,11} = 0.809$	0.388
	Experience * State	$F_{1,11} = 0.003$	0.958

Table 2.

Experimental condition main effect on the proportion of three levels of expression intensity of MeCP2 protein in the auditory cortex using a one-way ANOVA test.

	Factor	F Statistic	P-value
Cell Density	Experimental Condition	$F_{3,11} = 1.182$	0.361
H-Score	Experimental Condition	$F_{3,11} = 0.428$	0.737
Proportion 1+	Experimental Condition	$F_{3,11} = 0.623$	0.615
Proportion 2+	Experimental Condition	$F_{3,11} = 0.328$	0.805
Proportion 3+	Experimental Condition	$F_{3,11} = 0.277$	0.841

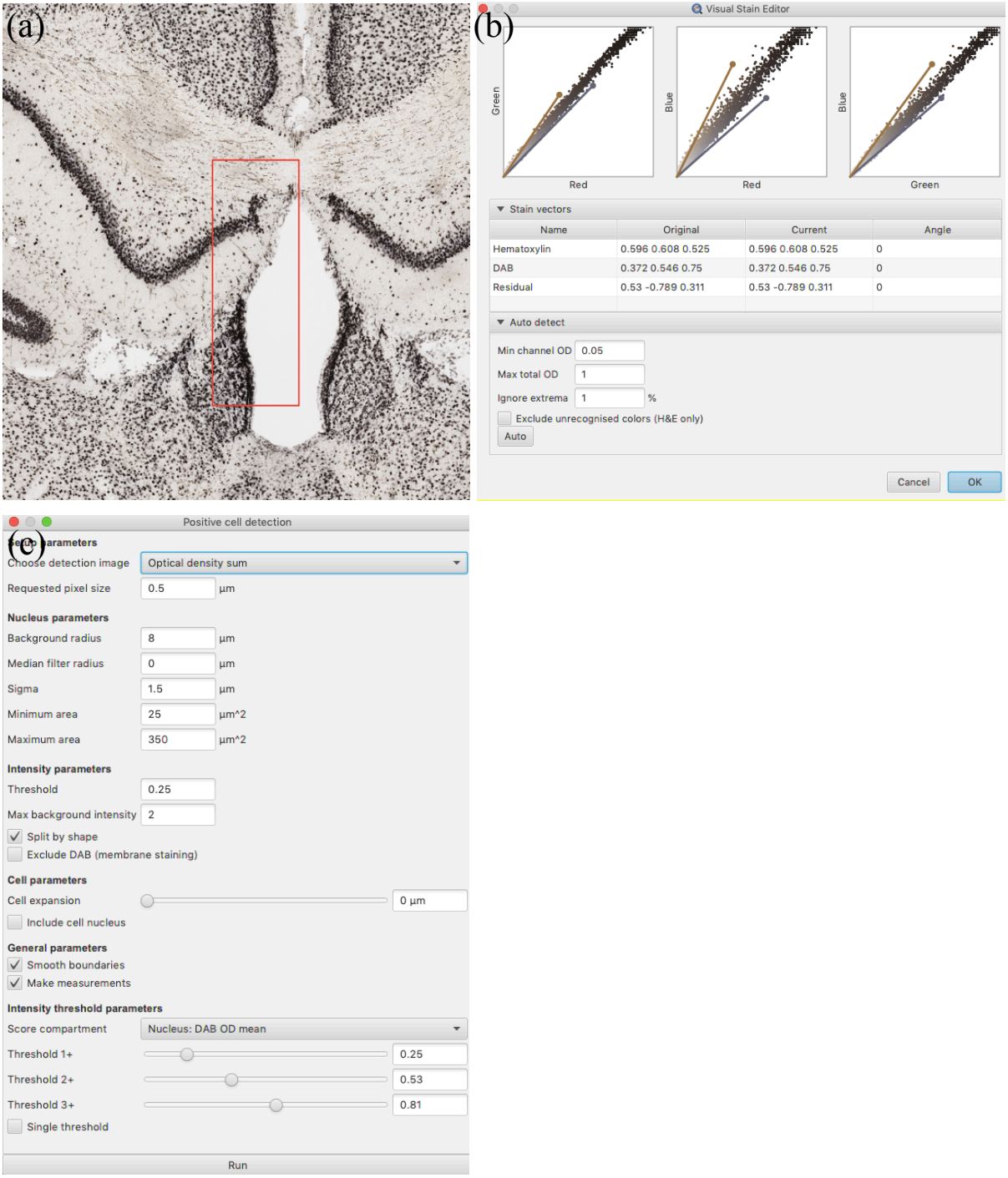

Figure 1.

Parameters and methodology used for QuPath analysis. (a) A region representative of stained cells and the background of a typical image for a stained batch was selected. (b) *Estimate stain vectors* tool was applied to the images prior to processing. (c) Parameters set for cell counting at weak, moderate, and strong thresholds in the auditory cortex.

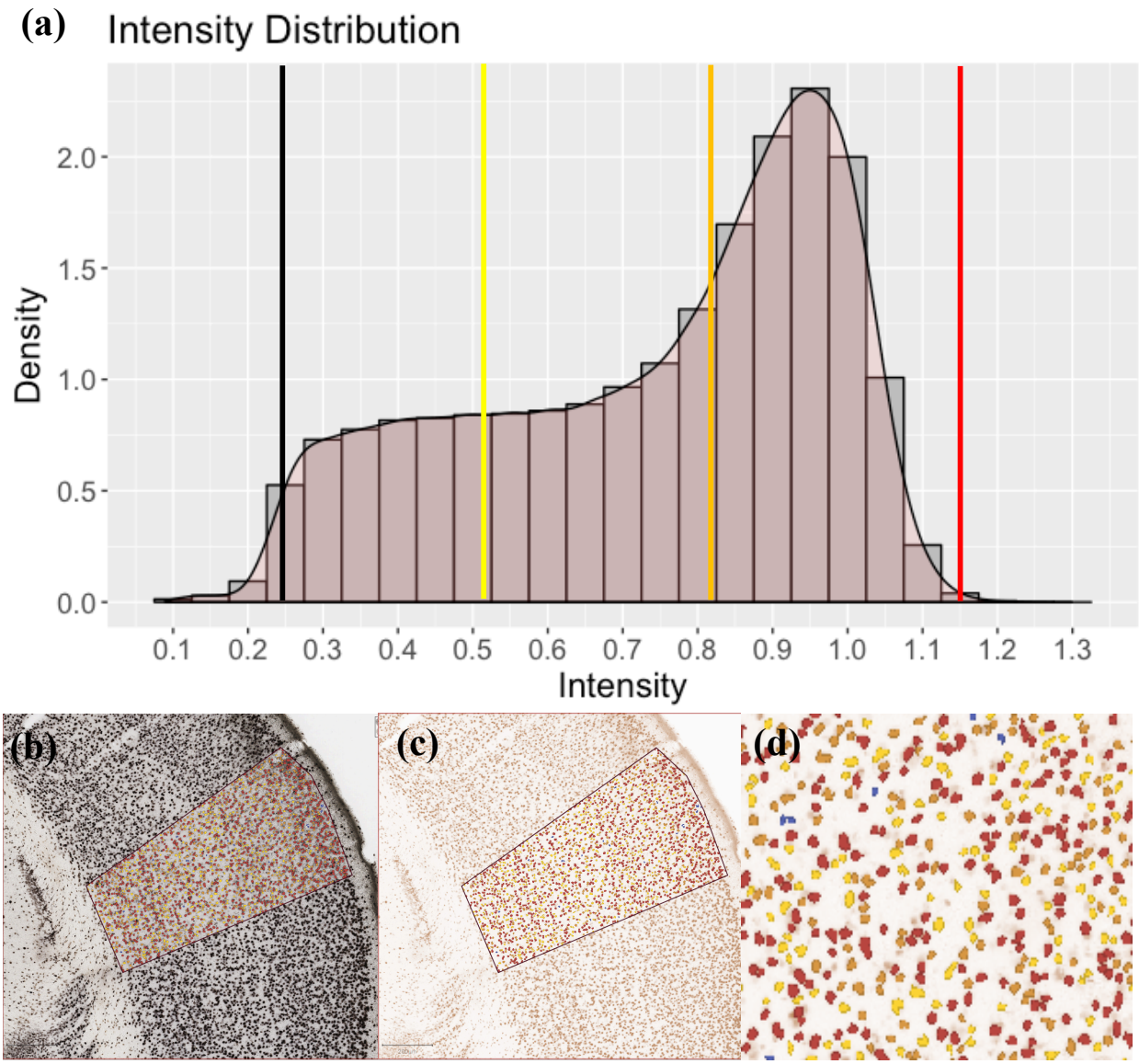

Figure 2.

(a) Density plot of the total intensity distribution of all cell bodies in the auditory cortex. The black line indicates the baseline threshold that the mean intensity value per cell body must pass to be counted as a positively detected cell. The remaining range of intensities were divided into three sections for weak (black to yellow line), moderate (yellow to orange line), and strong (orange to red line) intensity categories. Color deconvolution images separating the stains based on stain vectors. (b-c) Region of interest is outlined in red and cell bodies are marked in (d) red (strong intensity; >0.81), orange (moderate intensity; 0.53-0.81), and yellow (weak intensity; 0.25-0.53).

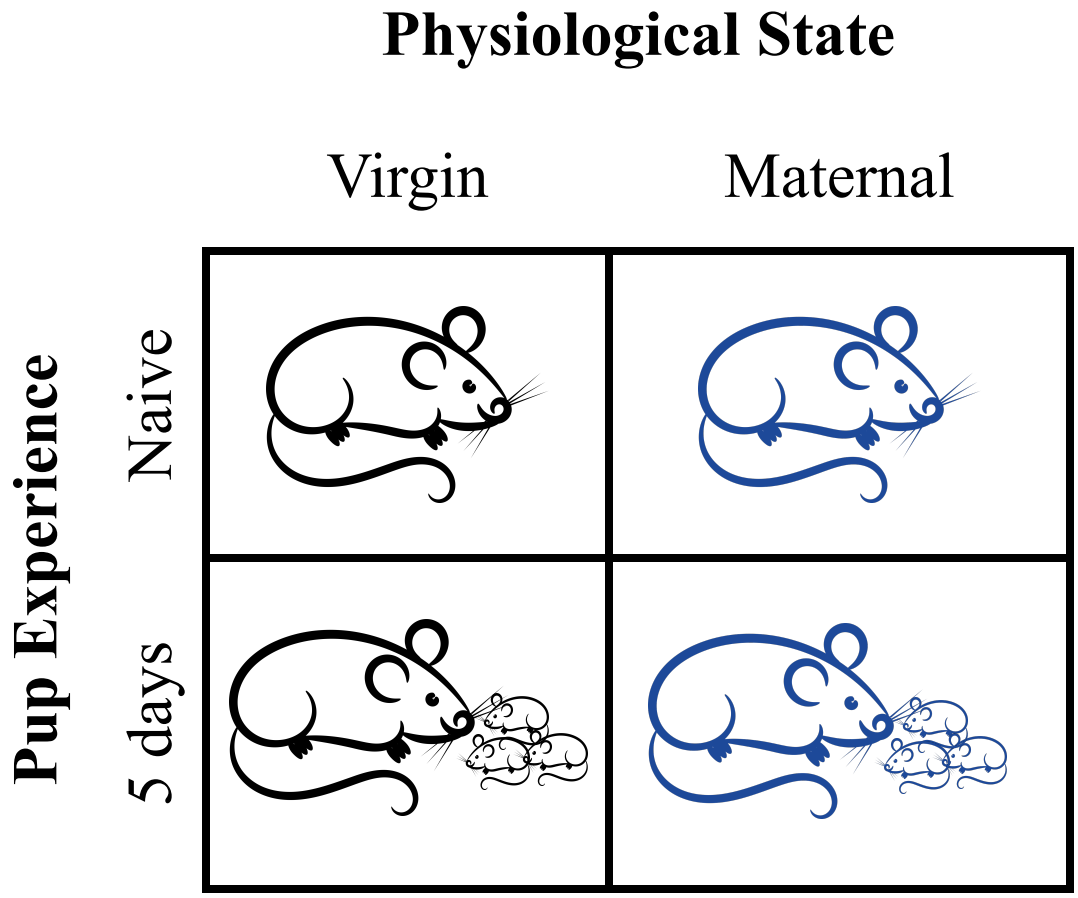

Figure 3.

Schematic of the four experimental groups used in this study. Rows represent pup experience. Columns represent physiological state (black = virgin, blue = undergone pregnancy).

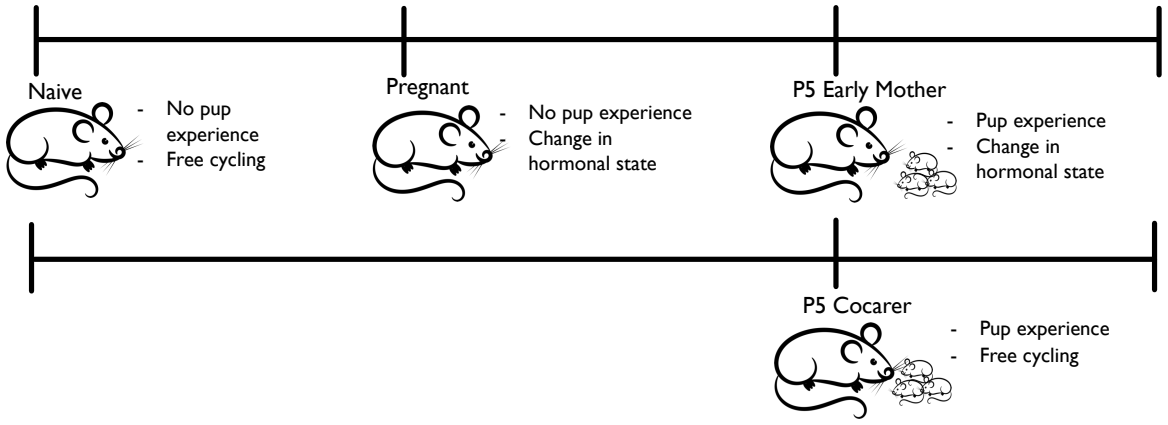

Figure 4.

Experimental design of physiological state, and pup experience of the four experimental groups in this study. All females were between 11-15 weeks old and were only tested for one experimental condition.

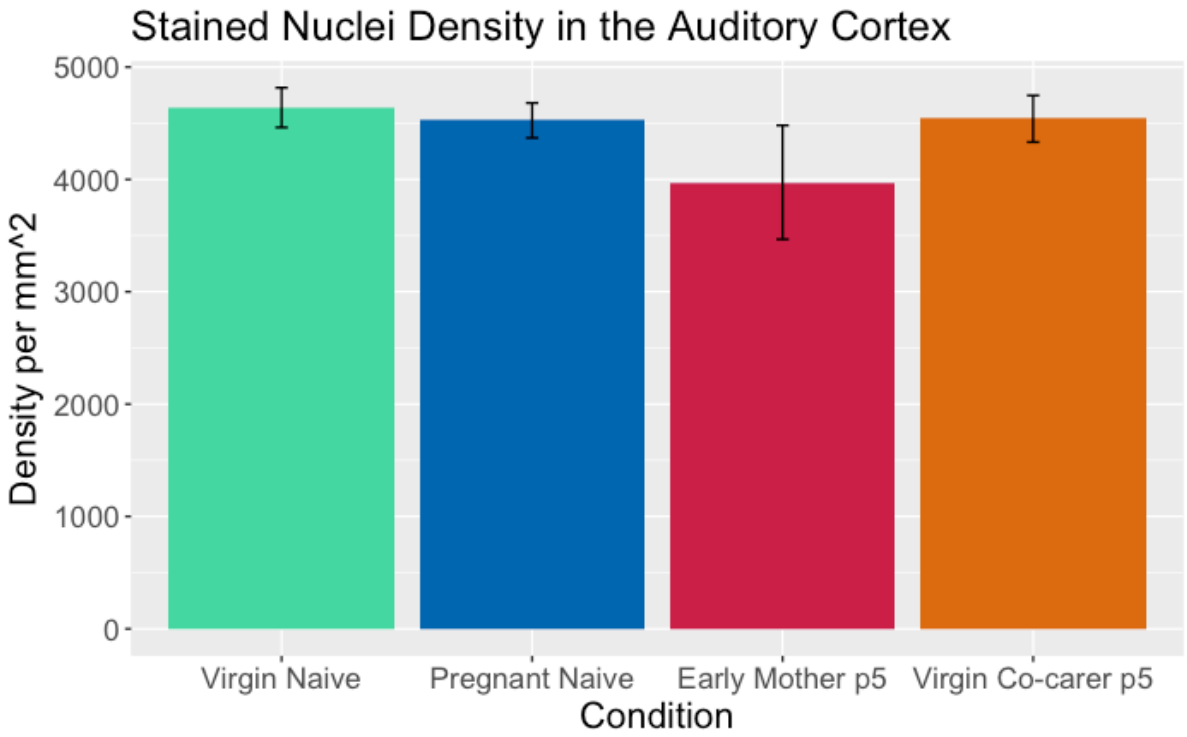

Figure 5.

Side-by-side bar plot of the density of cells per mm^2 expressing MeCP2 in the AC per experimental group. Error bars represent standard error. There were no significant differences of cell density in the AC (one-way ANOVA, $F_{3,11}=1.182$, $p=0.361$).

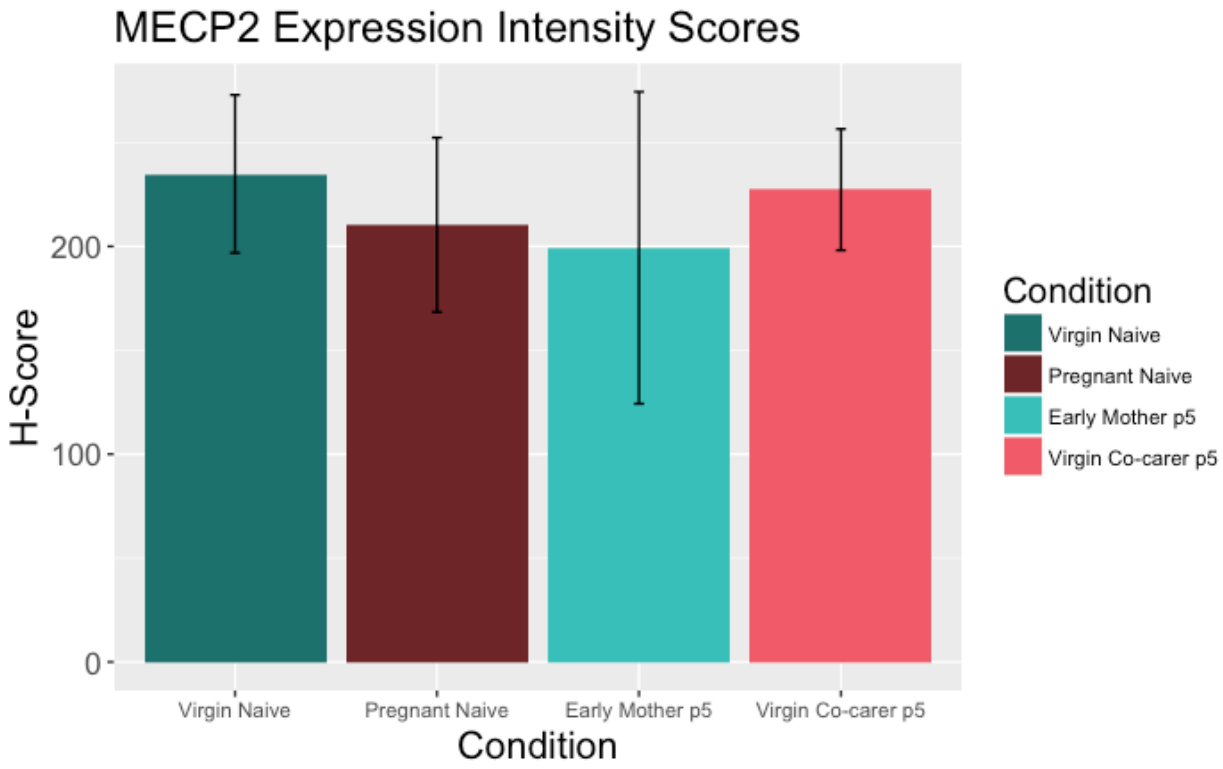

Figure 6.

Side-by-side bar plot of the H-Score calculated of the expression of MeCP2 per experimental group. Error bars represent standard error. There were no significant differences of H-score in the AC (2 x 2 ANOVA, main effect of pup experience $F_{1,11}$=0.093, p=0.766, main effect of physiological state $F_{1,11}$=1.87, p=0.299, interaction between the two factors $F_{1,11}$ = 0.005, p=0.944).

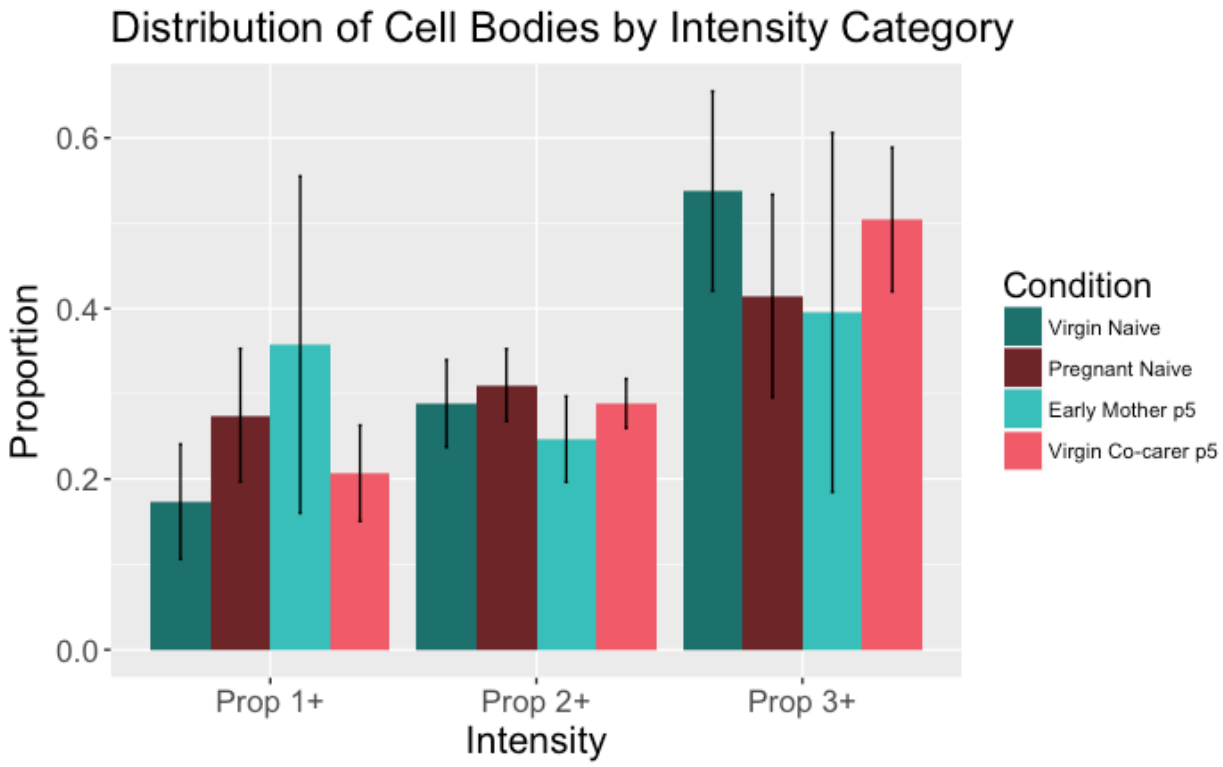

Figure 7.

Side-by-side bar plot of the distribution of nuclei by expression intensity of MeCP2 per experimental group in the AC. Error bars represent standard error. There were no significant differences in the proportion of nuclei in each intensity threshold (Prop 1+ = weakly stained, Prop 2+ = moderately stained, Prop 3+ = strongly stained) in the AC both in the two-way and one-way ANOVA.

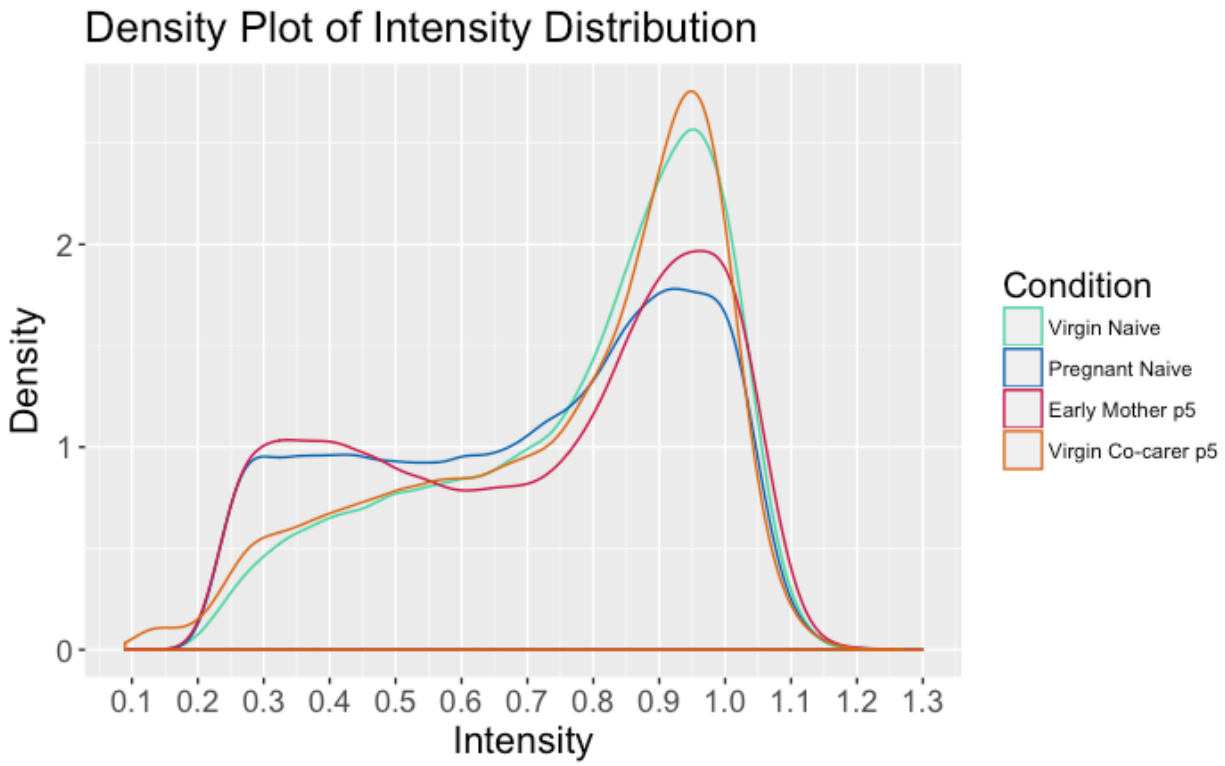

Figure 8.

Density plot of mean cell body stain intensity separated by experimental condition (Virgin Naive n = 4; Pregnant Naive, n = 4; Early Mother p5, n = 3; and Virgin Co-Carer p5, n = 4).

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