RISK AND PROTECTIVE FACTORS ASSOCIATED WITH EARLY ADVERSITY AND DEVELOPMENT: EVIDENCE FROM HUMAN AND ANIMAL RESEARCH

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RISK AND PROTECTIVE FACTORS ASSOCIATED WITH EARLY ADVERSITY AND DEVELOPMENT: EVIDENCE FROM HUMAN AND ANIMAL RESEARCH

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Editorial: Risk and Protective Factors Associated With Early Adversity and Development: Evidence From Human and Animal Research

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Editorial on the Research Topic

Risk and Protective Factors Associated With Early Adversity and Development: Evidence From Human and Animal Research

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The recent advances at the crossroad among behavioral science, psychoneuroendocrinology, neuroscience, genetics, and epigenetics are revealing how early environmental exposures—for good or ill—leave biochemical "scars" or signatures in our developmental biology. These biological and behavioral memorial processes of our precocious encounters with life constitute the interwoven mechanisms that contribute to the long-lasting programming of health and disease later in life. The quality of early caregiving (Weaver et al., 2004; Conradt et al., 2016; Lester et al., 2018) and the exposure to adverse life events (McCrory et al., 2010; Blaze et al., 2015; Provenzi et al., 2016)—even when the exposure occurs in previous generations (Yehuda et al., 2005; Moog et al., 2016)—are embedded into our developing phenotype and contribute to our health trajectories and disease risk from infancy to adulthood.

The rapid and consistent growth of this field is seen in the birth of new translationally relevant scientific areas, e.g., behavioral epigenetics (Lester et al., 2011), microbiome research (Robertson et al., 2019), and in new ramifications of existing and well-established areas, e.g., psychoneuroendocrinology (Heim et al., 2019), social and affective neuroscience (Cascio et al., 2019), behavioral neuroimmunology (Suárez-Álvarez et al., 2013). Future advances in these areas of scientific investigation will greatly contribute to our comprehension of the pathways of disease risk and inform smarter and more effective preventive and therapeutic early interventions, finally enabling us to use the environmental-sensitive nature of our genes and biology to serve neuroprotective goals (Heim et al., 2019).

From this perspective, in the present Research Topic, we aimed at collecting evidence from both animal models and human studies on these emerging research efforts which are directed at disentangling how these mechanisms (e.g., epigenetics, microbiome) may be "informed" by early environmental encounters and ultimately lead to specific health and disease outcomes. The 15 papers included in this issue vary widely in terms of subjects, exposures, mechanisms, and outcomes. The collection includes original research articles, theoretical viewpoints, systematic reviews, and methodological issues. Here, our hope was to make order out of this complexity by assuming a developmental view inspired by a dynamic system epistemological approach to early development (van Geert, 2008). The dynamic system approach is a theory of embodied

and embedded actions that considers developmental processes as emerging from the continuous coupling between the organism and the environment. More specifically, the early, continuous and fine-tuned micro-regulatory processes occurring between the individual and the environment result in the establishment of stable, yet flexible attractor states that further contributes to define the developmental trajectories later in life (Smith and Thelen, 2003). In other words, development is considered as the byproduct of messy and reciprocal connections (and disconnections) between an individual and the physical and conspecific environment in which he/she is situated (Tronick, 2017). Environmental encounters—such as the quality of parental caregiving or the exposure to traumatic events—play a critical role as they contribute to the shaping of the complex weaving of connections that result in the emerging behavioral phenotype of a given individual. Notably, from this viewpoint, environmental encounters cannot be defined as positive or negative *per sè*. Rather, encounters may provide beneficial or detrimental contributions for the developmental trajectory of a living being depending on the biochemical, cognitive, and socio-emotional that embed their effects in the developing biology and behavior of that given individual. Most prominently, reparation processes at many levels of the individual—molecular to behavioral—of messy or disruptive encounters with the human and physical environment assume a critical role by increasing the complexity and coherence of these processes which ultimately contributes to better and healthier outcomes later in life (DiCorcia and Tronick, 2011).

The original articles published in this Research Topic highlight the potential of early adversities occurring from pregnancy (Zietlow et al.) to the perinatal period (Gonzalez-Valenzuela et al.; Muntsant et al.) and from childhood (Stack et al.; Vilaseca et al.) up to adulthood (Liu et al.; Ranger et al.). Moreover, evidence of the impact of positive caregiving on infants' brain development (Hanford et al.) and cognitive and behavioral outcomes (Lejeune et al.) has is also reported. Taken together, these findings highlight the messiness of the dynamic developmental framework in which a living being grows in complexity as well as the openness of the developing phenotype to a wide and diverse set of potential environmental encounters. These encounters range from perinatal brain damage (Muntsant et al.) to postnatal music exposure (Lejeune et al.), from parental sensitive caregiving (Stack et al.;Vilaseca et al.; Zietlow et al.) to painful stimulations (Ranger et al.). The exposure to such a variety of environmental exposures is capable of shaping of cognitive development (Lejeune et al.; Muntsant et al.), brain connectivity (Hanford et al.), emotional (Stack et al.), and neuroendocrine regulation (Zietlow et al.) and even social and mating behavior in adult life (Liu et al.).

The theoretical, review, and methodological papers collected in this Research Topic further contribute by putting emerging research in the field into a broader context and by providing a different perspective for interpreting the research findings and highlighting clinical implications. Ludwig and Welch discuss controversies in the Darwinian theoretical construction. They ambitiously propose two testable theoretical arguments that move from lessons learned in clinical practice with at-risk babies which inform a relational and epigenetic model of emotional development that can be beneficial for early assessment and interventions in infancy and childhood. Two reviews focus on the embedding of early life stress into the developmental trajectories of behavioral, neuroendocrine, and immune system regulation. The mini review from Fogelman and Canli underlines the neuroendocrine and immune system physiological processes which contribute to the long-term embedding of early environmental encounters. Ylijoki et al. systematically reviewed evidence of the prenatal risk factors involved in setting the risk for detrimental health outcomes in preterm infants. Filippa et al. further focus on a specific potential mechanism—oxytocin secretion and its regulation—that may help us in understanding how early parental engagement may be beneficial for healthy development of preterm infants exposed to repeated painful stimulation during the hospitalization in the neonatal intensive care unit. Sacchi et al. present a brilliant study protocol that holds potentials for revealing how the post-natal caring environment may be beneficial for the socio-cognitive development in infants with intrauterine growth restriction, at least partially through neural mechanisms involved in social and non-social stimuli elaboration. Finally, a review of methodological approaches used in the field of developmental human behavioral epigenetics is provided by Provenzi et al., who highlight the pros and cons of different retrospective and prospective research architectures for the advancement of the field.

In conclusion, the present Research Topic successfully collected contributions from researchers and clinicians working in different European (i.e., Germany, Finland, Italy, Spain, Switzerland), Asiatic (i.e., China), and North American (i.e., Canada, United States of America) countries. The virtuous integration of expertise between the scientific and the clinical frameworks is hugely needed in order to advance the scientific knowledge in directions that can forcefully impact our capacity to provide smarter interventions and more efficient preventive strategies for at-risk individuals. We hope that the present Research Topic may be a step forward into this direction and that it can benefit future translational studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Impact of Caregiving on the Association Between Infant Emotional Behavior and Resting State Neural Network Functional Topology

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The extent to which neural networks underlying emotional behavior in infancy serve as precursors of later behavioral and emotional problems is unclear. Even less is known about caregiving influences on these early brain-behavior relationships. To study brain-emotional behavior relationships in infants, we examined resting-state functional network metrics and infant emotional behavior in the context of early maternal caregiving. We assessed 46 3-month-old infants and their mothers from a community sample. Infants underwent functional MRI during sleep. Resting-state data were processed using graph theory techniques to examine specific nodal metrics as indicators of network functionality. Infant positive and negative emotional behaviors, and positive, negative and mental-state talk (MST) indices of maternal caregiving were coded independently from filmed interactions. Regression analyses tested associations among nodal metrics and infant emotionality, and the moderating effects of maternal behavior on these relationships. All results were FDR corrected at alpha = 0.05. While relationships between infant emotional behavior or maternal caregiving, and nodal metrics were weak, higher levels of maternal MST strengthened associations between infant positive emotionality and nodal metrics within prefrontal ($p < 0.0001$), and occipital ($p < 0.0001$) cortices more generally. Positive and negative aspects of maternal caregiving had little effect. Our findings suggest that maternal MST may play an important role in strengthening links between emotion regulation neural circuitry and early infant positive behavior. They also provide objective neural markers that could inform and monitor caregiving-based interventions designed to improve the health and well-being of vulnerable infants at-risk for behavioral and emotional problems.

Keywords: infant brain, resting state, neural network nodal metrics, emotion behavior, observed caregiving

INTRODUCTION

The rapid development of the human brain in the first years of life (Knickmeyer et al., 2008; Gao et al., 2015b) is important for brain-behavior relationships that set the stage for future clinical and functional outcomes. Sensorimotor, auditory and visual networks develop first (Lin et al., 2008; Liu et al., 2008), followed by other large-scale networks important for higher-order regulatory processes (Gao et al., 2009, 2015a), including: the default mode network (DMN), implicated in self-referential processing (Amsterdam, 1972; Gusnard et al., 2001; Raichle et al., 2001; Ramenghi et al., 2009; Short et al., 2013; Alcauter et al., 2014; Ball et al., 2015); the salience network, underlying attention to personally salient stimuli (Seeley et al., 2007; Akazawa et al., 2016); and the frontoparietal executive control network, important for multiple cognitive control processes including emotional regulation (Phillips et al., 2008; Gao et al., 2016). Additionally, the normal adult pattern of inverse correlation of resting state functional connectivity between the DMN and the dorsal attention network (Corbetta and Shulman, 2002; Akazawa et al., 2016) emerges in the first year (Gao et al., 2013).

Yet, links between early alterations in neural circuitry structure and function and clinically relevant behavioral outcomes remain unclear. In normally developing infants, there is heightened neural sensitivity to environmental factors, including caregiving behaviors (Sur and Rubenstein, 2005; Roth and Sweatt, 2011). These early experiences influence the formation of synapses and dendritic projections, which alter the recruitment of specific brain regions in larger-scale networks, including those important for behavioral and emotional regulation (Huttenlocher, 1990; Kolb and Gibb, 2011). Although young infants have a rudimentary capacity to self-regulate, the expression and regulation of emotions is influenced by the provision of sensitive caregiving (Tronick and Gianino, 1986; Cirulli et al., 2003). Yet, the relationships between emotional network functioning and infant emotionality, and the impact of early caregiving on these relationships, are not well understood.

Positive and negative emotional behaviors (PE, NE) can be measured reliably within the first months of life (Worobey and Blajda, 1989; Rothbart et al., 2001; Dinehart et al., 2005). Infants displaying high levels of PE frequently smile or laugh, and experience high intensity pleasure (Tellegen, 1985; Watson et al., 1999). Some data suggest that low levels of PE in infancy precede behavioral inhibition (Park et al., 1997) and depression in childhood (Oldehinkel et al., 2004; Hayden et al., 2006; Anderson and Hope, 2008; Doucherty et al., 2010). Conversely, infants displaying high NE cry frequently and intensely in response to novelty and limitations, and are difficult to soothe (Henderson and Wachs, 2007). High NE in infancy is a relatively robust predictor of emotional problems later in childhood (Burgess et al., 2003).

Emotional regulation networks are well characterized in adults (Gray et al., 2002; Ochsner and Gross, 2005; Phillips et al., 2008), and include lateral and medial prefrontal cortical regions, specifically, anterior cingulate cortex (ACC), orbitofrontal cortex (OFC), and mediodorsal, ventrolateral, and dorsolateral prefrontal cortex (mdPFC, vlPFC, and dlPFC, respectively), within executive control and salience networks (Phillips et al., 2008; Sotres-Bayon et al., 2012; Nieh et al., 2013). Lower levels of functional connectivity between prefrontal cortical regions implicated in emotional regulation have been observed in children at risk for emotional disorders relative to healthy children (Singh et al., 2014; Manelis et al., 2015). By contrast, research examining relationships between large-scale network function and emotional behaviors in infancy is minimal. One study reported greater OFC activity to sad versus neutral vocalizations in healthy 3- to 7-month-old infants (Blasi et al., 2011), suggesting an early functional specialization for processing human auditory NE. More recently, connectivity patterns of the amygdala in early infancy have been associated with developmental changes in temperament (Graham et al., 2016).

Parental caregiving plays a critical role in the development of infant emotional behaviors and emotional regulation (Tronick and Gianino, 1986; Oldehinkel et al., 2006; Thompson and Meyer, 2007). Caregiving behaviors can be subdivided into three distinct categories; positive, negative and mental-state talk (MST) (Park et al., 1997; Sharp and Fonagy, 2008; Lipscomb et al., 2011; Meins et al., 2012). Positive aspects of caregiving, including warmth and sensitivity, predict lower NE in offspring (Park et al., 1997), whereas more negative caregiving behaviors, such as harsh and intrusive behaviors, are associated with greater infant NE (Lipscomb et al., 2011). Longitudinal studies support the *interaction* between infant emotional reactivity and maternal caregiving in shaping later clinical and functional outcomes in offspring (Kochanska, 1995; Boyce and Ellis, 2005; Oldehinkel et al., 2006; Pluess and Belsky, 2010). Thus, high NE infants, who experience negative caregiving are at highest risk for later emotional dysregulation (Morrell and Murray, 2003), while high PE may act as a buffer in negative parenting environments (Lengua et al., 2000; Prior et al., 2001; Feder et al., 2009). Another aspect of caregiving, the mother's capacity to 'read,' understand and attribute mental states to her infant, is considered a *prerequisite* for sensitive caregiving (Sharp and Fonagy, 2008; Meins et al., 2012). Declarative indices of maternal MST have been linked with subsequent social–cognitive abilities in the child, including heightened emotion understanding (Doan and Wang, 2010). Our own work and that of others, has shown maternal MST to be a distinct aspect of maternal caregiving (Hipwell et al., 2016) with particular relevance for shaping emotional behaviors in the child (Meins et al., 2012; Hipwell et al., 2015).

Family environment influences on infant network function have been only briefly studied. Greater connectivity at rest between anterior and posterior regions of the DMN in 6–12 months old infants was associated with higher levels of conflict between caregivers (Graham et al., 2015). This finding parallels connectivity patterns in depressed adults and children (Sheline et al., 2010; Gaffrey et al., 2012), suggesting a link between characteristics of the early caregiving environment, NE, and alterations in network connectivity in infancy. It further suggests negative caregiving may adversely affect network development in infancy and predispose to future mental health problems (Kochanska, 1995; Boyce and Ellis, 2005; Oldehinkel et al., 2006;

Pluess and Belsky, 2010). We are not aware, however, of any prior research that examined whether different types of caregiving behaviors impact neural networks, and relationships among networks and emotional behaviors, in infancy. Elucidating these relationships will be of clinical importance, as these relationships could help identify the most salient caregiving behavior targets in interventions designed to improve the health and well-being of vulnerable infants.

A robust method for establishing brain-behavior relationships is through network-based approaches, which can characterize the functional integration and segregation of large-scale networks (Supekar et al., 2009; Friston, 2011; Gao et al., 2015b). Two metrics commonly employed in such analyses are clustering coefficient (CC) and nodal efficiency (NEff). CC measures the degree to which nodes (neural regions) in a network segregate (Bullmore and Sporns, 2009; Rubinov and Sporns, 2010). Greater CC in network nodes is thought to provide more redundancy and render the network more resilient to insult to any single node (Bullmore and Sporns, 2009; Rubinov and Sporns, 2010). NEff measures the efficiency of communication of a single given node with other nodes in a network (Bullmore and Sporns, 2009; Rubinov and Sporns, 2010), and has been used to distinguish infants at-risk for future psychiatric problems including Autism Spectrum Disorder (Lewis et al., 2017). Using these network-based approaches and nodal metrics during resting state allows examination of the specific nodal contributions to the segregation and/or efficiencies of large-scale network functional topology without requiring the use of cognitive tasks, and is thus well-suited to studies in infants.

In the present study, we focused on infant network nodal metrics, infant displays of positive and negative emotional behaviors, and maternal caregiving behaviors at 3 months postpartum, as this represents a developmental window characterized by elemental forms of emotion regulation (Feldman et al., 1996; Horowitz-Kraus et al., 2017), and the appearance of early neural functional specialization for processing negative emotion (Blasi et al., 2011).

We aimed to:

(1) Identify relationships between infant emotional behaviors and nodal metrics in large-scale whole-brain networks. Based on behavioral data linking higher NE and lower PE in infancy with elevated risk for later emotional problems (Lonigan et al., 2003; Oldehinkel et al., 2004; Eisenberg et al., 2009; Doucherty et al., 2010), and observations of lower functional connectivity in large-scale networks important for emotional regulation in children at-risk for emotional problems (Singh et al., 2014; Manelis et al., 2015), we hypothesized that higher NE and lower PE in infancy would be associated with lower CC and NEff within emotion regulation networks. Specific nodes showing these relationships would include lateral and medial prefrontal cortical regions.

(2) Identify relationships between caregiving behaviors and nodal metrics in large-scale, whole-brain networks, and examine the extent to which maternal caregiving influences the relationships between infant emotional behaviors and nodal metrics in large-scale whole-brain networks. Based on findings linking positive caregiving and maternal MST with PE and self-regulation in the child (Meins et al., 2012), and findings linking higher NE and disrupted connectivity in emotional regulation networks in children at-risk for emotional problems (Singh et al., 2014; Manelis et al., 2015), we hypothesized that higher levels of positive caregiving and maternal MST would be associated with greater CC and NEff within emotion regulation networks. Specifically, we expected that these aspects of caregiving would strengthen positive relationships between infant PE, CC and NEff, and weaken inverse relationships between infant NE, CC and NEff, within these networks. By contrast, more negative caregiving would be associated with lower CC and NEff, and would strengthen inverse relationships between NE, CC and NEff in these networks.

MATERIALS AND METHODS

Participants

Mothers (aged 19–24 years) and their 3-month-old infants were recruited from the population-based Pittsburgh Girls Study (PGS), an ongoing longitudinal study which has conducted annual assessments on 2,450 girls followed from childhood through young adulthood (Hipwell et al., 2002; Keenan et al., 2010). Pregnant or recently delivered participants were identified from PGS interview data in waves 15–17, and study eligibility was confirmed by telephone. Following written, informed consent for their own and their infant's participation in the study, mother-infant dyads completed a research visit at the Children's Hospital of Pittsburgh. Procedures were approved by the University of Pittsburgh Institutional Review Board.

Exclusion criteria for the mother included prenatal or concurrent substance exposure (except for marijuana use), and provision of <2 h per day of care for her infant. Exclusion criteria for infants included: <37 weeks gestation, head circumference <32 cm, birth weight <5.5 lbs, APGAR score <7 at 5 min, extended hospitalization for any reason, or any MRI contradictions.

Demographic and Clinical Information

Mothers reported on infant age and gender. Mothers also reported on their own mood in the past week using the Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1987), given the potential impact of maternal depression on caregiving (Wisner et al., 2002; Logsdon et al., 2006). Household poverty was indicated by maternal report of ten potential sources of public assistance (e.g., food stamps, Medicaid, and WIC). Low levels of poverty were indicated by receipt of 0–2 types of assistance, whereas moderate to high levels were indicated by three or more types.

Behavioral Assessments

Each mother-infant dyad was filmed in face-to-face interaction. The mother was first asked to talk to her infant "in any way you want to" without the use of toys (2 min), and then to "help

your child to get interested" in a specific toy (3 min). Infant and caregiving behaviors during the two episodes were coded independently, using time-sampled global ratings, by pairs of trained coders at graduate and doctoral level who were unaware of all other information about the participants.

Positive and negative infant emotional behaviors were coded on 5-point behaviorally anchored Likert scales ranging from 1 (none) to 5 (frequent, intense, prolonged displays). Mean scores across the two observation periods were computed for infant PE and NE.

Maternal behaviors were coded on five global dimensions (hostility, intrusiveness, involvement, warmth and sensitivity) scored on 4-point Likert scales ranging from 1 (none/minimal) to 4 (many/high) adapted from the Early Parenting Coding System (Shaw et al., 2006; Hipwell et al., 2015). Mean scores on each dimension were computed from observations in the two episodes. A varimax rotation principal component analysis of these mean caregiving scores resulted in two components: positive maternal caregiving (warmth, involvement, sensitivity), and negative maternal caregiving (hostility, intrusiveness) that explained 74.9% of the variance.

Frequency of maternal MST was determined from verbatim transcriptions of maternal speech during the filmed interactions. Comments about infant internal states or intentionality (e.g., Do you think that's funny? and Do you want to go home?) were counted and reduced to 4-point scales (1 = none to 4 = five or more comments), and a mean maternal MST score from the two observation episodes was computed.

Inter-rater reliability for coded observations was examined in a random selection of 15 (26%) dyads. Intraclass correlation coefficients (ICCs) computed for absolute agreement on coded behavioral observations were high: 0.88 infant PE; 0.85 infant NE; 0.76 maternal hostility; 0.88 maternal intrusiveness; 0.91 maternal involvement; 0.85 maternal warmth; 0.70 maternal sensitivity; and 0.89 for maternal MST.

Resting State Data Acquisition and Image Processing

Infants were scanned during natural sleep without sedation (Haney et al., 2010; Windram et al., 2012). Mothers were asked to refrain from feeding or allowing their infant to nap for a few hours before the scan appointment, then at the allotted time the infant was swaddled and fed ('feed and bundle' approach) in a quiet, dimly lit room and then transferred to the MR scan table when asleep. Mothers without MRI contraindications could stay with the infant in the scan room.

Two 5-min resting state functional magnetic resonance images were acquired using a gradient-echo echo-planar imaging scan sequence with the following parameters: repetition time = 2020 ms, echo time = 32 ms, field of view = 256, 32 slices, and voxel size = $4 \times 4 \times 4 \times \text{mm}^3$.

Images were preprocessed using in-house routines, the Cincinnati Children's Hospital Imaging Processing Software (CCHIPS) (Schmithorst et al., 2000) written in IDL (Research Systems Inc., Boulder, CO), Statistical Parametric Mapping (SPM8) software[1], and the Brain Connectivity Toolbox (BCT) software (Rubinov and Sporns, 2010). Image preprocessing included slice time correction, motion correction, and a 2-step normalization procedure. Motion correction methods closely followed those laid out by Power and colleagues in order to avoid spurious results due to excessive motion (Power et al., 2014). Motion correction was performed using an affine transform to the best reference image within each resting state run. The reference image was selected by determining the frame within each run that best minimized an intensity-based cost function (Power et al., 2014). A two-stage procedure for spatial normalization was used: one, the reference image for each participant was normalized to an age-specific T2-weighted image (Shi et al., 2011) in MNI space, and two, all normalized reference images were then averaged to create a study-specific template, and then used as a normalization template for each participant's specific reference image. Finally, the entire run was transformed into MNI space (using a single transformation incorporating the motion correction parameters) and intensity normalized to grand mean = 1000. Frames with an rms deviation (DVARS) of greater than 25 from the previous frame, or a total movement from a framewise displacement (FD), as estimated from the motion correction parameters, of greater than 0.2 mm from the previous frame were rejected.

Data from each of the two resting state runs were combined into a single time course. Given the rigor of the above thresholds, a threshold of at least 4 min of total data (incorporating both runs), or 3.6 min of total data (with just one run) was used to include as many participants as possible. The number of usable frames was calculated and included as a covariate of no interest. An age-specific parcellation atlas (Shi et al., 2011) was used to extract average time courses from 90 bilateral cortical regions. Each time course was band-pass filtered ($0.009\ \text{Hz} < f < 0.08\ \text{Hz}$), and nuisance regressors including motion correction parameters, linear and quadratic drift, and global, average white matter and CSF signal, were used. Correlation matrices (90-X-90, absolute value) were computed based on the correlations of the time courses between two regions after removal of nuisance regressors and band-pass filtering.

The correlation matrices were thresholded (yielding a binarized graph, where each possible connection between nodes has a value of either zero or one) and computed to obtain specific nodal measures. The matrices were thresholded according to differing values of cost (the ratio of the number of connections in the graph to the number of possible connections). A cost-independent analysis was used according to fixed values of cost ranging from 0.05 to 0.45 (step size 0.05). Here, subject was included as a random variable within these random slopes models, since thresholding at different cost values would be expected to have a multiplicative and not just a linear effect on the graph metrics. These models were averaged to obtain a more stable value per subject.

The formulae for CC and NEff are given below, for the binarized graphs used here. The connection between nodes i and j in the graph is designated as G_{ij}, which has a value of zero or

[1] http://www.fil.ion.ucl.ac.uk/spm

one. The shortest distance (or path length) between nodes i and j is designated as D_{ij}; if the nodes are completely unconnected, the distance is infinite. The clustering coefficient of a node i is defined as:

$$CC_i = \frac{\sum_{j,k} G_{ij}G_{ik}G_{jk}}{\sum_{\substack{j,k \\ j\neq k}} G_{ij}G_{ik}}$$

and can be interpreted as the ratio of "triangles" (e.g., nodes i, j, and k are all connected to each other) to the number of possible "triangles" (e.g., node i is connected to nodes j and k). The nodal efficiency of a node i is defined as:

$$Neff_i = \frac{1}{N-1}\sum_j \frac{1}{D_{ij}}$$

where N is the total number of nodes in the graph, and $1/D_{ij} = 0$ for the case where D_{ij} is infinite (the nodes are unconnected). This metric is the average "efficiency" (reciprocal path length) of node i for communicating with all the other nodes in the graph.

Statistical Analyses

See **Figure 1** for study design and statistical analysis structure.

> **Aim 1:** Associations among specific nodal metrics (CC, NEff) and infant behaviors were examined in two separate regression models: where whole-brain nodal metrics were dependent variables, and either NE or PE, were independent variables.
>
> **Aim 2:** Associations among specific nodal metrics (CC, NEff) and positive caregiving, negative caregiving or MST were examined in three regression models: where whole-brain nodal metrics were dependent variables, and either positive caregiving, negative caregiving or MST were independent variables. Next, the moderating effect of maternal caregiving on associations between infant NE and PE and specific nodal metrics was explored in six regression analyses that each included an interaction term: NE x negative caregiving, NE x positive caregiving, NE x maternal MST, PE x negative caregiving, PE x positive caregiving or PE x maternal MST, covarying for main effects of the corresponding infant and maternal caregiving measures.

In each set of analyses, infant age, gender, maternal depressed mood, use of public assistance, and number of usable frames, were covariates of no interest (**Supplementary Materials** for main effects of these covariates). Results were FDR corrected at alpha = 0.05. A mixed-effects model with an unstructured correlation matrix was used to account for the varying values of cost.

All interactions that survived multiple comparisons correction were graphed using ggplot2 (Wickham, 2016) in R[2] to better display the relationships between infant emotional behavior, nodal metrics and maternal MST.

[2]https://www.r-project.org/

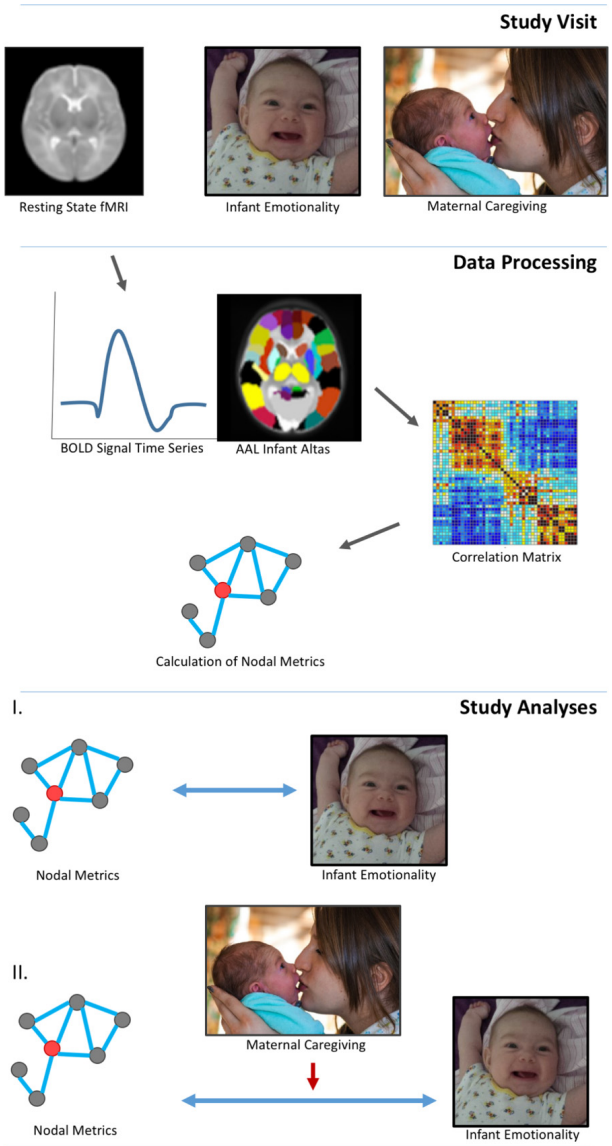

FIGURE 1 | Study design and data analyses. Three-month infants were scanned during natural sleep without sedation. Mother-infant dyads were filmed in a face-to-face interaction in order to code global ratings of infant emotional behavior and maternal caregiving. Data processing involved a standard pipeline: motion correction, normalization and smoothing. Images were segmented into 90 whole brain regions of interest (ROI) based on the aal neonatal atlas. Graph theory techniques defined the interactions between nodes, specifically, clustering coefficient and nodal efficiency. Analyses determined relationships among nodal network metrics and measures of infant emotionality, and the moderating effects of maternal caregiving on these relationships. Informed written consent was obtained for the publication of mother and infant images.

RESULTS

Resting state fMRI and observational measures of infant emotional behaviors and maternal caregiving were collected for 58 infants. Ten infants were excluded from analyses due

TABLE 1 | Demographic, clinical, behavioral, and other covariate information.

	Mean (SD)	Range
Infant demographics		
Age, in months	3.3 (0.8)	2–5
	***N* (%)**	
Gender, number of females	22 (48)	
Birth order, first child	32 (66)	1–5
Birth order, second child	13 (27)	1–5
Infant emotional behaviors		
Positive emotional behavior	2.2 (0.7)	1–5
Negative emotional behavior	1.3 (0.5)	1–2.5
Infant covariate information		
Number of usable frames	217.8 (58.9)	111–292
Scan length, in minutes	7.3 (2.0)	3.7–9.7
Maternal demographics		
	***N* (%)**	
Social assistance tally*, number of moderate/high users	35 (76)	
Maternal caregiving behaviors		
Positive caregiving (PCA component)	−0.06 (1.11)	−4.18–1
Negative caregiving (PCA component)	−0.20 (0.92)	−1.56–3.04
Maternal mental-state talk	2.9 (0.8)	1–4
Maternal clinical information		
Edinburgh Postnatal Depression Scale (EPDS) score‡	6.1 (5.6)	0–24

**Social assistance tally was measured by counting the number of public assistance services used, these were further dichotomized into low (0–2 counts) or high (3–7 counts).*
‡An EPDS score >10 was reported by 20% of mothers, indicating 'probable' depression, while an EPDS score of >13 was reported in 12.5%.

to excessive motion, and two mothers did not complete the EPDS. Thus, 46 infants were included in total (**Table 1**). For 67% of mothers, this was their first born infant, for 27% this was their second child. For the remaining 6%, the birth order ranged from third to fifth child. The majority of mothers (76%) reported using 3–7 counts of public assistance. 20% of mothers indicated 'probable' postnatal depression on the EPDS (a score of >10 on the EPDS), and 12.5% had EPDS scores indicative of postnatal depression (score of >13), consistent with population norms (O'hara and Swain, 1996; Wisner et al., 2002).

Infant emotional and maternal caregiving variables are in **Table 1**. Infant negative and positive emotional behaviors were uncorrelated ($r_s = -0.07$, *ns*), as were positive and negative caregiving behaviors with MST ($r_s = 0.29$, *ns* and $r_s = -0.14$, *ns*, respectively).

Network-Emotional Behavior and Network-Caregiving Relationships

No relationships between either PE or NE and CC or NEff survived FDR correction, nor did relationships between maternal positive caregiving, negative caregiving or maternal MST and CC or NEff (**Table 2**).

TABLE 2 | Network nodal metric associations with positive and negative infant emotional behaviors and with maternal caregiving indices.

Region	Metric	*T* statistic	*P* statistic
Positive emotional behavior			
Left superior orbitofrontal cortex	CC	−2.8	<0.01
Left inferior parietal lobule	CC	−3.6	<0.001
Left caudate	NEff	2.9	<0.01
Negative emotional behavior			
Left paracentral lobule	CC	−3.0	<0.005
Right middle occipital gyrus	NEff	2.7	<0.01
Positive caregiving			
No significant results			
Negative caregiving			
No significant results			
Mental-state talk			
Left parahippocampal gyrus	CC	−2.8	<0.01
Left amygdala	CC	−2.7	<0.01
Right hippocampus	NEff	−3.3	<0.005

CC, clustering coefficient; NEff, nodal efficiency.
No metrics survived FDR <0.05 correction for multiple comparisons.

Impact of Caregiving on Network-Emotional Behavior Relationships

There was one significant positive moderating effect of positive maternal caregiving on the relationship between PE and NEff in the left middle OFC. This PE-NEff relationship was less inverse with higher levels of positive maternal caregiving (**Table 3**). There was one significant positive moderating effect of negative maternal caregiving on the relationship between infant NE and CC in the right inferior parietal lobule. This NE-CC relationship was more positive with higher levels of negative maternal caregiving (**Table 3**).

Several highly significant FDR-corrected moderating effects of maternal MST were observed for the relationship between PE and CC. Here, there were robust positive moderating effects of MST on PE-CC relationships in bilateral OFC, left middle and inferior frontal gyri, bilateral olfactory cortex of the OFC, bilateral medial superior prefrontal cortex, left rectus gyrus, bilateral insula, left ACC, bilateral calcarine cortex, bilateral cuneus, left lingual, bilateral superior and middle occipital gyri, right inferior parietal lobule, bilateral precuneus, and left superior and right middle temporal pole. In each case, stronger positive PE-CC relationships were shown at higher levels of maternal MST, while these relationships were weakened, becoming less positive or inverse, at lower levels of MST (**Table 4** and **Figure 2**). Negative moderating effects of maternal MST were observed for the relationship between PE and CC in the right supramarginal gyrus and between PE and NEff in the left superior temporal gyrus. Here, inverse PE-CC relationships were weakened or became positive with higher maternal MST (**Table 4**).

The moderating effects of maternal MST did not survive FDR correction for relationships between infant NE and either CC or NEff (**Table 4**).

TABLE 3 | Moderating effects of positive and negative maternal caregiving on the associations between nodal metrics and positive and negative infant emotional behaviors.

Region	Metric	*T* statistic	*P* statistic
Positive emotional behavior by positive maternal caregiving			
Left inferior occipital gyrus	CC	−2.8	<0.01
Right angular gyrus	CC	−3.2	<0.005
Left middle orbitofrontal cortex	NEff*	4.6	<0.0001
Negative emotional behavior by positive maternal caregiving			
Right putamen	NEff	−2.9	<0.01
Right pallidum	NEff	−2.8	<0.01
Positive emotional behavior by negative maternal caregiving			
Left supplementary motor area	CC	2.7	<0.01
Right inferior frontal (pars triangularis) gyrus	NEff	2.9	<0.01
Left insula	NEff	−2.8	<0.01
Left superior temporal pole	NEff	−3.1	<0.005
Negative emotional behavior by negative maternal caregiving			
Right inferior parietal lobule	CC*	3.8	<0.0005
Left inferior parietal lobule	CC	3.2	<0.005
Left middle orbitofrontal cortex	NEff	3	<0.005

CC, clustering coefficient; NEff, nodal efficiency.
**Denotes results that survived FDR <0.05 correction for multiple comparisons.*
A positive interaction denotes that the relationship between nodal metrics and infant emotionality was more positive (or less negative) with greater level of the given maternal caregiving behavior. A negative interaction denotes that the relationship between nodal metrics and infant emotionality was more negative (or less positive) with greater levels of the given maternal caregiving behavior.

TABLE 4 | Moderating effects of maternal mental-state talk on the associations between nodal metrics and positive and negative infant emotionality.

Region	Metric	*T* statistic	*P* statistic
Positive emotional behavior by maternal mental-state talk			
Right superior orbitofrontal cortex	CC*§	4.7	<0.0001
Left middle frontal gyrus	CC*	2.3	<0.05
Left middle orbitofrontal cortex	CC*	2.3	<0.05
Left inferior frontal (opercular) gyrus	CC*	2.6	<0.05
Left inferior frontal (pars triangularis) gyrus	CC*	2.8	<0.01
Left inferior orbitofrontal cortex	CC*	2.8	<0.01
Right olfactory cortex	CC*	2.8	<0.01
Left olfactory cortex	CC*	2.7	<0.01
Right medial superior frontal gyrus	CC*	2.5	<0.05
Left medial superior frontal gyrus	CC*	3.0	<0.005
Left medial orbitofrontal gyrus	CC*	2.1	<0.05
Left rectus gyrus	CC*	2.8	<0.01
Right insula	CC*	3.2	<0.005
Left insula	CC*	2.9	<0.01
Left anterior cingulate gyrus	CC*	2.6	<0.05
Right calcarine cortex	CC*	2.4	<0.05
Left calcarine cortex	CC*§	3.9	<0.0005
Right cuneus	CC*	2.3	<0.05
Left cuneus	CC*	2.2	<0.05
Left lingual gyrus	CC*	2.2	<0.05
Right superior occipital gyrus	CC*	3.3	<0.005
Left superior occipital gyrus	CC*	2.3	<0.05
Right middle occipital gyrus	CC*	2.4	<0.05
Left middle occipital gyrus	CC*	3.0	<0.005
Right inferior parietal lobule	CC*	2.7	<0.01
Right supramarginal gyrus	CC*	−3.0	<0.005
Right precuneus	CC*	2.5	<0.05
Left precuneus	CC*	2.3	<0.05
Left superior temporal pole	CC*	2.7	<0.01
Right middle temporal pole	CC*	2.1	<0.05
Left superior temporal gyrus	NEff*	−3.7	<0.001
Right inferior temporal gyrus	NEff	−3.1	<0.005
Left inferior temporal gyrus	NEff	−3.1	<0.005
Negative emotional behavior by maternal mental-state talk			
Right middle temporal pole	CC	−3.1	<0.005
Right middle frontal gyrus	NEff	2.8	<0.01
Right calcarine cortex	NEff	2.9	<0.01
Left calcarine cortex	NEff	3.0	<0.005

*CC, clustering coefficient; NEff, nodal efficiency; *denotes results that survived FDR <0.05 correction for multiple comparisons. §Graphical representation of these metrics available in* ***Figure 1****.*
A positive interaction denotes that the relationship between nodal metrics and infant emotionality was more positive (or less negative) with greater maternal mental-state talk. A negative interaction denotes relationships between nodal metrics and infant emotionality that were more negative (or less positive) with greater maternal mental-state talk.

DISCUSSION

We aimed to identify large-scale neural network-emotion relationships in 3-month-old infants, and examine the impact of caregiving on these relationships. Our major findings indicate that maternal MST influenced associations between infant PE and CC predominantly in prefrontal and occipital cortical networks, where higher levels of maternal; MST were associated with stronger positive relationships between infant PE and CC within these networks. These results contrast with more limited effects of positive and negative components of caregiving on infant brain-emotional behavior relationships; and only weak relationships among nodal metrics and either maternal caregiving or infant emotional behaviors when examined without consideration of the caregiving context. These findings suggest a specific and critical role of maternal MST in shaping positive relationships between the extent of segregation, and resulting resilience, within prefrontal cortical and visual processing networks and PE in infants.

Higher levels of maternal MST were associated with stronger positive relationships, and weaker inverse relationships, between infant PE and CC among several lateral and medial prefrontal cortical regions implicated in emotional regulation (Gray et al., 2002; Ochsner and Gross, 2005). The lateral prefrontal cortex integrates emotional and cognitive information, and generates emotional responses (Gray et al., 2002). The medial prefrontal cortex, including the OFC, serves a critical role in memory retrieval, action selection, executive control and social cognition related to emotional regulation (Ochsner and Gross, 2005). Greater maternal MST was also associated with stronger positive relationships between PE and CC in bilateral insula, important for interoception and a key role in the salience network (Zaki et al., 2012; Simmons et al., 2013), and in visual cortical regions important for

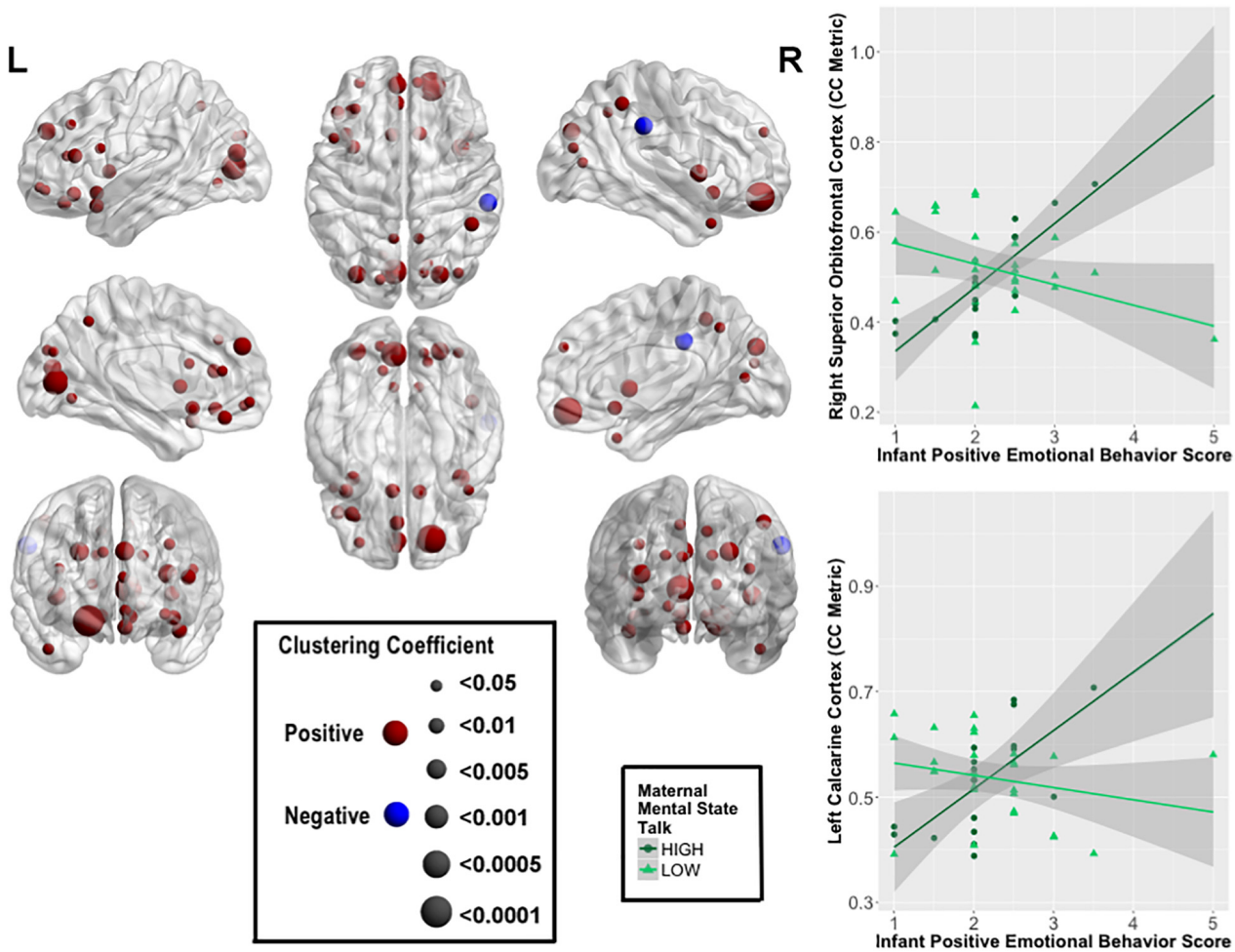

FIGURE 2 | Moderating effects of maternal mental-state talk on the associations between clustering coefficient (CC) and positive infant emotional behavior (PE). The most robust moderating effects were observed within bilateral prefrontal cortical and visual processing regions. The size of the node represents the strength of the moderating effect of maternal mental-state talk on PE-CC relationships. Graphical representations of these moderating effects on the right superior OFC and left calcarine cortex are displayed. In each case, stronger positive PE-CC relationships were shown at higher levels of maternal mental-state talk; these relationships were less positive or inverse at lower levels of mental-state talk.

higher-order visual processing. Mothers' capacity to engage in mentalizing behaviors is linked to behavioral and neural indices of empathy and reflection, and may also be a proxy for mother-infant dyadic attunement (Meins et al., 2012). We show the importance of observed maternal MST in facilitating infant positive emotion and higher levels of segregation and built-in redundancy and resilience in emotional regulation, interoception, salience and visual processing networks in early infancy.

There were negative moderating effects of maternal MST on CC and NEff in the right supramarginal gyrus and the left superior temporal cortex, respectively, where higher levels of maternal MST were associated with stronger inverse relationships between infant PE and CC and NEff. The right supramarginal gyrus is important for somatosensory processing and empathy (Reed and Caselli, 1994; Carlson, 2012; Silani et al., 2013), while the left superior temporal gyrus is critical for language, speech, and higher-level auditory processing (Bigler et al., 2007). Given the impact of maternal MST on infant brain-PE relationships described above, maternal MST may obviate the need for either the early segregation of neural networks implicated in somatosensory and empathic processing, or the integration of neural networks subserving higher-level auditory processing. Instead, this characteristic of caregiving may promote involvement of large-scale prefrontal executive control and visual networks in the shaping of infant brain-PE relationships.

There were two significant relationships relating to moderating effects of positive and negative caregiving. More positive maternal caregiving weakened the inverse relationship between PE and NEff in the left OFC, indicating that greater, rather than lower, engagement of this key emotional regulation prefrontal cortical region may help to promote positive emotion in infants in supportive caregiving environments. More negative

maternal caregiving rendered the relationship between NE and CC in the right inferior parietal lobule positive. The latter region is important for directing attention to salient new or alerting environmental stimuli (Lynch et al., 1977; Singh-Curry and Husain, 2009), and thus a positive relationship between NE and CC in this region likely reflects enhanced attention to potentially alerting or threatening stimuli in infants with higher levels of NE. That this relationship was strengthened in more negative caregiving environments parallels reports showing an effect of negative caregiving in promoting greater vigilance and NE in infants (Lipscomb et al., 2011).

The absence of strong relationships among maternal caregiving behaviors and nodal metrics *per se*, or among infant emotional behaviors and nodal metrics outside the caregiving environment, indicate a specific effect of maternal caregiving in shaping early infant brain-emotional behavior relationships. Our findings also parallel those of other studies indicating effects of positive and sensitive caregiving in providing normative social, cognitive and emotional contexts for the development of self-regulatory capabilities, and greater higher-order functioning within these domains (Bick and Nelson, 2016). Studies examining the absence of positive caregiving, including maternal separation studies, and subsequent interventions, help to elucidate caregiving effects on neural networks subserving emotional regulation (Brett et al., 2015; Bick and Nelson, 2016). Here, studies of institutional care and adoption highlight the importance of caregiving on development of child neural networks (Gee et al., 2013; Bick and Nelson, 2016). The critical role of maternal caregiving in shaping infant behavior is further highlighted in rodent studies that provide links between levels of maternal attention, through extensive licking and grooming behaviors and typical rat pup development, and the development of anxiety-like behaviors in rat pups who are ignored by their mothers (Meaney, 2001). Moreover, cross-fostering studies show these latter behavioral changes can be reversed by reintroducing pups to highly attentive foster mothers (Francis et al., 1999; Weaver et al., 2004). Non-human primate studies demonstrate that social behaviors and amygdala gene expression are directly impacted by quality of maternal care (Sabatini et al., 2007). While these studies highlight the sensitivity of the early developmental period in humans, non-human primates and rodents to effects of caregiving on emotional behaviors and neural network development, our findings are the first to show a specific impact of caregiving on shaping critical neural network-emotion relationships in human infants.

The study has some limitations. The cross-sectional nature of analyses does not provide information on the direction of relationships between maternal caregiving, infant emotion and nodal metrics. Given that this is the first study to explore any relationships among these variables, however, identifying associations is a logical first step. The relatively small sample size limited our ability to examine the influence of additional environmental factors (e.g., maternal psychiatric history) on relationships among neural measures and emotional behavior. Similarly, our sample was predominantly of low SES, which limited our ability to generalize to other populations. Low SES samples are often under-represented in research, however, and thus inclusion of such samples in future infant studies will allow examination of a greater range of emotional developmental outcomes. We did not include birth order as a covariate. This can be an additional focus of study in future research. Longitudinal studies can establish the temporal ordering of associations among infant behavior, brain functional topology and caregiving within a larger sample.

We are the first to show that maternal MST, a key component of parenting linked to emotional behavior in offspring, influences the association between infant positive emotion and functional topology in prefrontal cortical and visual processing networks. Moreover, these associations were weak in the absence of moderating effects of any caregiving components. These findings thus highlight the importance of MST, above positive or negative aspects of caregiving, on development of infant brain-positive emotional behavioral relationships. These findings are clinically important, as they can provide objective neural markers to monitor the effectiveness of caregiving-based interventions targeted at strengthening infant brain-positive emotion relationships, to improve the health and well-being of vulnerable infants at risk for behavioral and emotional problems.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Pittsburgh Institutional Review Board. The protocol was approved by the University of Pittsburgh Institutional Review Board. All mothers gave written informed consent their own and their infant's participation in the study in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MP and AH conceived and designed experiments. JR, AH, and LH performed all the experiments. LH, VS, AH, and MP analyzed the data. LH, AH, and MP wrote the manuscript with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2018.01968/full#supplementary-material

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METHODS
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Methodological Challenges in Developmental Human Behavioral Epigenetics: Insights Into Study Design

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Developmental human behavioral epigenetics (DHBE) holds potential for contributing to better understanding of how early life exposures contribute to human developmental trajectories and to inform clinical practice and early interventions. Nonetheless, DHBE research to date is challenged by two major issues: (a) the frequent use of retrospective study designs; and (b) the major focus on epigenetic variations associated with early life adversities, rather than protective care exposures. In order for DHBE research to maintain its promises, these issues need to be addressed in a systematic way according to a careful methodological planning of study design. In this contribution, we provide pragmatic insights on methodological aspects that should be dealt with while designing DHBE studies. We propose different study designs for the retrospective and prospective investigation of both adversity- and care-related epigenetic variations. Examples from available scientific literature are provided to better describe the advantages and the limitations of each study design.

Keywords: behavioral epigenetics, developmental science, methodology, study design, DNA methylation

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INTRODUCTION

Behavioral epigenetics (BE; Lester et al., 2011) refers to the study of epigenetic mechanisms and variations in association with exposures to early environmental conditions and phenotypic developmental outcome. The emergence of BE as a relevant field of research is due to the pioneering work by Meaney and Szyf (2005) who reported on epigenetic variations (i.e., DNA methylation) observed in rats exposed to varying levels of caregiving quality within a normal range. Notably, exposures to early environmental conditions might include both the experience of adversities (e.g., prenatal and post-natal stress exposures; Hyman, 2009) as well as protective care conditions (e.g., high-quality caregiving; Curley and Champagne, 2016). On the one hand, rats exposed to low quality of caregiving (i.e., low frequency of linking and grooming and arch-back nursing) showed high levels of methylation at the exon 1F of a specific stress-related gene (i.e., glucocorticoid gene, *nr3c1*), which in turn was predictive of developmental outcomes including behavior regulation, social interactions and stress reactivity (Turecki and Meaney, 2016). On the other hand, when rats born by mothers rated as low-quality caregivers were subsequently cross-fostered to mothers rated as high-quality

caregivers their *nr3c1* methylation patter was reversed to levels similar to those of rats born and raised by high-quality mothers and the detrimental effects on phenotype were no more observed (McGowan et al., 2011).

THE EMERGENCE OF DEVELOPMENTAL HUMAN BEHAVIORAL EPIGENETICS

The fascination arising from these original animal model research intrigued researchers involved in human developmental science to test whether such epigenetic mechanisms might be involved in the processes through which early experience is embedded into the phenotype (Lester et al., 2016). The gene X environment approach was providing insightful support to the notion that—at some point in life—our genetic predisposition and the environmental encounters might interact resulting in observable behaviors (Belsky and Pluess, 2012; Mileva-Seitz et al., 2016). Nonetheless, BE holds promise to reveal the biochemical processes through which this interaction actually occurs in a developmental framework (Lester et al., 2011; Conradt, 2017).

Developmental human behavioral epigenetics (DHBE) studies—i.e., the application of BE to the study of human development—rapidly emerged in scientific literature. To date, this research has revealed that early experiences of prenatal stress (Oberlander et al., 2008; Devlin et al., 2010; Sosnowski et al., 2018), maltreatment and abuse (Beach et al., 2010; Blaze et al., 2015; Tyrka et al., 2016), neonatal pain (Provenzi et al., 2015; Montirosso et al., 2016a; Fumagalli et al., 2018), socio-economic disadvantage (Essex et al., 2013; Swartz et al., 2017) and other adversities might lead to altered patterns of DNA methylation that contribute to an individual's phenotype programming.

Nonetheless, very few DHBE studies to date focused on the epigenetic vestiges of protective care environmental conditions. For example, a retrospective study investigated the effect of maternal stroking on glucocorticoid receptor gene expression in infants that were exposure to maternal depression during pre and postnatal period (Murgatroyd et al., 2015). In this study, infants who were previously exposed to postnatal maternal depression showed a decrease of methylation after maternal stroking at 5 weeks of life. Consistent with this result, in a prospective study, the methylation correlates of physical maternal contact were measured in 4-to-5-year-old infants: those exposed to more frequent affectionate contact in the first 5 days of life showed greater methylation during preschool age (Moore et al., 2017).

CHALLENGES IN DHBE RESEARCH

The misbalance between adversity- and care-focused BE research in human subjects is particularly evident. Different reasons might be invoked for such a predominant interest in adversity-related epigenetic variations in HBE. First, findings from animal models BE were more easily translated into study designs in human developmental science aimed at understanding the biological effects of early-in-life exposures to less-than-optimal caregiving contexts (Blaze et al., 2015). Second, the study of care-related epigenetic variations in animals can be relatively easy to set up with experimental manipulation, which is not the case of human subjects (Lester et al., 2011). Third, in humans protective and care interventions are delivered concurrently or—more often—after the occurrence of stressful, traumatic or adverse conditions and the timing of adversities and protective care is only partially predictable. As a result, in the absence of a conjoint and integrated DHBE research on both adverse and protective exposures, limited implications can be derived from this field of research that might have clear and insightful implications for clinical practice.

Moreover, the majority of the available DHBE research is characterized by retrospective study designs. The prevalence of correlational research adopting retrospective investigations might be at least partially related to the fact that when Meaney and Szyf's findings on rat's caregiving and DNA methylation variations began to be published, researchers involved in human studies started to assess DNA methylation by applying epigenetic investigation *post hoc* to ongoing cohorts. Another reason to set up more retrospective and cross-sectional studies than prospective and longitudinal ones in DHBE might be due to limitations of experimental manipulation in human subjects. As stressful or adverse conditions might not be "administered" to infants and children as well as to adult human subjects for ethical reasons, in most of the cases epigenetic mechanisms can be investigated only at *post hoc*. Notably, this limitation mostly applies to DHBE focused on early adversities, whereas it is less valid for BE research on the epigenetic correlates of protective care interventions in human subjects. Whereas retrospective and cross-sectional designs allow to set up DHBE research to reveal potential associations between environmental exposures and variations in DNA methylation, to date the lack of prospective and longitudinal research leads to reduced capacity of developing causal interpretations and theoretical modeling.

THE PRESENT CONTRIBUTION

In the present study, we provide an in-depth discussion of different study designs in DHBE, we describe in details the advantages and limitations of different methodological architectures and we explore how different designs can be affected or solve major challenges in human BE research. When available, examples from research to date are also reported to better highlight the benefits and the challenges inherent to each specific methodological approach.

RETROSPECTIVE AND CROSS-SECTIONAL STUDY DESIGNS

Type 1: Concurrent Assessment of Epigenetics and Outcomes

Description

As reported in **Figure 1** (Type 1), this kind of study design implies the presence of an adverse exposure which has occurred before the study began (T_0) and that can be only measured or accounted for through retrospective investigations such as self-report, medical charts, socio-demographic records and other

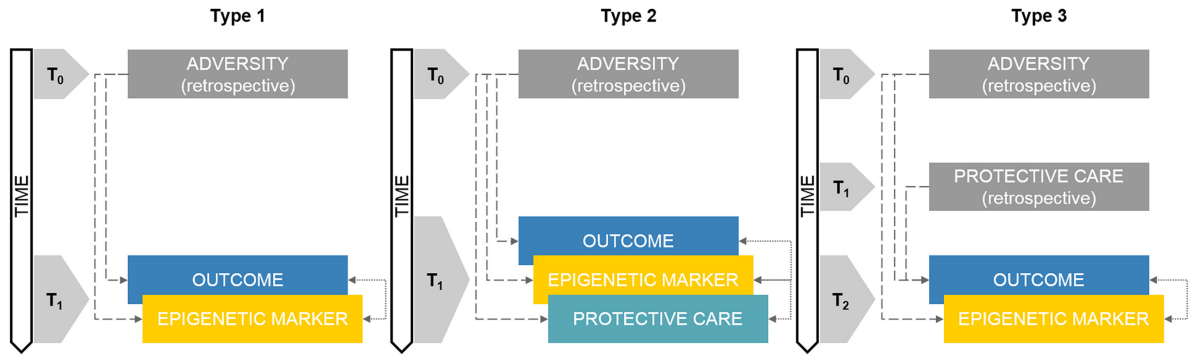

FIGURE 1 | Schematic representations of retrospective developmental human behavioral epigenetics (DHBE) study designs. Note. Type 1: concurrent assessment of epigenetics and outcomes; Type 2: concurrent assessment of epigenetics, care exposures and outcomes assessment; Type 3: retrospective exposure to adversity/care and concurrent assessment of epigenetics and outcomes. Gray boxes reflect retrospective measurements. Double-headed arrows reflect correlational nature of the association. Colored boxes reflect non-retrospective assessments. The annotation t_n refers to progressive time-points in the research study design.

proxies. The core focus of this study design is on the assessment of both epigenetic variations and the identified outcome(s) at T_1.

Example

In a recent article, Cicchetti and Handley (2017) reported on the retrospective investigation of the link between maltreatment, glucocorticoid receptor gene (i.e., *NR3C1*) methylation and several outcomes (i.e., emotional lability, self-control, behavioral problems, depressive symptoms) in a cohort of more than 500 school-aged children. Maltreatment information was obtained through the consultation of healthcare services records, classified in subtypes (i.e., neglect, emotional maltreatment, physical and sexual abuse) and characterized in terms of severity, age at exposure and chronicity. Cross-sectional assessment of *NR3C1* methylation and outcomes occurred in a single research session. Children with history of early-onset maltreatment presented significant greater methylation of the *NR3C1* gene when compared to non-maltreated counterparts. The cumulative exposure to more than one maltreatment subtype and to chronic maltreatment was significantly linked with *NR3C1* hyper-methylation, which, in turn, was associated with greater emotional lability, lower self-control, more externalizing behavioral problems and depressive symptoms.

Advantages

The cross-sectional nature of the study is generally associated with a less expensive research in terms of human resources. Moreover, researchers do not have to wait for environmental conditions to develop in time in order to obtain assays of epigenetic biomarkers and outcomes, as the stressful conditions are already occurred prior to the study onset. Additionally, no experimental manipulations of environmental conditions are required, thus partially limiting the potentials of ethical issues for at-risk or fragile human subjects with a history of adverse exposures. This type of retrospective studies is well suited to investigate potential associations which have never been previously tested, providing preliminary evidence of putative significant correlations and co-variations between specific epigenetic markers and developmental outcomes.

Limitations

As the information on adversity exposure and stressful conditions can only be obtained indirectly, many efforts should be devoted in controlling as many confounding sources as possible. Nonetheless, the absence of direct account of stressful exposures (i.e., the actual duration or accurate observation of specific stress conditions) is a major flaw of this kind of DHBE study design. Additionally, the kind of depicted relationship among variables (i.e., retrospective adversity indexes, epigenetic markers and outcomes) can only be correlational and no assumption can be proposed on the direction of the effect between epigenetic marker and outcome measures. Finally, in absence of two sequential measures of epigenetic markers, there is no possibility to obtain a variability index assessing the actual effect of adversity on the quantitative change in the selected epigenetic marker.

Type 2: Concurrent Assessment of Epigenetics, Care Exposures and Outcomes Assessment

Description

In this type of study design, adversity exposure already occurred before the subjects were enrolled in the research project (T_0) whereas epigenetic markers, protective care conditions and outcomes are obtained within the same cross-sectional research session (T_1; see **Figure 1**, Type 2). Compared to Type 1 study design, this methodological setting adds the integrated investigation of adversity and protective care exposures in a single DHBE research program.

Example

Conradt et al. (2016) investigated the association among the exposure to maternal post-natal depression, DNA methylation of *NR3C1* and placental 11β-hydroxysteroid dehydrogenase Type 2 gene (i.e., *11β-HSD-2*), salivary cortisol reactivity to socio-emotional stress (i.e., the five-episode Face-to-Face Still-Face; Provenzi et al., 2016) and maternal sensitivity in 128 4-month-old infants. Retrospective self-report measure of maternal

depressive symptoms was obtained through questionnaire administration. Infants' DNA methylation, hypothalamic-pituitary-adrenal (HPA) axis regulation in response to the FFSF procedure and maternal sensitivity were all measured in the same research session at 4-month-age. As such, this study design is cross-sectional and retrospective, but has the merit of providing a preliminary account of the concurrent associations of adversity (i.e., maternal depression) and protective care (i.e., maternal sensitivity) with infants' DNA methylation and developmental outcome (i.e., stress regulation). Indeed, the authors showed that infants from depressed mothers who exhibited high levels of sensitivity during the face-to-face interaction occurring just before the still-face episode of the FFSF procedure had lower *NR3C1* and *11β-HSD-2* methylation levels compared to counterparts from depressed mothers with lower sensitivity ratings. Consistent with previous animal model research (Curley et al., 2011), this study suggests that the quality of maternal caregiving behavior might be a significant protective buffer in the face of early adversity exposure in human infants.

Advantages

The limited impact on human resources and the possibility to obtain in relative short amount of time information on a whole cohort of subjects also applies to Type 2 study design. Also, limited manipulation of experimental conditions is allowed and care dimensions usually include naturally available protective factors such as socio-economical conditions or normal variations in parental care. These studies also have the advantage of testing uninvestigated hypotheses on the potential buffering or mediating role of protective care factors on the association among early adversities, epigenetic markers and selected developmental outcomes.

Limitations

Two major flaws affect this kind of study design: first, the indirect and retrospective account of adversities, as in Type 1 research; second, the concurrent assessment of protective factors and outcomes, which do not allow the interpretation of the direction of potentially significant associations. As such, while Type 2 research increases the complexity and provides more information compared to Type 1 study design, this DHBE research is still characterized as correlational and more careful interpretations of the study results should be done. Still, only epigenetic markers—but not variability indexes associated with adverse and protective exposures—are usually obtained in Type 2 research.

Type 3: Retrospective Exposure to Adversity/Care and Concurrent Assessment of Epigenetics and Outcomes

Description

In this kind of study design, three time-points are considered (**Figure 1**, Type 3). Adversity exposure occurred in T_0, usually well before (e.g., previous generation) the actual assessments included in the research program. Protective factors occurred in T_1, which is still antecedent to the study onset, but subsequent to adversity in T_0. The research assessments occur in T_2 and usually include at least one of the following measurements: (a) epigenetic marker (e.g., DNA methylation) and (b) developmental outcome (e.g., health-related indexes). As consequence, in this kind of studies, both adverse and protective exposures are measured indirectly through retrospective investigations, whereas epigenetic and outcome measurements occur usually concurrently in one research session.

Example

The project Ice Storm is a well-suited example of the Type 3 study design (Cao-Lei et al., 2018). In this research, adversity (T_0) consists in the well-known ice storm disaster that occurred in Quebec in 1998 and which offers a unique condition to assess intergenerational effects of early stressful conditions (King and Laplante, 2005). Ice storm exposure was quantified through retrospective questionnaire measuring the "objective hardship" of the exposure, including threat, loss, scope and change. Protective factor (T_1) consisted in the maternal capacity of cognitive reappraisal coded as "negative" *vs.* "neutral and positive" meaning making and coping strategies. At the time of assessment (T_2), enrolled subjects from the generation subsequent to that exposed to the 1998 ice storm averaged 13.6 years old and they were assessed for C-peptide secretion in blood T cells and for DNA methylation at selected genes associated with risk of Type 1 and 2 diabetes. The main merit of this study is the possibility to test for independent effects of adversity (i.e., objective hardship) and protection (i.e., maternal cognitive reappraisal) as well as for mediation models including both environmental exposures and epigenetic markers (i.e., DNA methylation at target sites within selected genes). Indeed, the authors showed that a direct marginal effect emerged only for objective hardship, whereas DNA methylation of diabetes-related genes significantly mediated the effects of objective hardship (positive mediation) and cognitive reappraisal (negative mediation) on risk for diabetes (i.e., C-peptide secretion) in children.

Advantages

Although both adversities and protective care exposures are quantified at *post hoc*, this study design allow researchers to build a more complex model of the interactions as well as of the individual and joint contributions that environmental conditions exert on infants' epigenetic regulation and developmental outcomes. More specifically, the presence of a temporal sequence that organizes stress and protection conditions in the past of enrolled individuals adds to the possibility to develop a theoretical framework in which the buffering effect of protective factors can be appreciated.

Limitations

In the light of advantages listed above, major limitations still apply to this complex, yet retrospective study design. First, in the absence of an initial epigenetic assay at T_0—or even before—there is no way researchers can observe and report on epigenetic regulation and variations in time and on the

effects of environmental conditions for bad and for good on this time-related change. Second, despite a temporal sequence is hypothesized in the past occurrence of adversity and protective factors, careful interpretation of findings still applies to this kind of research, as it cannot be entirely excluded that other protective factors might have intervened concurrently or even before T_0. Other flaws previously reported in relation to the retrospective assessment of adversity and protection (see Type 1 and Type 2 above) still apply to the present DHBE research program and only correlational interpretations of the relationship between epigenetic markers and developmental outcomes are sustained.

PROSPECTIVE AND LONGITUDINAL STUDY DESIGNS

Type 4: Prospective Assessment of Adversity-Related Epigenetic Variations on Longitudinal Outcomes

Description

The key feature of this research architecture is the presence of at least two assessments of epigenetic markers at two different time points (see **Figure 2**, Type 4). Between the first (T_0) and the second (T_2) epigenetic assay, a prospective quantification of an adverse-related exposure is carried on (T_1). A longitudinal assessment of a developmental outcome (T_3) can be also included at least once after the second epigenetic assessment.

Example

Recent research applying epigenetic research principles and methodology to the study of early stress exposure in preterm infants during the hospitalization in the Neonatal Intensive Care Unit (NICU; Montirosso and Provenzi, 2015) represents a prototypic research context to describe Type 3 study design (Provenzi et al., 2017a). In this research program, epigenetic markers are obtained at birth (T_0) and at NICU discharge (T_2), whereas during the NICU stay (T_1) specific variables related to stress exposures (e.g., pain-inducing skin-breaking procedures, mechanical ventilation, maternal separation) are quantified in a prospective way, usually on a day-by-day basis. Outcomes (e.g., socio-emotional stress regulation; Montirosso et al., 2016a) are measured at specific post-discharge time-points (T_3). In a recent article from this emerging field of research (Fumagalli et al., 2018), structural equation modeling was applied to assess the mediation role of epigenetic variation (e.g., difference in the methylation levels of the serotonin transporter gene, *SLC6A4*, from birth to discharge) on the association between NICU-related stress and anterior temporal lobes volumes at 12-month corrected age in preterm infants.

Advantages

The presence of two epigenetic measurements in different time-points allow researchers to move from epigenetic markers to epigenetic variations. Ratios and/or differential indexes of this variation in time can be obtained and are warranted to be tested in association with the target environmental exposure. Second, the kind of association that can be observed between adversity and epigenetic variation might also be characterized in terms of direction and hypothesis on the effect of adversity on epigenetic variation can be advanced. Third, a long-term chain of action-reaction sequences can be tested in complex models including all the variables, from the first epigenetic assay at T_0 to the outcome assessment(s) at T_3. More specifically, a prospective evaluation of the mediation of epigenetic variation on the association between adversity and outcome can be tested consistent with the temporal sequence in which environmental exposures and assessments occurred.

Limitations

Limitations of retrospective designs (Type 1–3) do not apply to prospective studies. Nonetheless, specific issues and challenges should be highlighted. First, these studies are much more expensive in terms of research procedures and human resources. Second, the longitudinal nature of the study exposes the research plan to moderate-to-high risk of sample attrition, as subjects enrolled at T_0 might not be available for the subsequent research sessions from T_1 to T_n due to many different reasons. Third, mainly for ethical reasons, contrary to what happens in animal model research, the adversity exposure cannot be experimentally manipulated or induced. As such, human populations who are naturally exposed to less-than-optimal developmental conditions featuring stress exposure are the best eligible subjects for this kind of research. In other words, this study design should be considered as quasi-experimental and careful interpretation of the direction of the effects is recommended.

Type 5: Clinical Trials of Care-Related Epigenetic Variations on Longitudinal Outcomes

Description

This study design differs from Type 4 basically because of the type of environmental exposure which is assessed, namely protective care conditions (see **Figure 2**, Type 5). Indeed, between the first (T_0) and the second (T_2) epigenetic assay, some kind of protective care intervention is promoted and offered to at least one sub-group in the study sample (T_1). A longitudinal assessment of a developmental outcome (T_3) can be also included at least once after the second epigenetic assessment.

Example

Research on the effects of brief psychotherapy interventions appear to be among the best examples of the Type 5 prospective DHBE study design. Roberts and collaborators (Roberts et al., 2014) assessed *SLC6A4* methylation before (T_0) and after (T_2) the exposure to a cognitive-behavior therapy (T_1) in children with anxiety disorder. Post-treatment effects were measured in terms of a reduction of anxiety symptoms occurring at 6-month follow-up (T_3). The findings suggested that *SLC6A4* methylation partially explained the difference in the reduced anxiety symptomatology between children who improved and those who did not after the psychotherapy.

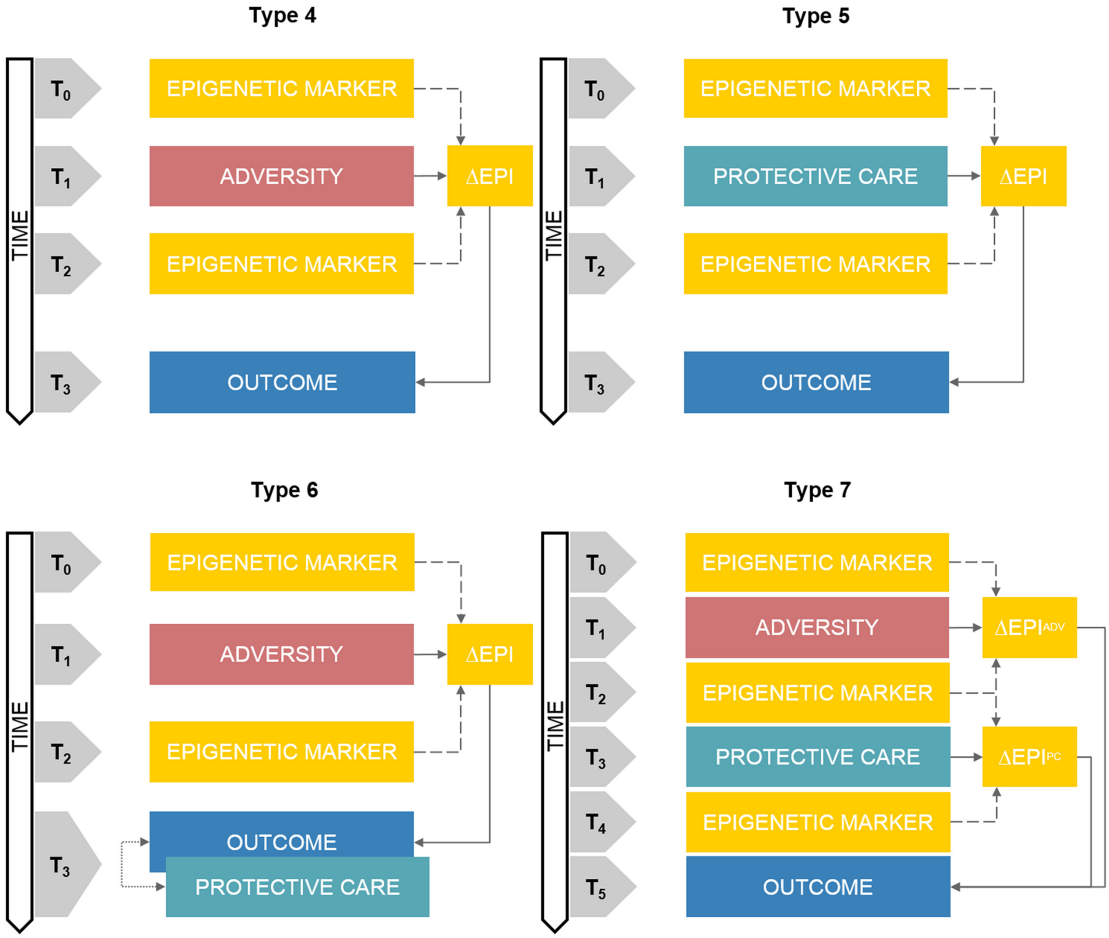

FIGURE 2 | Schematic representations of prospective DHBE study designs. Note. Type 4: prospective assessment of adversity-related epigenetic variations on longitudinal outcomes. Type 5: clinical trials of care-related epigenetic variations on longitudinal outcomes. Type 6: prospective assessment of adversity-related epigenetic variations on longitudinal outcomes with concurrent exposure to protective care. Type 7: prospective and sequential assessment of adversity- and care-related epigenetic variations on longitudinal outcomes. ΔEPI box, Epigenetic variation; ΔE^{ADV} box, Adversity-related epigenetic variation; ΔE^{PC} box, Protective care-related epigenetic variation. Double-headed arrows reflect correlational associations. The annotation t_n refers to progressive time-points in the research study design.

Advantages

The inclusion of a protective care intervention instead of an adverse condition in T_1 is not only a mere difference in terms of the object of investigation. Rather, the possibility to set up an intervention implies much more control for researchers and this kind of study design actually can be described as a clinical trial, with variable degrees of control and randomization. As such, this is probably one of the most robust methodological architectures for DHBE research. At the same time, this field of research holds potentials for the most direct implications and insights to advance evidence-based clinical practice and to inform early healthcare interventions for at-risk infants and children.

Limitations

The focus on prospective exposure and measurement of protective care interventions requires the setup of an integrated multi-professional research team and an accurate standardization of the intervention. At greater degrees of methodological control of confounders, more than one group of subjects is needed and infants/children included in the groups need to be matched for a number of socio-demographic, clinical and task-relevant variables. At lower degrees of control and randomization, only one group can be included, but less robust interpretations are supported.

Type 6: Prospective Assessment of Adversity-Related Epigenetic Variations on Longitudinal Outcomes With Concurrent Exposure to Protective Care

Description

Many variants of Type 4 and 5 study designs can be developed to include an integrated assessment of both adversity and protective care exposures into a DHBE research project. One of this variant presents the inclusion of a concurrent assessment of a specific protective exposure at T_3—together with the target outcome

measurement—increasing the complexity of Type 4 study design (see **Figure 2**, Type 6).

Example

Preterm birth and its consequences in terms of early hospitalization and developmental outcomes represent an elite condition for designing Type 6 research projects. Using this kind of methodological architecture, Provenzi and colleagues (Provenzi et al., 2017b) assessed the moderation role of maternal sensitive interactive behavior during a face-to-face interaction (T_3) with full-term and preterm infants on the association between NICU-related (T_1) variations in *SLC6A4* methylation (T_0 and T_2) and infants' behavioral stress regulation (i.e., negative emotionality; T_3) at 3 months of age (corrected for prematurity). Notably, this study suggested that a significant moderation emerged only for full-term infants, in which mothers rated as highly sensitive appeared to protect their infants from the positive association between high *SLC6A4* methylation and heightened negative emotionality. By converse, no significant moderation emerged for preterm infants, indirectly supporting the need of DHBE research focused on an integrated investigation of NICU-related adversity and specific early interventions occurring well before outcomes' assessment (see Type 7 below).

Advantages

Type 6 study design combine the benefits of a prospective and longitudinal research project reported above (see Types 4 and 5) with the opportunity to appreciate the role of protective factors as post-adversity buffering mechanisms on developmental outcomes. This methodology is highly indicated and potentially insightful for areas of clinically applied research in which high individual variability is documented as a consequence of early stress exposure. In other words, this study can reveal the biological pathways that contribute to the fact that some individuals seem to cope adequately and develop well in the face of a developmental history of adversities, whereas others do not.

Limitations

Despite the many advantages of this study design, two major flaws should be highlighted. First, this kind of research can be applied only to those human developmental conditions that are inherently distressful and in which infants or children are naturally exposed to stressful environments (see Type 4 limitations). Second, the concurrent evaluation of outcomes and care variables do not allow researchers to assess the independent role of protective factors as contributors to epigenetic regulation.

Type 7: Prospective and Sequential Assessment of Adversity- and Care-Related Epigenetic Variations on Longitudinal Outcomes

Description

This is probably the most complex study design within the landscape of DHBE research, as at least seven time-points are included and complex relationship among exposures, epigenetic variations and outcomes are addressed (see **Figure 2**, Type 7). Epigenetic assessments occur before (T_0) and after (T_2) an adverse event exposure (T_1). After that, protective care intervention is promoted and quantified prospectively (T_3) and a third epigenetic assessment occurs after that (T_4). At least one post-intervention time-point is included for follow-up evaluation of the developmental outcomes (T_5). Unfortunately, to the best of our knowledge, there is no evidence of such research projects in the available DHBE literature.

Advantages

The sequential order of exposures and epigenetic as well as outcome assessments allows researchers to develop specific and methodologically sound hypotheses that can tested and falsified with a modular approach. These can include simple hypotheses regarding two or three subsequent time-points as well as more complex modeling hypotheses including the integrated assessment of different environmental exposures, epigenetic variations and outcomes. The assessment of the competitive, additive or independent effects of adversity and protective care on the same targets of epigenetic variations and developmental outcomes is a specific plus of this study design which cannot be pursued with other methodological architectures.

Limitations

Whereas this study design maximizes the benefits at the methodological level, it should also be clear that resource costs, the risk for longitudinal sample attrition and the involvement of a multi-professional team of researchers and clinician imposes relevant challenges so that risks and potential solutions need to be carefully planned before and revised during the research project. At the same time, these research projects can be only applied to naturally occurring adversities for which protective care interventions are already available and provided some levels of evidence for the specific selected developmental outcomes. From this point of view, research on preterm behavioral epigenetics (PBE) still represents an elite field of applied research for DHBE (Maddalena, 2013; Provenzi and Barello, 2015). Nonetheless, it should be noted that a simplified version of Type 7 study design can be also suggested, in which a mixed retrospective assessment of adversity exposure and a prospective evaluation of care-related epigenetic variation together their effects on developmental outcomes of are featured. In this mixed design, $T_{0\text{-to-}2}$ would be collapsed into one single retrospective assessment, reducing the interpretive potentials of the model, but allowing its application to many different adverse conditions and reducing the risk of longitudinal sample attrition.

DISCUSSION

DHBE is one of the most promising and rapidly growing areas of developmental science in human psychobiological research field. These studies are well-recognized as holding promises of advancing our comprehension of the biological

mechanisms underlying the association between environmental exposures and the developmental phenotype (Szyf et al., 2008; Griffiths and Hunter, 2014). Additionally, the growing body of information on the epigenetic targets and markers of early environmental exposures—for bad and for good—is going to inform smarter and effective evidence-based clinical practice for infants and children who present genetic and/or environmental risk factors (Bianco-Miotto et al., 2017; Murgatroyd and Spengler, 2011; Notterman and Mitchell, 2015). Nonetheless, DHBE presents specific challenges and issues, when it comes to make methodological choices and produce a robust study design. In the present contribution, we have proposed several different prototypical and didactically organized study designs to cope with these challenges.

One major distinction among different methodological approaches regards the use of retrospective or prospective measurements of environmental exposures, either adversities or protective conditions. Retrospective approaches clearly have the advantage of being applicable to a number of adverse conditions which can be faced by human beings and that cannot be experimentally induced or manipulated (e.g., abuse and maltreatment; Beach et al., 2013; Parent et al., 2017). Moreover, retrospective study designs represent a compromise solution that balances the amount of resources needed to the research project and the strength of methodological procedures and robustness of findings. Indeed, retrospective designs are usually correlational and no reliable interpretation can be supported for what pertains predictive effects and the direction of potential significant associations. As such, these study designs are better suited for exploratory research projects and to test innovative and previously uninvestigated hypotheses that hold the potential to pave the way for more research in the field.

By converse, prospective approaches usually implies more complex study designs which increase the robustness of findings' interpretation, allow the formulation of more specific hypotheses on the direction of predictive associations and effects. At the same time, they imply new challenges such as the high risk of sample attrition and subjects' loss and the careful planning of multiple research sessions within specific time frames. Prospective study designs are highly indicated when environmental conditions—for bad and for good—can be longitudinally followed-up, monitored and quantified and/or when greater opportunities for experimental manipulation and control is granted. For instance, this is the case of PBE research (Provenzi et al., 2018a), in which the early exposure to adverse conditions (e.g., NICU-related pain-inducing procedures) and care interventions (e.g., skin-to-skin contact support) are expected and can be easily measured on a daily basis for a relatively long period (Provenzi et al., 2015; Montirosso et al., 2016a). Prospective DHBE studies are particularly well suited for the investigation of specific hypotheses regarding the predictive effects of adversities and protective care interventions on target epigenetic regulation.

Another major difference between retrospective and prospective study designs regards the kind of epigenetic indexes that can be obtained. On the one hand, retrospective and cross-sectional study designs provide the context for the assay of punctual epigenetic markers, which can be considered as potential endophenotype associated with specific environmental exposures (Radley et al., 2011). On the other hand, prospective research allows the measurements of epigenetic variations from pre- to post-exposure, which further increases the possibility to provide stronger interpretations on the direction of the effects and supports the development of more complex mapping of the reciprocal interactions among environmental conditions, epigenetic regulation and developmental outcomes. In other words, while retrospective approaches to DHBE provide photograph-like accounts of the relationships among environmental exposures, epigenetic markers and individuals' phenotype, prospective study designs provide the methodological requisites to depict a movie-like systematic landscape of such relationships.

In light of considerations reported above, we would like to highlight specific implications for future studies in DHBE research field. First, a more explicit account of the limitations of the adopted study design and their advantages and disadvantages for direct and indirect implications and interpretations should be provided to readers in DHBE studies. As previously suggested, the "seductive allure" (Miller, 2010) of BE research and its application to human developmental science might pave the way for misinterpretations in the absence of scientifically sound supervised and guided readership (Richardson et al., 2014; Provenzi and Montirosso, 2015). Second, as more and more DHBE research get published in the scientific community, there is a need to provide systematic accounts of this rapidly growing body of evidence, to weight them and to highlight the gray areas of under-investigated yet clinically relevant areas of BE applications for human developmental research. For example, the imbalance between DHBE research focused on adversities and on protective care exposures is evident and more efforts should be devoted to care-related DHBE studies in the years to come. Third, in the face of the described challenges inherent to the application of BE research principles to the study of human development, more direct and frequent contacts between animal model and human epigenetics research should be pursued. Investing in this multi-professional communities of BE researchers is warranted to provide a more systematic account and robust rationale for the applied and basic evidence that can support our improved knowledge of developmental mechanisms and our capacity to provide smarter and more effective early interventions in at-risk conditions. Additionally, it should be highlighted that the use of peripheral tissues for measuring DNA methylation variations is the only available proxy in DHBE, whereas epigenetic research on animal models rely on central nervous system tissues. Recent research reported on partial correspondence in DNA methylation measured from peripheral blood and saliva (Thompson et al., 2013; Staunstrup et al., 2017), but still methylation status appears to be tissue- (Smith et al., 2015; Forest et al., 2018) and gene-specific (Di Sante et al., 2018). Also, Walton et al. (2016) reported only 7.9% significant correlation between methylation measures in blood and brain tissue obtained in

post-mortem patients: despite this proportion was greater than predicted by chance, it is still a small proportion. As such, a direct translation of findings from animal model studies to humans is discouraged, in absence of multiple evidence and cross-tissue reports (Lester et al., 2011). Finally, as previous DHBE research mainly focused on DNA methylation, in this article we limited our discussion and examples to this epigenetic mechanism. Nonetheless, it should be noted that DNA methylation can have direct and indirect effects of the transcription machinery (Curradi et al., 2002). Moreover, other epigenetic mechanisms have been documented in associations with early adversities, at least in animal models (e.g., histone regulation, Cittaro et al., 2016; non-coding RNA, Daskalakis et al., 2018; telomere length regulation, Provenzi et al., 2018b) and might interact with each other in contributing to establish chromatin availability to transcription and gene silencing (O'Leary et al., 2017).

In sum, it should be highlighted that when it comes to apply BE principles to human developmental science, there is no "best approach" and that the balance between benefits and challenges should be considered for each specific research project based on a conjoint evaluation of researchers' aims, objects of investigation and available resources. Although non-exhaustive, this article provides a wide overview of study designs and offers methodological support and guidelines to researchers who are interested in integrating BE methods and investigation in their developmental science research. A well-designed research program is key to develop robust methodology for future DHBE studies that can be beneficial for increasing and deepening our knowledge of developmental mechanisms and inform early interventions for at-risk infants and children.

AUTHOR CONTRIBUTIONS

LP conceived the idea. LP and MB drafted the manuscript. RM and RB critically revised the manuscript for important intellectual content. All authors gave final approval of the final version and are accountable for this work. The corresponding author attests that all listed authors met authorship criteria and that no others meeting the criteria have been omitted.

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Socio-Emotional and Cognitive Development in Intrauterine Growth Restricted (IUGR) and Typical Development Infants: Early Interactive Patterns and Underlying Neural Correlates. Rationale and Methods of the Study

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Intrauterine growth restriction (IUGR) is defined as a fetal growth retardation, resulting in an estimated fetal weight less than the 10th centile for gestational age. IUGR developing brain is affected by the atypical fetal growth, presenting altered structure and connectivity and increased risk for neurodevelopmental impairments. Behaviorally, IUGR infants show reduced responsiveness and engagement with human faces during mother-child exchanges. The neural mechanisms of these patterns of interactions remain unexplored, as well as their potential role in shaping socio-cognitive trajectories of development. Aim of this research project will be to longitudinally investigate mother-infant interactions and infant's event-related potential (ERP) components of face processing (infant N170, P400, Negative central) in 4 and 9 months IUGR as potential early markers of expected atypical cognitive and behavioral outcomes observed at 12 months. Thirty IUGR participants will be recruited after receiving the *in utero* diagnosis (>28th gestational week). Thirty healthy infants will be enrolled as the control group. Maternal environment will be assessed via Emotional Availability Scales (EASs), with child responsiveness and maternal sensitivity as variables of interest. Infants' scalp-recorded cortical activity in response to social and non-social stimuli will be investigated using a high-density EEG system (EGI Geodesic system). Neurodevelopment will be measured at 12 months of child's life, using Bayley Scales for Infant Development (BSID), while the possible presence of emotional-behavioral problems will be rated via Child Behavior Checklist (CBCL). We expect that being IUGR significantly affects cognitive and behavioral outcomes, through mediation effects of both infants' neural and behavioral capacity to respond to social stimuli. Indeed, we expect an altered response to social stimuli in IUGR infants, resulting in smaller ERP components amplitude in response to

human faces compared to healthy matched peers. A significant association between neural response to social stimuli and infants' responsiveness to maternal stimulation during interactions is expected, with impoverished performances on the interactive domain in IUGR, compared to healthy peers. This study will enhance understanding on neural mechanisms underpinning the interactive patterns sustaining socio-cognitive development in IUGR and healthy infants. The study will help in clarifying the role of postnatal environment in buffering the vulnerability experienced by children delayed in their fetal growth.

Keywords: intrauterine stress, fetal growth restriction, face processing, socio-cognitive development, mother-child interactions

INTRODUCTION

Intrauterine growth restriction (IUGR) is defined as a fetal growth retardation, resulting in an estimated fetal weight (postnatally confirmed by birth weight) on the lowest 10th percentile for gestational age (Alfirevic and Neilson, 1993). By affecting 5% to 7% of pregnancies, IUGR is the second leading cause of perinatal mortality and morbidity worldwide, representing a major public health problem (Murray et al., 2015). A fetal growth unable to reach its genetic potential is a risk factor for later neurodevelopmental outcomes (Kok et al., 2007; Baschat, 2011). In fact, functional impairments have been observed at birth and early in life; specifically, immature attention-interaction scores and impaired visual recognition memory performances are described in 7-month-old infants (Gotlieb et al., 1988; Tolsa et al., 2004), while at 1 year of life, significantly lower scores on Bayley Scales are reported (Fernandez-Carrocera et al., 2003; Batalle et al., 2013). Moreover, growth-restricted infants show poor use of environmental stimuli, reduced social responsiveness, more insulated cry states, and poor motor performance as compared with normal birth weight infants (Padilla et al., 2011). Persisting and long-term outcomes are also observed, with cognitive impairments (e.g., executive functioning; Geva et al., 2006) and behavioral problems described in childhood (Sung et al., 1993); motor problems, learning difficulties and lower academic achievements during school age period (Leitner et al., 2007; Esteban et al., 2010), as well as increased risk for neurodevelopmental disorders, such as ADHD (Heinonen et al., 2013). Apart from evidence of neurodevelopmental and cognitive outcomes, socio-emotional development still appears as unexplored in the developmental context of growth restriction, although few signs of early atypical social interactions are described (Watt, 1990; Feldman and Eidelman, 2009), as well as later poor socio-cognitive performances at school age and mood disorders (Fischi-Gómez et al., 2015).

Literature evidenced several structural and functional brain abnormalities potentially linking fetal growth rate to the detrimental neurodevelopmental and socio-cognitive outcomes. Indeed, IUGR infants show reduced brain volumes as well as delayed and diminished myelination (Dubois et al., 2008; Padilla et al., 2011; Ramenghi et al., 2011). The alterations seem to persist in long term deficits, since motor and cortico-striatal-thalamic networks impairments are observed in 6 years old IUGR children, and delayed myelination as well as disrupted white matter integrity last up to adulthood (Fischi-Gómez et al., 2015). Despite this evidence, the early neural mechanisms sustaining the socio-emotional competencies and the socio-cognitive development in IUGR infants are still underexplored. However, it is of the highest importance to provide comprehension on early markers, both in terms of behavioral and brain mechanisms, of the developmental cascade that begins with early fetal abnormal experience and might potentially result in socio-emotional difficulties and neurodevelopmental outcomes observed later in life. Indeed, in a preventive perspective, targeting a potential early vulnerability in IUGR development, particularly when born at term, could be highly convenient and rewarding. Despite literature extensively reports altered quality in antenatal environment, quite few studies investigated IUGR developmental trajectories, thus neglecting the opportunity to tailor interventions and to develop *ad hoc* follow up mental health care. In addition, the urgency for identifying potential targets for interventions should consider a multifaceted approach, where infant and caregiver are parts of a mutually influencing complex and interrelated system (Sacchi et al., 2018). Taking into account infant and mother's variables, different potential mechanisms to target could arise from this study. First, detecting vulnerability in processing social stimuli might guide behavioral intervention sustaining parenting abilities to use multi-modal channels of stimulations during interactions. Second, fostering a protective effect of parenting behavior on brain functionality would potentially have an effect on infant socio-emotional development, hopefully compensating for the suspected reduced early communication abilities of IUGR infants. Third, a longitudinal investigation would allow to study different potential windows of plasticity both for typical and atypical development. This could lead to more focused interventions aware of the most susceptible periods and the most rewarding processes to target.

With this theoretical and clinical perspective in mind, we propose a longitudinal investigation of two interrelated mechanisms that might be detected across the first year of life as early markers of potential atypical socio-emotional and cognitive outcomes of IUGR developmental trajectories: infant behavioral responsiveness in social interaction and infant early neural face processing.

Infant Behavioral Responsiveness in Social Interaction

Within the first year of life, typically developing infants display an amazingly sophisticated set of social behaviors, which foster learning processes in a broad collection of developmental domains (McDonald and Perdue, 2018). These socio-emotional competencies involve the abilities to interact, communicate and deal with emotions, which are primarily experienced in early interactive exchanges with the mother (Bowlby, 1978). Within this affectionate bond, the child receives not only protection, care and the recognition of his/her needs, but also an encompassing environment for physical, cognitive, social and affective development (Britto et al., 2017). Indeed, the mutuality of exchanges between mother and child represents not only a source of stimuli for the child but also an environment sensitive to activities and modifications (van den Bloom and Hoeksma, 1994). In the case of atypical development, infant characteristics can deeply expose the quality of mother child interactions (Kiff et al., 2011). Indeed, the few available studies evidenced that IUGR infants are likely to display difficulties in orientating to social and non-social environment (Watt, 1990) and tend to look at people less frequently than age-matched healthy infants; also, higher levels of negative affect are reported, evidencing an early vulnerability in communication skills (Watt and Strongman, 1985; Watt, 1987). As regards, interactive abilities, the very limited findings on IUGR or small for gestational age (SGA) children suggest those infants are more passive during mother-child interactive exchanges, smiling and looking at their mothers' face less than normal birth weight matched newborns, being less rhythmic and synchronous in daily interactions (Feldman and Eidelman, 2006), and thus appearing as less rewarding interactive partners. A similar interactive pattern is displayed in preterm infants and their mothers, where a scarcity of communicative signals on infant's side could activate some compensatory behaviors in parents (Miles and Holditch-Davis, 1995; Montirosso et al., 2017). This parenting response can be highly adaptive but can eventually result into intrusive and non-attuned behaviors (Howe et al., 2016). Therefore, on the one hand, atypical development in the domain of diminished early communicative abilities can disrupt mother child exchanges leading to an additional impoverishment of infant's environment. On the other hand, the quality of mother-child interactions can potentially buffer the negative effect of infant scarce interactive abilities on child development (Baker et al., 2007). In fact, some evidences show the moderating role of maternal sensitivity on infant developmental trajectories. More specifically, recent evidences show the role of mother child interactions on infant's brain functionality, confirming the relevance of considering mother and child as a broad interrelated system where infant development takes place.

Infant Neural Face Processing

Among early neural competences displayed by newborn and infants, the ability to recognize and direct the attention to faces appear as highly relevant for socio-cognitive development. Face perception represents an experience-expectant and activity-dependent function (Nelson, 2001; Young et al., 2017); that is critical in the development of higher level social and cognitive functions (Parker and Nelson, 2005). Indeed, the human face provides the infant with a wealth of socially and affectively relevant information and humans appear to be inherently interested in faces, displaying from infancy a strong interest in facial-like figures (Johnson, 1991; Morton and Johnson, 1991). Early disruption or delay in this low-level process can negatively impact infant's ability to interact with the social environment (Elsabbagh et al., 2015) and disturb natural mutuality in social interaction with potential detrimental effects for child development (Wan et al., 2013). Indeed, huge part of early interactive exchanges rely on the use of face and facial expression are early used to understand others emotion and thought, to make others understand themselves, and to share emotional states (Beebe et al., 2010, 2016). Studies observed that different early stressors and risk factors, such as prolonged institutionalization or risk for autism, are likely to affect this infant capacity that is considered a strong candidate for being one of the mechanisms of the association between early stress and socio-emotional difficulties (Nelson and McCleery, 2008). More specifically, Parker and Nelson (2005) found that the amplitude of the event-related potential (ERP) responses to familiar and unknown faces were lower in institutionalized children, while Swingler et al. (2010) found ERP latencies to be associated with infant behavioral response to maternal separation. Mesquita et al. (2015) showed altered ERP components magnitude in response to faces in children with atypical social behaviors and recently (Kungl et al., 2017) found an association between attachment security and face brain responses. In addition, recent studies show that in healthy children the quality of the maternal environment is related to the magnitude of ERP components in response to emotional faces (Carlsson et al., 2008; Taylor-Colls and Pasco Fearon, 2015), confirming the association between early interactive experience and brain development of face perception. As a consequence, it is possible that an early dysfunction of the relevant circuitry of neural face processing could affect the quality of the interactions, and probably also decrease the quality of the child environment, contributing to activate a negative developmental pathway. Up to date limited information is available on how infants process and respond to social stimuli in early at-risk conditions. In particular, no study investigated whether early human face processing is susceptible to antenatal growth and/or might be affected by fetal growth restriction. Indeed, in the study of IUGR, researches are needed in order to ensure that facial processing is not compromised by their antenatal adversities slowing down the fetal growth. In fact, in the light of studies on clinical groups (Parker and Nelson, 2005; Nelson and McCleery, 2008), it appears as of highest clinical importance to understand the role of early adversities on neural face processing and how altered face processing could be conceived as early marched on possible risk on socio-emotional development.

With the aim of bridging the above-described research focuses and objectives, the present study protocol attempts to open

a new research perspective on early development of IUGR infants, following their interactive and neural developmental pathways across the first year of life. By comparing IUGR with healthy children, we study the effect of antenatal adversity on brain functionality and interactive abilities. Specifically, aim of the study will be to investigate whether growth restriction significantly affect socio-cognitive developmental at 12 months both directly and thought the mediation of behavioral and neural response to social stimuli as displayed at 4 and 9 months. In particular, mediation hypotheses cover the following pathways:

- *Infant behavioral responsiveness in social interaction*: since studies on IUGR population support IUGR infants' greater passivity, communicative difficulties in early mother-child exchanges and an early disinclination to be engaged by human faces, we investigate a group difference (IUGR—Control), expecting IUGR lower levels of responsivity to maternal stimulation during free-play exchanges. Worse communicative abilities in the IUGR group can lead to an impoverished environment for the infant and therefore fewer opportunities for stimulation and learning. In turn, this could affect the cognitive development and therefore could represent a mechanism linking the stress experienced during intrauterine life to later adaptation.
- *Infant neural social processing*: many evidences showed that neural competence in face processing is significantly altered in clinical populations, advocating a likely role of this neural domain in sustaining and worsening the effects of early stress on child development. Since research on IUGR show their difficulties in engaging with faces and social situations, we suggest a potential role for face processing neural correlates in the association between antenatal growth restriction and cognitive outcomes. Therefore, we aim to explore the role of the scalp-recorded cortical activity, in terms of ERPs, in response to social and non-social stimuli in IUGR and non IUGR infants. The following ERP components will be selected in accordance with current evidence of the literature on infants' face processing—i.e., the infant N170 at around 290 ms after the stimulus onset and P400 (de Haan and Nelson, 1999; de Haan et al., 2003; Moulson et al., 2009), and emotional/attentional processing—i.e., Negative central (Nc; Moulson et al., 2009; Taylor-Colls and Pasco Fearon, 2015). Indeed, by considering several ERP components, the potential role of intrauterine growth adversity on social processing will be linked to specific features of neural processing. Specifically, we expect to find reduced amplitude (N170, Nc) and latency (P400) in the IUGR group. In addition, we hypothesized that these alterations in brain functionality in response to human faces can represent an early marker of the later cognitive deficit of IUGR infants, therefore suggesting a mediation effect.

Then, cortical response to social stimuli will be investigated as possible underpinnings of reduced responsiveness to maternal environment in IUGR infants, compared to matched healthy controls. Therefore, positive correlations will be expected, highlighting this link.

Last, along with the mediation roles expected for child behavioral and neural social responsivity, maternal environment, in terms of sensitivity, will be investigated at an explorative level as exerting a moderating role in the association between infant responsivity on cognitive and behavioral development assessed at 12th months. Indeed, although no specific evidence suggests that maternal sensitivity can modulate the developmental trajectories in IUGR samples, this effect has been shown in other at-risk population such as premature infants. Therefore, we aim at considering moderator effects in order to detect potential buffering or detrimental roles of maternal environment in infants experiencing fetal stress.

Overall, research design and hypotheses are graphically summarized in **Figure 1**.

METHOD

Participants

For the IUGR group, 30 pregnant women will be recruited at the Department of Women's and Child's Health, University of Padova (Italy). Healthy control pregnant mothers ($N = 30$) will be recruited from birth-preparation courses of the Obstetrics and Gynecological Clinic of Padua Hospital. All pregnant women are orally presented with a longitudinal study on the role of IUGR on child socio-emotional development by a Gynecologist and a Psychologist, while waiting their visit or the birth-preparation class. For mothers with pregnancy complicated by IUGR; research will be proposed at the first obstetrical visit following diagnosis. All mothers interested in the study will receive a detailed informative module, describing stages and tasks on the study. In particular, they will be informed on the study length and procedures; absence of risk for both behavioral and EEG assessment is declared, and a potential tolerable level of discomfort is reported for the EEG cup wearing procedure. Participants will also be informed on the possibility to withdraw their participation at any time without giving an explanation and that their decision would not affect future healthcare encounters. In accordance with the Declaration of Helsinki, prior to first assessment, parents agreeing to be involved in the present study will sign written informed consent. Mothers will sign an informed consent as participants; while two other different consent forms are required to be signed by both parents for infant's participation, namely in the behavioral and neuroimaging assessments. The present study received ethical approval from the Ethic Committee of the University of Padua (protocol reference number: 2293).

Participants will be mother-infant dyads who received *in utero* IUGR diagnosis (verified by Doppler ultrasound and estimated birth weight below the 10th percentile of growth), confirmed by birth weight below the 10th percentile of growth curve. Infants exclusion criteria will be: genetic disorders, unrelated comorbidities, presence of fetal infections, congenital malformations (i.e., congenital heart disease), metabolic and chromosomal disorders at birth, as well

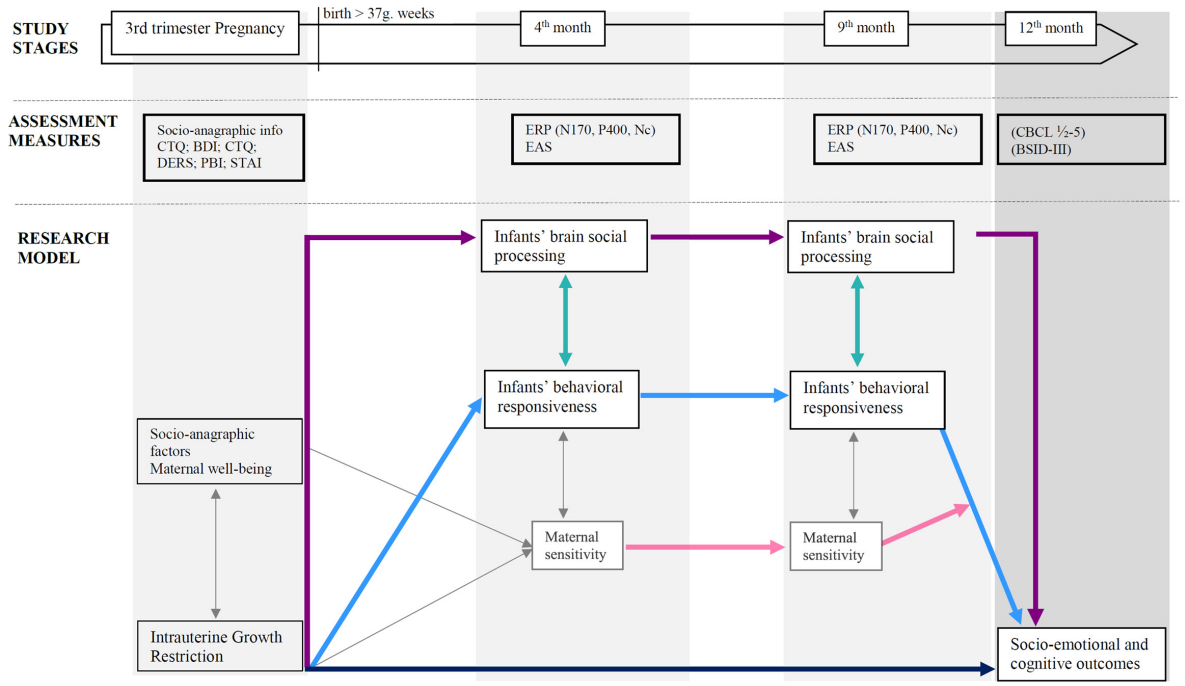

FIGURE 1 | Research design and hypothesized associations between variables. Colored arrows depict research hypotheses; gray thin arrows refer to expected paths not considered in this study protocol for sake of brevity. *Note*: PBI, Parental Bonding Instrument (Parker, 1989); CTQ, Childhood Trauma Questionnaire (Bernstein et al., 2003); BDI-II, Beck Depression Inventory-II (Beck et al., 1996); STAI, State Trait Anxiety Inventory (Spielberger, 1983); DERS, Difficulties in Emotion Regulation (Scale Gratz and Roemer, 2004); ERPs, Event-related potentials, EASs, Emotional Availability Scales (Biringen, 2008); CBCL ½-5, Child Behavior Checklist ½-5 (Achenbach and Rescorla, 2000); BSID-III, Bayley Scales for Infant and Toddler Development—Third Version (Bayley, 2006).

as infant neurological pathologies, brain abnormalities, or preterm delivery (<37th gestational week). Mothers' exclusion criteria will be IUGR diagnosis before the 7th month of pregnancy and complicated pregnancies, non-Italian nationality, mother age <18 years, psychiatric disorders' risk as defined by clinical score (namely a Global Symptom Index >65) in the Symptom Checklist 90-Revised (Derogatis, 1975), neurocognitive disorders, drug addiction, single mothers.

Procedure

This project describes a longitudinal research, articulated in four stages over the first year of the child's life. During recruitment at the pregnancy stage demographical information will be collected through a detailed paper-and-pencil evaluation. Indeed, an *ad hoc* socio-demographic assessment has been designed in order to collect comprehensive information about maternal age, cohabitation, marital status, education, work, parity and presence of previous abortion or at-risk pregnancies.

In addition, psycho-social and clinical-psychological status of the mother and of the whole family system will be assessed, by applying the following self-report questionnaires: Childhood Trauma Questionnaire (CTQ)—Short Form (Bernstein et al., 2003; Sacchi et al., 2017); Parental Bonding Instrument (PBI; Parker, 1989); Difficulties in Emotion Regulation Scale (DERS; Gratz and Roemer, 2004); State Trait Anxiety Inventory (STAI; Spielberger, 1983); Beck Depression Inventory-II (BDI-II Beck et al., 1996).

After recruitment and the first assessment during pregnancy taking place at the Hospital, all participants agreeing to take part into the study will be telephonically contacted at the 4th month of child's life for behavioral and neuroimaging assessment. In particular, mother-child couples will be invited to visit the Department of Developmental Psychology and Socialization at the University of Padova. At 4 and 9 months, assessment procedure will involve free play interactions and EEG recording at the Inter-departmental High-density EEG lab; while at 12 months, developmental outcomes will be measured with children assessed on cognitive development by a structured procedure performed by trained psychologist, and emotional-behavioral problems rated by mothers.

Cognitive and Socio-Emotional Development at 12 Months of Life

Cognitive assessment will be performed at 12 months of child's life using Bayley Scales for Infant Development—Third Version (BSID—III; Bayley, 2006); which evaluates five different domains: cognitive, language, motor, socio-emotional behavior and adaptive behavior. Evaluation of the first three domains consist of a direct observation of the child performance on different task, while socio-emotional and adaptive behaviors are parent rated. For direct assessment, each item is assessed on a dichotomous scale, with 1 given to child's ability

to perform the targeted behavior and 0 to the absence of such behavior. After five consecutive missing behaviors the scale's administration is interrupted. Cognitive scale is composed by 91 items assessing: sensorimotor development, exploration and manipulation, object relatedness, concept formation, and memory. Language scale is composed by 49 items referring to receptive communication (i.e., pre-verbal behavior, vocabulary development, morphological development, understanding morphological markers, social referencing and verbal comprehension), and 48 items assessing pre-verbal communications (i.e., vocabulary development and morpho-syntactic development). Motor scale examines fine motor and gross motor domains. In particular, fine motor subtest is composed by 66 items about: prehension, perceptual-motor integration, motor planning and speed, visual tracking, reaching, object grasping, object manipulation, functional hand skills, responses to tactile information. Gross motor subtest refers to 72 items covering movement of the limbs and torso, static positioning (e.g., sitting, standing), dynamic movement (including locomotion and coordination), balance, and motor planning.

Socio-emotional scale represents and adaptation from the Greenspan social-emotional growth chart (Greenspan, 2004) assessing child self-regulation, communicating needs, the ability to establish relationship and the use of emotions for interactive purposes or to solve problems. Last, Adaptive Behavior assessment refers to child's social, motor, pre-academics, home living, self-care, self-direction, community use, leisure, communication, health and safety skills.

Each of the 5 scales provide a raw score, and a scaled score ($M = 10$, $SD = 3$). For Cognitive, language and motor scales also allow to compute composite scores, referred to a mean value of 100 and a standard deviation of 15. Composite scores lower than 85 were considered as abnormal performances (Albers and Grieve, 2007). Examinations will be performed by a trained psychologist with enduring experience in the BSID-III.

Socio-emotional development will be parent rated via Child Behavior Checklist ½-5 (CBCL ½-5; Achenbach and Rescorla, 2000), a checklist of 113 questions, scored on a three-point Likert scale (0 = Not True, 1 = Somewhat or Sometimes True, 2 = Very True or Often True, based on the past 6 months). CBCL provides scores for eight syndromes, three broadband domains (Internalizing, Externalizing, and Total Problems), and six DSM-oriented scales. Although CBCL was originally designed for child assessment from 18 months of age, previous studies showed its good psychometrical properties with 12 months infant and encouraged its downward extension (Van Zeijl et al., 2006a,b; Ramchandani et al., 2013).

Child Responsiveness and Maternal Sensitivity

Mother-child interactions will be video-recorded during free-play interactive exchanges lasting about 15 min. At this purpose, a quiet and silent room will be equipped with a kid rug, pillows and age-appropriate toys; namely: rattles, puppies, and soft activity books for 4 months infants; pop-up surprise box, soft telephone, blocks box, activity book, and rock-a-stuck for 9 months. Mothers will be instructed to freely interact with their baby as they are used to do at home; they are kindly asked to remain within camera focus, unless their baby show signs of distress and need to calm them down.

Emotional Availability Scales (EASs; Biringen, 2008) will be applied to code interactive behaviors following the coding system of the EA Third Edition. EAS constitutes of four parental dimensions: adult sensitivity, adult structuring, adult non-intrusiveness, adult non-hostility; and two child scales: child responsiveness and child involvement. Each EA dimension produces score on a 7-point scale, where higher ratings stand for more optimal features. Values between 5 and 7 are representative of an emotionally available dyad and considered index of a healthy relationship. Scores around 4 indicate complicated emotional availability, that is behaviors that are appropriate in some ways but that are not optimal. Scores around 3 indicate less optimal aspects while the range between 1 and 2 concerns more problematic behaviors (Biringen, 2008). According to EAS Third Edition, the 6 scales can also be scored on seven subscales each; this allows to observe and detect specific behaviors composing the six macro-categories. Among the six dimensions, Adult sensitivity and Child responsiveness will be selected for the purposes of the present study. Indeed, maternal sensitivity represents an early indicator of the quality of infant's postnatal social environment. Child responsiveness will be selected as behavioral correlates of child's early responsiveness to social stimuli, investigated as cortical response. Video-recorded interactions will be coded by two independent judges, trained on the EAS system, who will be blind with respect to objectives and design of the study.

EEG Recording, Signal Processing and ERP Components Selection

Infants' cortical activity will be continuously recorded using a Geodesic EEG system (EGI) through a pre-cabled high-density 128-channel HydroCel Geodesic Sensor Net (HCGSN-128) referenced to the vertex. While infants are placed on their mother's legs in front of a screen at about 50 cm of distance, brain activity will be registered, through the use of the elastic sensor nets fitting each participant's head size. Each electrode channel of the net is enveloped by a sponge and protected by a soft, plastic pedestal; this guarantee participants' skin contact is only with sponge and plastic parts. Before assembly, Sensor Net is immersed in a shampoo, potassium chloride and distilled water solution for 5 min. After disassembly, all the non-disposable material used during the experiment (net, electrodes), is always disinfected before a subsequent re-use.

The electrophysiological data collection will last about 30 min per each infant, including equipment assembly and disassembly; also, to maximize infant comfort, skin pressure points and overturned sensors are checked before data acquisition, in accordance with EGI recommendations. While seating on mothers' legs in the overshadowed room, both social and non-social stimuli will be presented. Specifically, the experimental paradigm employed will be adapted from a previous study (Mento and Valenza, 2016), and will involve the use of real female faces as social stimuli. Images of unfamiliar toys will be selected as the visual non-social stimuli. A total of

100 trials per condition will be delivered. During the procedure, infants' behavior will be continuously monitored via a video camera, in order to allow the experimenter to decide when deliver on the screen attention-getter audio-visual stimuli (cartoon scenes) as soon as infants attention on the screen will be loose. The electrical signal will be filtered with a 0.1-Hz to 100-Hz band-pass with a sampling rate of 500 Hz.

Consistent with previous studies on face and emotion processing in infants (de Haan and Nelson, 1999; de Haan et al., 2003; Taylor-Colls and Pasco Fearon, 2015; Guy et al., 2016), component timings will be selected as follows: the infant N170 component will be selected as the early correlate of specialized face processing in infants (infant 170), reflecting structural features of face processing. This component has been consistently shown to exhibit greater amplitude in response to faces as compared to visual noise in 3 month-old infants (Halit et al., 2004) and also to familial vs. non-familial faces at 9 months (Scott et al., 2006). The infant N170 will be expected to peak negative in amplitude 290–350 ms after stimulus onset in posterior electrodes (de Haan et al., 2003). Second, the P400 will be considered as involved in high-order face processing; the P400 represents a positive component peaking between 390 ms and 450 ms after stimulus onset and maximal over occipital electrodes (de Haan et al., 2003). Last, the "Nc," component will be considered as relevant components of late face-processing (de Haan et al., 2003). The Nc will be defined as the negative EEG deflection occurring between 350 ms and 750 ms after stimulus onset over frontal and central midline electrodes (Guy et al., 2016). The Nc component is thought to reflect the activation of attentional processing linked to the appraisal of the motivational significance of emotional expressions (Taylor-Colls and Pasco Fearon, 2015).

The EEG recordings will be processed offline using MATLAB toolboxes EEGLAB and ERPLAB. EEG signal will be segmented into epochs beginning 100 ms before stimulus onset and ending 800 ms after. Prior to epoching procedure, videos will be visually inspected off-line in order to reject EEG segments where participants did not look at the screen. In order to identify, reject or correct bad channels, artifacts, eye blinks and eye movements, the Independent Component Analyses (Stone, 2002) will be applied on individual epoched EEG dataset. As the last step, data will be averaged and re-referenced to average reference. Only participants showing a minimum of 30 artifact-free trials per condition will be included in the grand average.

DATA ANALYSIS

To answer the first research question about the social stimuli processing in IUGR infants in terms of amplitude and latency of ERP components, analysis will involve repeated measure models, with group (IUGR vs. Controls) as between factor and developmental stage (4–9 months) and stimuli condition (social vs. non-social) as within factors. No previous study is available to obtain an estimate of the target effect size; however, we can refer to Parker and Nelson (2005) work on institutionalized children compared with non-institutionalized children to obtain an estimation of the effect of clinical conditions on ERP components in response to human faces. Even if it is unlikely that a perinatal condition such being IUGR is comparable with a complex relational stressor as being raised in an institution, this study can provide a rough estimation of the effect involved in the present protocol. Indeed, they found differences in N170, Nc, PSW and P250 amplitude between groups that range from intermediate to large. In the present study, considering the planned sample size, we should be able to obtain a 0.98 power to detect a small effect (repeated measures ANOVA within-between interaction, G*Power 3.1.9.2, Faul et al., 2009), which seems satisfactory in relation to the previous findings.

Second, to test the mediation effect of both neural response to social stimuli and behavioral child responsiveness on cognitive and neurodevelopment outcomes, Hayes approach will be followed (Hayes, 2013). The power to detect a direct an intermediate effect of IUGR condition on 12 months outcome is above 0.80 (difference between two independent means, G*Power 3.1.9.2). For what concerns indirect effects, mediation models have usually larger effect sizes than main effects (Kenny and Judd, 2014). Last, at a more explorative level a path analysis will be conducted to study the moderation role of maternal sensitivity in the previous mediation models. In particular, the moderation effect on the direct association between IUGR condition and later outcome as well as on the association between IUGR condition and child responsiveness will be explored.

EXPECTED RESULTS

For the developmental outcomes at 12 months, in line with previous studies (Fernandez-Carrocera et al., 2003; Batalle et al., 2013), we expect poorer cognitive and behavioral performances in IUGR infants, compared to control peers, as result of both a direct effect of being IUGR and a mediation of neural and behavioral responding to social stimuli.

On the behavioral domain, lower levels of child responsiveness during mother-child interactions are expected within the IUGR group, evidencing poorer behavioral responses to social stimuli, in accordance with evidence of IUGR greater passivity during social exchanges (Feldman and Eidelman, 2006). Then, significant positive correlations between ERPs amplitude for social stimuli and behavioral responsiveness to maternal stimulations are expected across groups, suggesting that early face processing might be conceived as a neural correlate of child responsiveness during mother-child interactive exchanges.

As regards the investigation of the neural mechanisms sustaining infants processing of social stimuli, temporal resolution given by the application of the EEG will allow to test the potential effect of being IUGR on different steps of face processing. Specifically, differences in infant N170 amplitude will allow to detect a potential role of being IUGR on basic structural features of face processing, while differences in P400 latency between groups, expected in the direction of longer latency for IUGR performances, will allow to detect an atypical IUGR processing regarding more complex steps of face processing. Last, between-groups difference in the Nc component will be tested in order to highlight atypical attention engagement in IUGR infants.

Considering in details the potential differences in ERP components in response to social and non-social conditions, we first expect faces to elicit greater amplitude in infant N170 and Nc and shorter latency in P400 than toys across groups (IUGR vs. Controls), in line with previous studies on infants face processing (Taylor-Colls and Pasco Fearon, 2015; Guy et al., 2016). Second, we expect that the prenatal stress experienced by IUGR infants results in smaller ERPs amplitude for social stimuli in the IUGR group, similarly to other at risk populations exposed to early adverse conditions, such as institutionalized children and young children with autism (Nelson and McCleery, 2008). Third, we expect an interaction effect Group × Condition, resulting in a reduced difference in amplitude between the social vs. non-social conditions for the IUGR group. Moreover, at an explorative level, the longitudinal design of the study will offer the opportunity to investigate whether neural social processing is susceptible to different pathways of specialization across groups (IUGR vs. Controls), as displayed by potential between-groups differences in neural face responses across steps (4 vs. 9 months). No specific results are expected, but a tendency toward stability across stages of the hypothesized detrimental effect of prenatal stress on neural face processing would suggest the presence of an atypical developmental trajectory for the IUGR population. On the contrary, a tendency toward a decreasing gap between groups would point toward considering face processing in IUGR as a stage-dependent mechanism limited in time, even if the potentially negative effect on long term outcomes could remain.

However, the limited knowledge in the functionality of IUGR brain in response to social stimuli does not ensure that group differences can be found in the hypothesized components or that they are located in the same brain regions of typically developed children. In this respect, subsequent exploratory analyses can enrich the quality of the investigation by means of data driven approach able to study the overall brain functionality (i.e., Maris, 2004).

Last, about the role of infants' postnatal environment, high maternal sensitivity, considered as a proxy of the overall maternal environment quality, is expected to buffer the effect of adversities in fetal growth on later developmental outcome by enhancing child's engagement and responsivity to social environment.

DISCUSSION

Recent approaches to the study of early brain development are shifting backward sensitive epochs, emphasizing the role of antenatal life and fetal growth. Framed in this context, Barker's (1998) hypothesis of fetal programming suggests that adverse influences during intrauterine life, such as growth restriction, can result in permanent long-term changes in physiology and metabolism, increasing the risk for adult diseases and health problems. The present study pursues the objective of broadening this research field providing new insights on the interconnected role of both antenatal and postnatal life on cognitive and emotional-behavioral development. In particular, results deriving from this research project will enhance understanding on early neural mechanisms underpinning the interactive-relational patterns sustaining socio-cognitive development in infants with IUGR. Indeed, this study represents a first contribution to understand whether antenatal stress in terms of fetal growth delay is likely to affect early neural competences of face processing and whether this capacity represents a neural correlate of altered behavioral-interactive development along the first year. In particular, the evidence of a role of intrauterine life experiences in affecting later face processing would enhance our understanding of the development of this fundamental ability and its experience-expectant and activity-dependent nature. In addition, the study will also help in clarifying the role of (prenatal and postnatal) mother-child exchanges in buffering the vulnerability experienced by children delayed in their fetal growth. Indeed, even if it is difficult to disentangle the direction of the effects, it is clinically relevant considering infant's face processing and environmental quality in the study of developmental trajectories of children experiencing early adversities, such as alterations in the antenatal growth. The present study also aims to develop the perspective proposed by Taylor-Colls and Pasco Fearon (2015) on the role of parental quality on infants' neural response to emotional faces, in which further studies on clinical and at-risk populations and longitudinal designs are claimed. However, in the study of IUGR, before considering the neural response to emotional cues, a step back is needed in order to ensure that facial processing is not compromised by the antenatal adversities that slow down the fetal growth. Therefore, the present study will provide a preliminary link, opening the way for further studies on early social processing in IUGR infants.

As a first attempt in the study of IUGR socio-emotional fragility, our protocol still presents some potential limitations. First, the aforementioned lack of knowledge on the specificity of brain functionality of IUGR infants does not ensure that ERP components can be found with the same localization and characteristics to be compared with typically developing children. Second, the selection criteria of excluding IUGR infants born before the 37 gestational week ensures a specific focus on the unique role of being IUGR as a source of antenatal stress, apart from the stress and physical pain experienced by premature infants after birth (Montirosso et al., 2018). However, future studies could explore potential differences between term IUGR and preterm IUGR, in order to disentangle the specific contribution of ante and post-natal stress in infant development. Third, IUGR disorder could result associated with highly severe maternal conditions during pregnancy such as infections, toxins, prescriptions drugs, substances abuse, that affect both intrauterine and postnatal environment (Brancato and Cannizzaro, 2018). In the present protocol, severe maternal conditions were excluded in order to study the specific effect of being IUGR, but future investigations with a similar methodology could consider whether IUGR is one of the mechanisms involved in the child detrimental outcomes of these maternal conditions.

CONCLUSION

In conclusion, very few is known on the effect of antenatal growth on socio-emotional development during early infancy.

Studies investigating early pattern of social processing (Tronick and Beeghly, 2011), both in terms of neural and behavioral features in clinical or at-risk groups, have the potential to early inform on underpinning mechanisms exposing vulnerable infants to different developmental pathways (Fumagalli et al., 2018). Overall, the clinical relevance of the present research protocol lays in designing a longitudinal research perspective where, despite the laboratory setting, the selected tasks rely on processes (i.e., face perception and mother child interactions) relatively ecological for infants. More importantly, infants neural and behavioral competences are combined to at least partially switch on a light on one of the potential pathways through which antenatal adversities translate into development fragilities, before fragility becomes a clinical outcome. Addressing this question has the clinical relevance to translate results into applicative guidelines in order to potentially generate effective and empirically-driven interventions in early infancy. Indeed, considering possible difficulties of IUGR infants in face processing and behavioral interactions might help in developing early *ad hoc* interventions aimed at supporting mothers in sensitive and multimodal communications, thus hopefully constraining the effect of infant's social processing deficits on later socio-emotional development. Last, a second-order implication of the present protocol is that it might be generalizable to several developmental risks' population deriving from decreased or altered antenatal growth trajectories (i.e., prematurity, congenital heart disease; maternal substance abuse) in order to identify differential trajectories starting form specific etiopathological conditions, or rather common mechanisms predisposing to multiple outcomes.

AUTHOR CONTIBUTIONS

CS designed the study and drafted the manuscript. PDC contributed to the manuscript draft, planned the analyses, and performed the power analysis. GM designed the experimental tasks and revised the manuscript critically for important intellectual content. TF contributed to the design of the experimental tasks and revised the manuscript critically for important intellectual content. SV contributed to the study design. AS mentored the first author in designing the study, revised the manuscript critically for important intellectual content. All the authors carefully read and approved the final version of the manuscript.

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Adverse Behavioral Changes in Adult Mice Following Neonatal Repeated Exposure to Pain and Sucrose

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Sucrose is recommended for the treatment of pain during minor procedures in preterm infants in the neonatal intensive care unit (NICU) and is currently used worldwide as the standard of care. We recently reported that adult mice repetitively exposed to sucrose compared to water during the first week of life, irrespective of exposure to an intervention, had significantly smaller brain volumes in large white matter, cortical and subcortical structures (e.g., hippocampus, striatum, fimbria). These structures are important for stress regulation and memory formation. Here, we report the effects of repeated neonatal exposure to pain and sucrose on adult behavior in mice. Neonatal C57BL/6J mice (N = 160, 47% male) were randomly assigned to one of two treatments (sucrose, water) and one of three interventions (needle-prick, tactile, handling). Pups received 10 interventions daily from postnatal day 1 (P1) to P6. A single dose of 24% sucrose or water was given orally 2 min before each intervention. At adulthood (P60-85) mice underwent behavioral testing to assess spatial memory, anxiety, motor function, pain sensitivity, and sugar preference. We found that mice that had received sucrose and handling only, had poorer short-term memory in adulthood compared to water/handling controls ($p < 0.05$). When exposed to pain, mice treated with repetitive sucrose or water did not differ on memory performance ($p = 0.1$). A sugar preference test showed that adult mice that received sucrose before an intervention as pups consumed less sugar solution compared to controls or those that received water before pain ($p < 0.05$). There were no significant group differences in anxiety, motor, or pain sensitivity. In a mouse model that closely mimics NICU care, we show for the first time that memory in adulthood was poorer for mice exposed to pain during the first week of life, irrespective of sucrose treatment, suggesting that sucrose does not protect memory performance when administered for pain. In the absence of pain, early repetitive sucrose exposure induced poorer short-term memory, highlighting the importance of accurate pain assessment.

Keywords: sucrose, pain, prematurity, NICU, mouse model, neurodevelopmental outcomes

INTRODUCTION

An estimated 15 million infants are born preterm (<37 weeks gestational age [GA]) each year according to a recent report by the World Health Organization, and this number is rising (World Health Organization, 2018). In North America this represents ∼8% of all live births, of these about 1% are born very preterm (24–32 weeks GA) 2 to 4 months early (Hamilton et al., 2015). Although the survival rate has increased substantially, over one-quarter of surviving infants experience moderate to severe neurodevelopmental problems, including poor motor and cognitive outcomes (Kuban et al., 2016; Heeren et al., 2017). Very preterm birth is coupled with an array of significant early-life stressors such as maternal separation, as well as exposure to pain, inflammation and pharmacological treatments. During their extended stay in the neonatal intensive care unit (NICU), very preterm infants undergo ∼200 painful procedures (Grunau et al., 2007), averaging 10 invasive and stressful procedures per day (Roofthooft et al., 2014). The negative effects of early untreated pain on brain development and behavorial outcomes have been demonstrated in both rodents (Anand et al., 1999; Dührsen et al., 2013), and humans (reviewed in Ranger and Grunau, 2014; Vinall and Grunau, 2014). Our longitudinal cohort studies in humans found short-term (Brummelte et al., 2012; Zwicker et al., 2013) and long-term adverse (Ranger et al., 2013, 2014, 2015; Vinall et al., 2014) effects of repetitive exposure to early pain-related stress on brain development and neurodevelopmental outcomes in preterm children, after accounting for clinical risk factors related to prematurity.

Effective pain management is essential to help mitigate these negative consequences of early pain exposure in very preterm children. However, the optimal strategy to achieve this goal is unclear. Oral sucrose, known as a non-pharmacological agent, is now used worldwide as the standard of care in neonatal units to alleviate acute procedural pain, but its safety relative to neurobehavioral outcomes remains to be determined (Gao et al., 2016; Stevens et al., 2016). To date, only one clinical study (albeit short term) has examined neurodevelopment after repeated sucrose administration in very preterm infants. Johnston and colleagues found that in infants born below 31 weeks GA, more than 10 sucrose doses per day given in the first week of life was associated with poorer attention and motor function at term-equivalent age (Johnston et al., 2007). Effects of repetitive sucrose on longer-term neurobehavioral development has not been studied, and this lack of safety data was highlighted in two recent reviews (Gao et al., 2016; Stevens et al., 2016). There is a growing concern regarding the use of sucrose in this population. Recently, the American Academy of Pediatrics cautioned use of sucrose for infant pain management until appropriate dose, mechanisms of action, and long-term effects of this treatment are addressed; sucrose should be viewed as a prescribed medication that must be tracked (Committee on Fetus and Newborn and Section on Anesthesiology and Pain Medicine, 2016).

Mechanisms of sucrose-induced analgesia are well-established in rodent models. Oral administration of sweet substances such as sucrose inhibits pain by mediating endogenous opioid peptide and μ1-opioid receptor actions (de Freitas et al., 2012). Others have suggested additional involvement of the 5-HT2A-serotonergic receptors in the antinociception effect of sweet solution administration (Rebouças et al., 2005). Key brainstem sites critically involved in descending pain modulation have been shown to be activited by intraoral sucrose administration in neonatal rats (Anseloni et al., 2005). Studies in adult rodents have shown that repeated sucrose doses lead to higher levels of the neurotransmitters dopamine and acetylcholine (Hajnal et al., 2004; Spangler et al., 2004; Rada et al., 2005). Dopamine plays a key role in motor and cognitive functions (Dreisbach et al., 2005), and acetylcholine in attention, memory, learning, and pain (Lagercrantz et al., 2010). In the developing preterm brain, it is not known whether increases in dopamine and/or acetylcholine levels would have positive or negative effects, either short or long-term, on related functions (Holsti and Grunau, 2010).

To our knowledge, only two pre-clinical studies have examined the effects of early repetitive exposure to pain and/or sucrose on adult memory (Nuseir et al., 2015, 2017). However, in one of those studies, neonatal sucrose and pain exposure were induced over far longer periods than would be developmentally relevant as a model of preterm NICU care (Nuseir et al., 2015). Using a mouse model of pain and sucrose administration which closely mimics the exposure of preterm infants in the NICU, we previously reported widespread long-term alterations in white and gray matter brain volumes in adult mice repeatedly exposed to sucrose compared to water in the first week of life (Tremblay et al., 2017b). In that study, irrespective of pain exposure, repetitive sucrose induced smaller brain volumes mainly in white matter regions of the forebrain, cerebellum, and hippocampus. Consistent with our findings in mice, in human preterm infants, higher exposure to glucose for pain relief in the NICU was associated with lower thalamic volume on neonatal MRI (Schneider et al., 2018). There appear to be no animal studies of effects of neonatal repeated sucrose in the context of pain on neurobehavior functions such as anxiety, motor, and cognition, that accurately models the duration and frequency of exposure in humans following preterm birth.

Given that sucrose treatment is currently administered to thousands of preterm infants for minor procedural pain relief, it is crucial to determine the long-term consequences of repetitive sucrose for pain management on neurodevelopment. Therefore, we examined effects of neonatal repetitive sucrose exposure on behavioral and cognitive outcomes in adulthood in a mouse model that closely mimics pain of minor procedures during NICU care.

MATERIALS AND METHODS

Animals

All animal procedures were approved by The University of British Columbia Animal Care Committee and conform to the guidelines outlined by the Canadian Council on Animal Care. Animals were maintained on a 14/10 h light/dark cycle with food and water *ad libitum*. Mice were provided with nestlets and Plexiglas igloo-style houses as part of standard enrichment. Cellulose bedding was used to minimize discomfort of inflamed

paws in the pups (1/4-inch pelleted cellulose; Biofresh). C57BL/6J and ICR (CD1) mice used in this study were obtained from the Goldowitz Laboratory mouse inbred and outbred colonies, respectively. A non-nursing ICR female mouse was added to the litter to prevent rejection of pups from nursing C57BL/6J dam and improve survival rate of treated mouse pups (Tremblay et al., 2017a). Postnatal day 0 (P0) was defined as the day of birth. Pups were left with their nursing dam and non-nursing ICR female until the age of weaning, on P21. After weaning, mice were ear-notched to allow for the identification of individual mice and housed together with appropriate nesting materials, enrichment, and cellulose bedding (5 mice/cage, sex-matched cages) during aging and behavioral testing (study endpoint P85-P95).

Experimental Design

On the day of birth (P0) newborn mouse pups were randomized to one of six groups; each litter included at least two different groups and nearly equal distribution by sex per group. For individual identification during infancy, pups were tattooed on their paw using a 30G needle prick. Experiments were started on the following day (P1) and continued until P6. Pups received either sterile water or sucrose 24% (Vehicle; Treatment) given orally by a micropipette 2 min before one of three interventions: paw needle-prick, light paw tactile pressure with a cotton-tipped swab (Tactile), or only being handled by simply picking up the pup (Handling). Treatment/Intervention sequence duration was less than 5 min per interval. Treatment/Intervention was administered 10 times per day per mouse pup over a 10-h period (from 8 AM until 6 PM) during the day (light) cycle from P1 to P6 inclusive. Each Treatment/Intervention was spaced by a minimum of 30 min to allow enough time for feeding, mother-pups interaction/care, and recovery from the interventions.

The treatment consisted of a solution of sucrose 24% w/v [Sucrose $\geq$ 99.5% (GC) Sigma, United States, cat#S7903] in sterile water filtered with 0.22 μm filter, or sterile water, administered intraorally 2 min before the intervention. The treatment was administered either on the anterior part of the tongue or inner-cheek of the mouth using a micropipette and sterile tips. The dose of sucrose was based on the standard guideline recommended dose for human neonates, which is 0.5 mL per dose for very preterm infants (24 to 32 weeks of gestation), corresponding to 0.08–0.2 g of sucrose per body weight (kg) (Stevens et al., 2016). Pups received 0.1–0.2 g of sucrose per kg of body weight and the dose was adjusted daily based on the pups' weights (i.e., ∼1 to 4 μl/dose during the course of the experiment). Mouse pups in the vehicle treatment groups received an equivalent volume of oral sterile water. For each intervention, the forepaws and hindpaws were alternated and only one paw was touched (tactile) or pricked. Needle-pricks were performed using a 30G sterile needle (0.3 mm outer diameter) angled at 10–15 degrees from the skin to carefully pierce only the surface of the skin, with special attention in avoiding penetration of deeper layers, such as tendon or bone. Mouse pups were returned to the dams between each hourly Treatment/Intervention sequence. To minimize heat loss by pups, all procedures were conducted on a circulating water heating pad. Prior to each procedure, C57BL/6J nursing females and ICR non-nursing females were transferred to a clean cage and housed in a separate room to prevent possible stress induced by sight, sound or smell from the procedures. At P7, pups remained in their home cage with their dam until weaning (P21). A total of 160 mouse pups (47% male) were treated and survived to adulthood with an overall survival rate of 93% (non-survival due to cannibalism, rejection of dam, and/or poor feeding during the first 2 days of life). Mice were weighed daily from P1 to P7, at weaning on P21, at the start and end of behavioral testing (P60, P80-85), and finally at experimental protocol endpoint (P85-95). The timeline of the experimental design is shown in **Figure 1A**.

At P0, mouse pups (75 males; 85 females) were randomly allocated to one of six groups: Vehicle/Handling (n = 27), Vehicle/Tactile (n = 25), Vehicle/Needle-prick (n = 26), Sucrose/Handling (n = 27), Sucrose/Tactile (n = 28), or Sucrose/Needle-prick (n = 27) (**Table 1**). During the first days of life, from P1 to P3, pups in the needle-prick groups showed visible paw inflammation at the site of skin-breaks as exposure to the intervention accumulated throughout the 10 h. In each case, local inflammation disappeared by the following morning. No signs of infection or other complications occurred (**Figure 1B**).

Behavioral Evaluation During Adulthood

Behavior testing took place when adult mice reached P60 and lasted 2 to 3 weeks (endpoint ∼P80-85). The mice were transferred to the behavioral testing suite 2 weeks before testing to acclimate to the room. All testing sessions were conducted during the light cycle, between 7 AM to 6 PM. All groups went through the same non-randomized behavioral testing sequence: Open-Field, Elevated Plus Maze, Morris Water Maze, Rotarod, Hot Plate, and Sugar Preference. Males and females were tested separately; female rats were tested first, followed by the males.

General Locomotor Activity Assessment

The Open-Field test was used to assess locomotor activity, anxiety and behavior in a novel environment (Gould et al., 2009). A plexiglass chamber of 50 cm × 50 cm × 12 cm was used. Individual adult mice were placed into the chamber and allowed to explore the environment for 10 min while locomotion was recorded by a camera placed directly above the chamber and analyzed by an image tracking system (Noldus Ethovision XT software; Noldus Information Technology, United States). Measures included total distance traveled, percent time spent in movement and mean velocity.

Anxiety-Like Behavior Assessment

The Elevated Plus Maze was used to assess general anxiety behavior (Balsevich et al., 2014). A maze was placed 55 cm above floor with four black Plexiglas arms, two open arms (67 cm × 7 cm) and two enclosed arms (67 cm × 7 cm × 17 cm) which formed a cross shape opposing each open area. Adult mice were placed in the center of the maze and allowed to explore for 5 min. A camera mounted directly above the maze recorded the behavior of the animal and was analyzed by the image tracking system (Noldus Ethovision XT software; Noldus

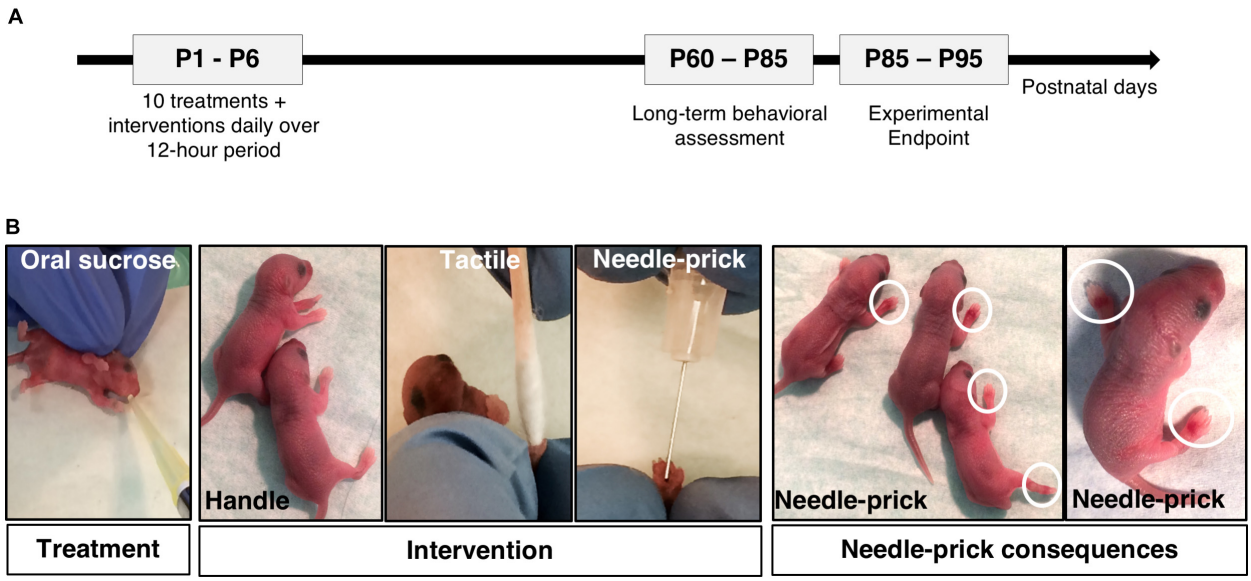

FIGURE 1 | Neonatal mouse model of repetitive sucrose exposure given for procedural pain [adapted from Tremblay et al. (2017b)]. **(A)** Schematic illustrating the experimental protocol timeline. Long-term behavioral testing were performed between postnatal day (P) 60–85 following neonatal treatment/intervention period. **(B)** Images showing C57Bl/6J mouse pups receiving oral sucrose or water treatment preceding 10 daily interventions: Handle, Tactile, or Needle-prick. White circles are highlighting ecchymosis and inflamed paws seen on pups after needle-prick intervention. P, postnatal day.

Information Technology, United States). Measurements included time spent in open and enclosed arms and number of entries in open areas.

Gross Motor Function, Balance, and Coordination

The Rotarod test was used to measure balance, motor coordination, and motor learning on an accelerating rotarod (Ugo Basile, Italy) (Buitrago et al., 2004). Mice were placed on a rotating rod in individual compartments (up to five mice during one session); distance from the rotating rod to the base of the apparatus was approximately 10 cm. The rotation speed of the rotating rod was accelerated from 4 to 40 rpm over 5 min. Each trial lasted a maximum of 300 s or when the mouse fell off the rotating rod. The duration of time spent on the rotating rod was recorded for each trial. Trials were conducted over 3 days including five trials on the first day followed by three trials per day with a minimum of 30-min inter-trial. After each completed trial, the mice were returned to their home cage.

Spatial Learning and Memory Assessment

Spatial learning and memory abilities were tested by the Morris water maze test (Morris, 1984; Vorhees and Williams, 2006; Hamre et al., 2007). Latency to find the platform was recorded manually as well as quantified using an automated video tracking system located above the pool and analyzed with Noldus Ethovision XT 7.0 software (Noldus Information Technology, United States). The pool was filled with room temperature water (1 m in depth) and made opaque with white non-toxic water-soluble paint. A platform was located in an arbitrarily defined quadrant of the maze and 2 cm below the surface of the water so that mice could not see the platform when swimming. Mice were trained for 3 days to assess learning (2 tests/day- in AM and PM for 3 days) and two probe trials in the absence of the platform were used to test short (on fourth day) and long-term (7 days post-training) memory (Tremblay et al., 2017a). Between each session, mice were resting in a heated standard cage with paper towel and water gel for a 10 min inter-trial period. In each of the trials, mice were given 60 s to locate the platform. Probe trials were performed without the platform and mice were recorded over a 60 s period prior to being removed from the pool. Measures included a learning curve over 3 days, distance covered and latency to reach the hidden platform, average swim speed and percent of time spent in their respective platform quadrant. Manually recorded measures of latency to find platform during the 3-day training period were used in our analysis to assess learning, all other reported measures were quantified using the automated video tracking system (Noldus EthovisionXT7.0 Software).

Pain Threshold

A Hot-plate test for pain threshold was performed with an analgesiometer. The apparatus consists of 25 by 25 cm metal hot-plate surface set at 52°C, a Plexiglas cage to restrain the animal fitting over the hot plate, and a foot-switch operated timer. Pain threshold was measured by the latency to nociceptive responses (paw lift, limb shaking, or paw lick) with a maximum cut-off time of 30 s to avoid any tissue injury. Latency to pain was calculated from averaging the results from three trials spaced by 15 min between each trial. The surface temperature (i.e., 52°C) and 30 s cut-off time assured that none of the mice endured any kind of skin injury during this test.

TABLE 1 | Study sample group allocation and weight trajectory.

Treatment pre-intervention	Intervention	*Mean weight gain during treatment (P7 weight–P1 weight)*	*Mean body weight at behavioral testing*	*n (%Males)*	Overall distribution (*N* = 160)
Water	Handling	2.1 ± 0.1 g	21.3 ± 0.6 g	27 (44.4%)	*N* = 78
Water	Tactile	2.1 ± 0.1 g	21.9 ± 0.7 g	25 (56.2%)	
Water	Needle-prick	1.9 ± 0.1 g	21.9 ± 0.7 g	26 (50.0%)	
Sucrose 24%	Handling	1.8 ± 0.1 g	21.6 ± 0.6 g	27 (44.4%)	*N* = 82
Sucrose 24%	Tactile	1.8 ± 0.1 g	21.2 ± 0.6 g	28 (42.9%)	
Sucrose 24%	Needle-prick	1.9 ± 0.1 g	22.2 ± 0.7 g	27 (38.9%)	

Neonatal mice (n = 160) were assigned randomly across treatment/intervention groups (total of six groups). Table provides the distribution/sex ratio, mean weight gain during treatment period, and mean body weight at behavioral testing. Values are means ± SEM. P, postnatal day.

Sugar Preference

At the end of the behavior testing period, mice were challenged with a sugar preference test (Bachmanov et al., 1996a,b; Avena et al., 2004). Mice were housed in single-plex cages (with bedding, housing and chow) with *ad libitum* access to two bottles containing 50 ml each of either water or 10% sucrose water placed side by side for 48 h (bottles were switched position at 24 h interval). Mice and chow were weighed pre- and post-sugar preference test; amount of liquid in each bottle was measured pre- and post-test. Percent of water versus 10% sucrose water consumed was calculated.

Statistical Analysis

Group comparisons were carried out using analysis of variance (ANOVA) or MANOVA, repeated-measures ANOVA, Mauchly's test, and Multivariate test Pillai's Trace to examine differences for all neurobehavioral outcome measures (i.e., open field, elevated plus maze, Morris water maze, rotarod, hot plate, and sugar preference) across the three treatment and three intervention groups followed by Fisher's least significant difference (LSD) or Tukey's honestly significant difference (HSD) test *post hoc* tests, unless otherwise specified. Sex was also examined in our statistical models. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 22 (IBM, Somers, NY, United States); p-values < 0.05 were considered statistically significant. Exclusion of outliers from behavioral and structural analyses was made prior to statistical analysis; outliers were defined by being below 1.5x the interquartile range from the 25th percentile or above 1.5x the interquartile range from the 75th percentile. Data were graphically organized using GraphPad Prism version 7.0 (San Diego, CA, United States). Data are presented graphically as means ± SEM.

RESULTS

Repeated Pain and/or Sucrose Does Not Affect Weight Trajectory Throughout Life

Daily weights were captured from P1 to P7 and prior to behavioral testing at P60 (**Table 1**). One-way ANOVA analysis showed that mean weight gain during the treatment period, measured by the difference between P7 and P1 weights, was significantly different between groups ($F_{(5,154)} = 2.825$, $p = 0.02$). However, the significant difference between groups was not evident after correcting for multiple comparisons using Tukey's HSD *post hoc* test. At adulthood prior to behavioral testing (P60), there were no significant differences in mean body weight between groups ($F_{(5,154)} = 0.369$, $p = 0.87$). No sex effect was found.

Long-Term Behavioral Alterations of Adult Mice Exposed to Repetitive Sucrose and/or Interventions Over the Neonatal Period

Neonatal Repetitive Treatment and/or Intervention Exposure Did Not Alter General Locomotor Activity, Anxiety-Like Behaviors, or Gross Motor Function in Adult Mice

Repeated exposure to sucrose and/or intervention did not have a significant effect on any of the general locomotor activity measures during the open-field test (**Figures 2A–C**). One-way ANOVA analyses did not show significant differences between groups for the total distance traveled during recording ($F_{(5,156)} = 0.429$, $p = 0.83$), the mean velocity ($F_{(5,156)} = 0.493$, $p = 0.78$) and the percentage of time spent in movement ($F_{(5,156)} = 1.386$, $p = 0.23$) (**Figure 2**). No sex effect was found.

Gross motor function assessed by the rotarod test revealed a highly significant trial effect on overall mean time spent on rotarod across the 3 days of training (repeated measure ANOVA multivariate test Pillai's Trace $V = 0.12$, $F_{(2,153)} = 10.45$, $p < 0.0001$) (**Figure 2D**). Mauchly's test indicated that the assumption of sphericity was violated ($\chi^2_{(2)} = 25.8$, $p < 0.0001$), multivariate (MANOVA) tests are reported ($\varepsilon = 0.87$) for within subject analysis. We did not find any significant effect of group ($F_{(5,154)} = 0.76$, $p = 0.58$) or interaction between trial/training and group (multivariate test Pillai's trace $V = 0.054$, $F_{(10,308)} = 0.85$, $p = 0.58$). Here, we found that overall, adult mice showed improvement performance on the rod across trials, irrespective of group allocation. No sex effect was found.

Similarly to the general locomotor assessment, exploratory and anxious behaviors in a novel environment examined by the elevated plus maze test revealed no significant difference between the six groups. The percentage of time spent in the central zone of the open-field arena ($F_{(5,156)} = 0.821$, $p = 0.55$) along with the amount of time spent in the open arm of the elevated plus

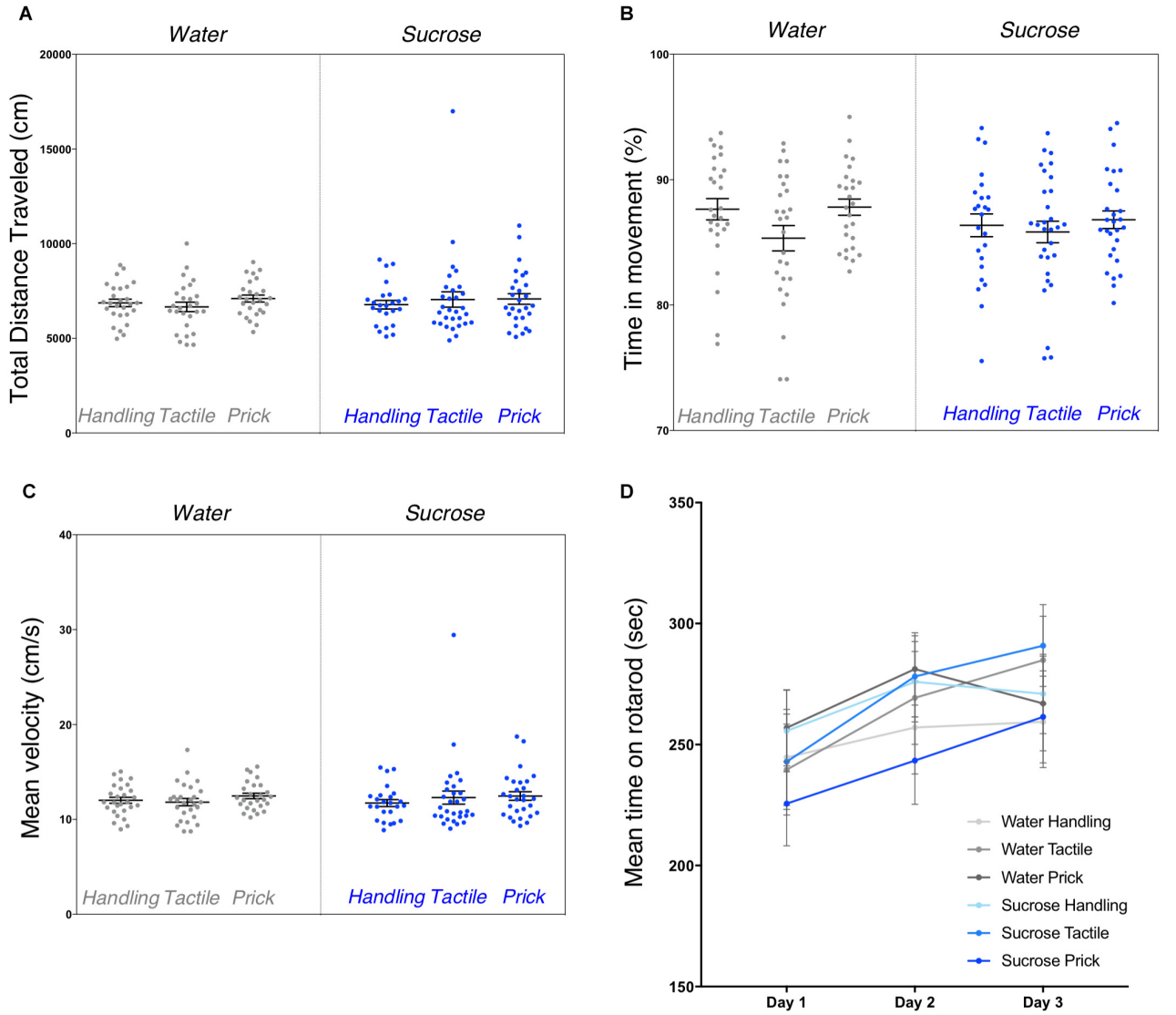

FIGURE 2 | Assessment of general motor function and coordination after neonatal repetitive exposure to treatment prior to an intervention. Open-field testing performed to assess locomotion capacities in adult mice from six experimental groups. Box plots show the total distance traveled ($p = 0.83$) **(A)**, the percentage of time spent in movement ($p = 0.23$) **(B)** and mean velocities ($p = 0.78$) **(C)** measured between groups. Data presented as scatter plots with mean ± SEM; horizontal bars represent mean values; black asterisks and bars denote statistical significance using ANOVA. Graph in **(D)** shows the mean time adult mice spent on rotarod (i.e., latency to fall) learning curves between the six experimental groups. All groups learned to stay longer on the rod over the 3 days ($p < 0.0001$), but there were no significant differences in time to learn over days between experimental groups ($p = 0.58$), or interaction between trials and groups ($p = 0.58$). $n = 24$–27 per group.

maze ($F_{(5,154)} = 1.187$, $p = 0.32$) were not statistically different (**Figures 3A,B**). No sex effect was found.

Neonatal Repetitive Pain and Sucrose Impacts Short-Term Memory in Adulthood

Learning on the Morris water maze showed a highly significant trial effect on overall mean time to locate the platform across the 3 days of training (multivariate test Pillai's trace $V = 0.67$, $F_{(5,143)} = 56.93$, $p < 0.0001$) (**Figure 4A**). Mauchly's test indicated that the assumption of sphericity was violated ($\chi^2_{(14)} = 85.6$, $p < 0.0001$), multivariate (MANOVA) tests are reported ($\varepsilon = 0.82$) for within subject analysis. We did not find any significant effect of group ($F_{(5,143)} = 0.77$, $p = 0.57$) or interaction between trial and group (multivariate test Pillai's trace $V = 0.22$, $F_{(25,735)} = 1.33$, $p = 0.13$) on learning. Thus, showing that overall, adult mice learned and improved their time to locate the platform across trials irrespective of group allocation. No sex effect was found.

Short-term memory during the probe test, 1 day after 3-days training, revealed an overall effect of group on time to reach the area where the platform was during training ($F_{(5,147)} = 4.12$, $p = 0.002$). *Post hoc* test, shown here as mean differences (confidence intervals), uncovered that mice in the Water/Needle-Prick group (pain) took significantly longer to reach the "platform" compared to those in the Water/Handling

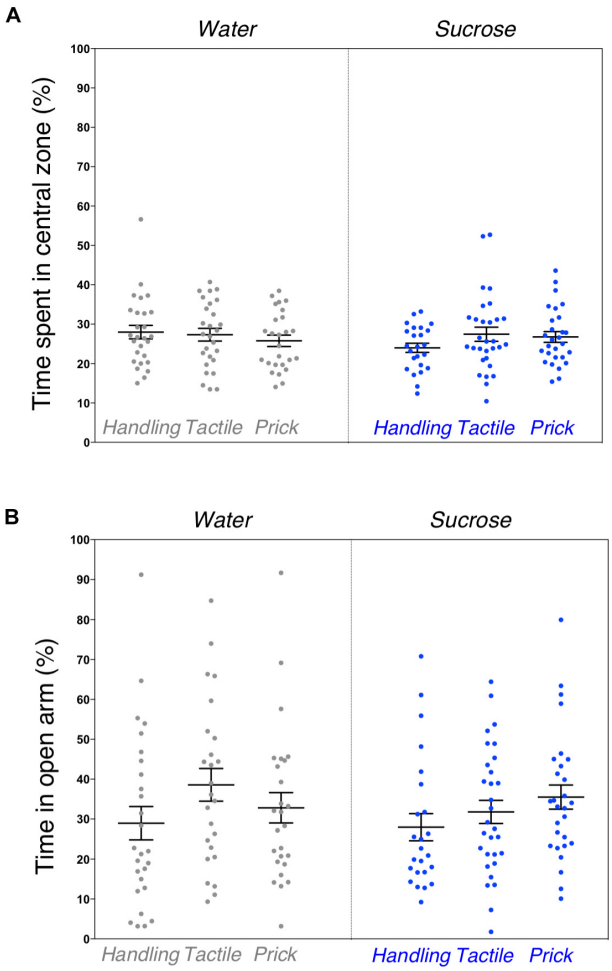

FIGURE 3 | Assessment of anxious behavior after neonatal repeated exposure to treatment prior to an intervention. Box plots comparing six experimental groups **(A)** showing the time spent in central zone of a novel environment measured during the open field test ($p = 0.55$). **(B)** Percent of time spent in open arms of an elevated plus maze ($p = 0.32$). Data presented as scatter plots with mean ± SEM; horizontal bars represent mean values; black asterisks and bars denote statistical significance using ANOVA. $n = 24$–27 per group.

(10.68 [3.71, 17.65], $p = 0.003$), Water/Tactile (12.92 [5.87, 19.96], $p < 0.001$), and Sucrose/Tactile (11.14 [4.31, 17.98], $p = 0.002$) groups. Mice in the Sucrose/Handling group took significantly longer to locate the "platform" compared to Water/Handling (7.5 [0.46, 14.54], $p = 0.04$), Water/Tactile 9.74 [2.63, 16.85], $p = 0.01$), and Sucrose/Tactile (7.96 [1.06, 14.87], $p = 0.02$). Adult mice that received repetitive needle-pricks preceded by water or that received sucrose but were simply handled during the first week of life had poorer short-term memory at adulthood. There was no significant difference in the time to locate the "platform" between mice in the Water/Needle-prick and Sucrose/Needle-prick groups ($p = 0.1$) (**Figure 4B**). When exposed to needle-pricks, mice that were treated with repetitive sucrose or water did not differ on their short-term memory performance at adulthood. The significant effects of sucrose and/or pain on short-memory were no longer present at the second testing probe (i.e., long-term memory; $p = 0.8$). Male and female mice did not differ significantly in their short or long-term memory performances.

Sugar Preference Was Altered in Adults Exposed to Sucrose During the Neonatal Period

Due to time constraints, the sugar preference test was conducted on a smaller sample of mice ($n = 118$). A one-way ANOVA revealed an overall significant group difference on percentage of 10% sucrose water consumed during the 48 h sugar preference test ($F_{(5,117)} = 2.36$, $p = 0.044$). LSD *post hoc* test group comparisons showed significant mean differences between Water/Handling (controls) compared to Sucrose/Tactile (−9.28 ml [1.58,16.97], $p = 0.019$) and Sucrose/Needle-prick (−7.97 ml [0.09, 15.85], $p = 0.047$) groups, as well as between Water/Needle-prick compared to Sucrose/Tactile (−10.7 ml [2.9, 18.5], $p = 0.008$) and Sucrose/Needle-prick (−9.39 ml [1.41, 17.37], $p = 0.021$) groups (**Figure 5**). Overall during the 48 h sugar preference, adult mice that were repetitively exposed to both an intervention (tactile or needle-prick) and treatment (sucrose) as pups consumed significantly less sucrose water compared to those that received water as treatment during the first week of life. Mice that were simply handled and exposed to sucrose in early-life did not significantly differ from any other group in the amount of sucrose water consumed at adulthood. No sex effect was found.

Pain Threshold Was Not Altered

We found no significant differences between the groups on mean time for paw lift from hot plate ($F_{(5,154)} = 0.72$, $p = 0.61$). There was no difference in pain threshold between male and female mice (**Figure 6**).

DISCUSSION

This is the first study to examine the effects of early repetitive pain and sucrose exposure on long-term behavioral and cognitive outcomes using a model that closely mimics NICU pain and treatment of the very preterm neonate. Our key findings were that memory in adulthood was poorer for mice exposed to pain during the first week of life, regardless of sucrose treatment, suggesting that sucrose is not protective for memory performance when administered for pain. We also found that in the absence of pain, when mouse pups were only handled, early sucrose exposure induced poorer short-term memory in adulthood. Finally, sugar preference in adult mice was lower in those exposed to early sucrose before an intervention, indicating possible conditioned memory and anhedonic behavior.

These important findings on adult memory are consistent with our earlier findings that mouse pups exposed to neonatal repetitive sucrose had smaller adult brain volumes in important regions involved in memory formation (Tremblay et al., 2017b). Specifically, in that study, sucrose exposure during the first week of life induced smaller volumes of the hippocampus, dentate gyrus, and stratum granulosum of the hippocampus and the fornix along with other brain regions including cortical, subcortical gray and white matter structures in adulthood.

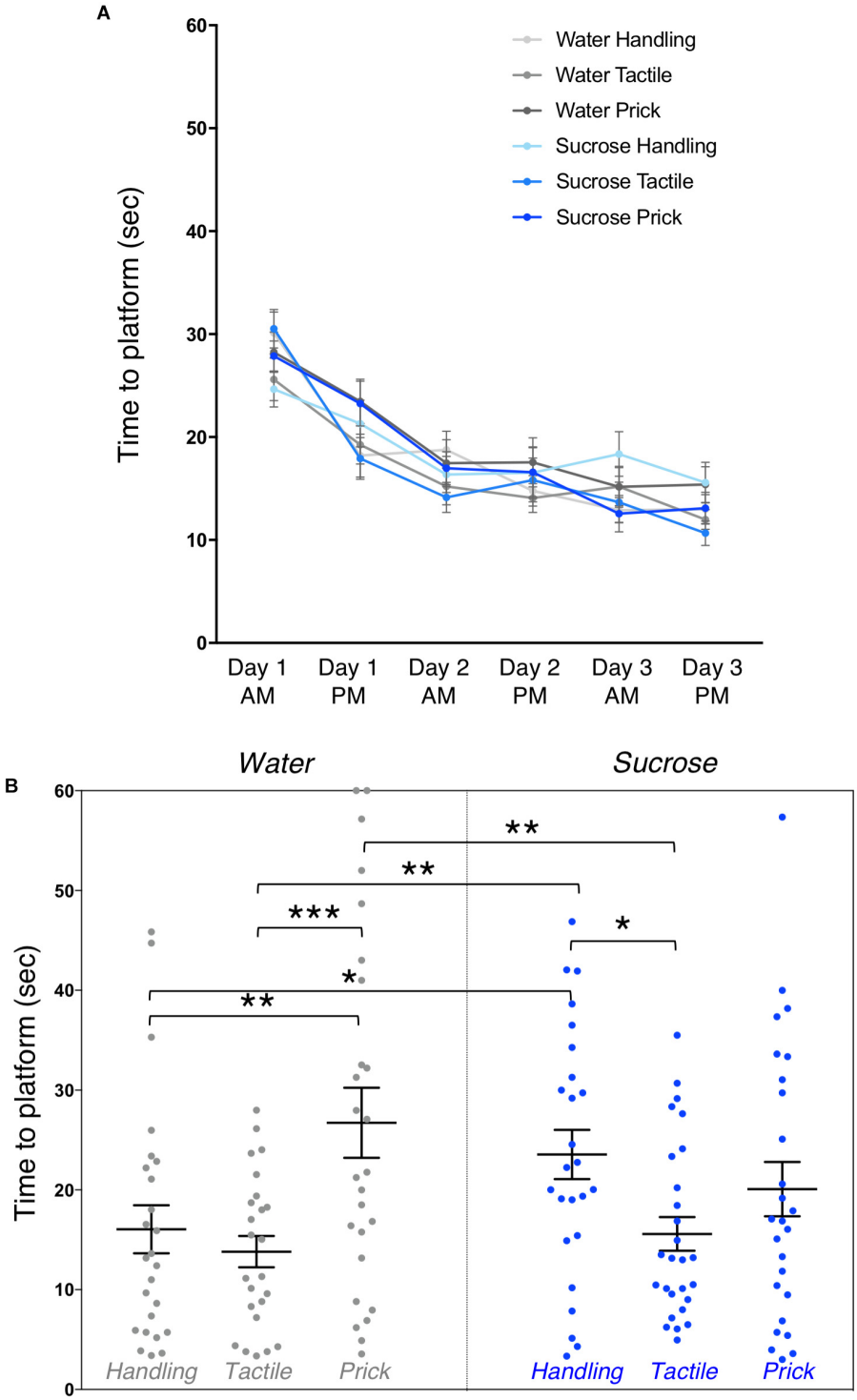

FIGURE 4 | Effects of treatment and intervention on short-term memory in adulthood (Morris water maze test). **(A)** Training. Morris water maze learning curves are shown from testing day 1 to day 3. All groups learned to locate the platform over their six trials ($p < 0.0001$), but there were no significant differences in time to learn over days between experimental groups ($p = 0.57$), or interaction between trials and groups ($p = 0.13$). **(B)** Short-term memory. Group comparison of adult mice time to reach the area where the platform was during training during the first testing probe (1 day post-training). Compared to adult mice in the Water/Needle-prick group, mice in the Water/Handling ($p = 0.003$), Water/Tactile ($p < 0.001$) and Sucrose/Tactile ($p = 0.002$) groups took significantly less time to locate the "platform" on the MWM test. Compared to mice in the Sucrose/Handling group, those in the Water/Handling ($p = 0.04$) and Water/Tactile ($p = 0.01$) and Sucrose/Tactile ($p = 0.02$) took less time to locate the "platform." Data presented as scatter plots with mean ± SEM; horizontal bars represent mean values; black asterisks and bars denote statistical significance using ANOVA. $n = 24$–27 per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

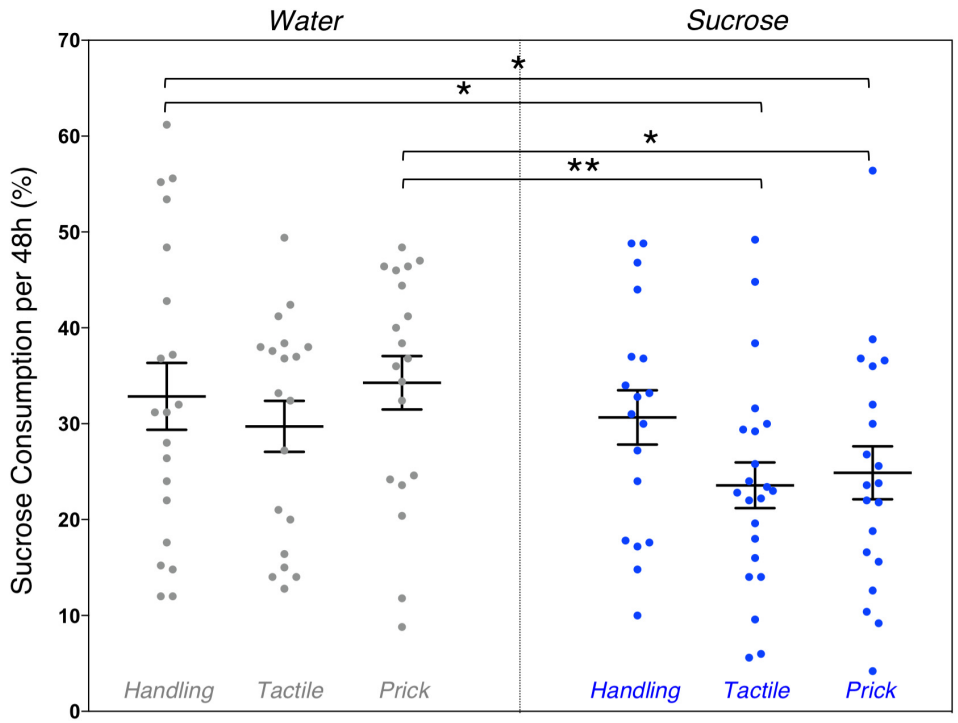

FIGURE 5 | Effects of treatment and intervention on sugar preference test. Group comparisons of percent of 10% sucrose water consumed from the total liquid consumption during the 48 h sugar preference test. Compared to adult mice in the Water/Handling group (controls), those in the Sucrose/Tactile ($p = 0.019$) and Sucrose/Needle-prick ($p = 0.047$) groups consumed significantly less 10% sucrose water. Adult mice in the neonatal Sucrose/Tactile ($p = 0.008$) and Sucrose/Needle-prick ($p = 0.021$) groups consumed significantly less 10% sucrose water compared to those in the Water/Needle-prick group. Data presented as scatter plots with mean ± SEM; horizontal bars represent mean values; black asterisks and bars denote statistical significance using ANOVA. $n = 18$–22 per group. $^{*}p < 0.05$; $^{**}p < 0.01$.

These significant changes were found irrespective of the type of intervention (needle-prick, tactile, or handling) in the neonatal period consistent with the adult functional alterations affecting memory seen in our current study. Neurodevelopmental consequences of postnatal stress have been studied extensively in preclinical models. A recent review examining the developmental effects of early life stress exposure in mice, focusing on the maternal separation paradigm, found that the most robust effect was on memory performance (poorer spatial memory across all strains) (Tractenberg et al., 2016). In a longitudinal cohort study following very preterm infants exposed to oral glucose with limited sedative and analgesic medications for pain management, a higher number of invasive procedures was associated with slower growth of important brain structures such as the thalamus and basal ganglia (Schneider et al., 2018). Similar to the findings in our mouse model, glucose administration for procedural pain management did not mitigate the deleterious effect of procedural pain on the developing brain of these very preterm infants. Higher procedural pain and glucose exposure were associated with poorer psychomotor development at 18 months corrected age. This reinforces earlier findings by Johnston and colleagues that found that very preterm infants exposed to more than 10 doses of sucrose per day during the first week of life in the NICU showed poorer motor function at term-equivalent age (Johnston et al., 2007).

In our study, we assessed neurobehavioral changes in adulthood, but not earlier. We found gross motor function and coordination were not significantly affected. One explanation for the differences between effects of sucrose on human infant motor outcome at term-equivalent age and lack of such findings in our current study, may be that the damaging effect of repetitive exposure to sugar solutions on motor development may not persist into adulthood. In our previous work (Tremblay et al., 2017b), we found that adult mice exposed to sucrose as pups, irrespective of intervention exposure, had significantly smaller volumes in the cerebellum, specifically in the anterior lobules III–V, which are cerebellar regions involved primarily in motor control. We aimed here to measure a broad array of behaviors to examine the overall impact of early repetitive sucrose exposure. This broad strategy limited our ability to assess more detailed fine motor and cognitive functions, such as using the skilled walking assessment on the horizonatal ladder (Metz and Whishaw, 2002) or the beam walking assay (Southwell et al., 2009) for instance. It would be important to further examine the functional impact of neonatal repetitive sucrose exposure on cerebellar structures at various developemental ages in a rodent model.

Our sugar preference findings of reduced sucrose water solution consumption in mice exposed to early repetitive interventions and sucrose treatment support the established evidence that chronic mild stress exposure in rodent models

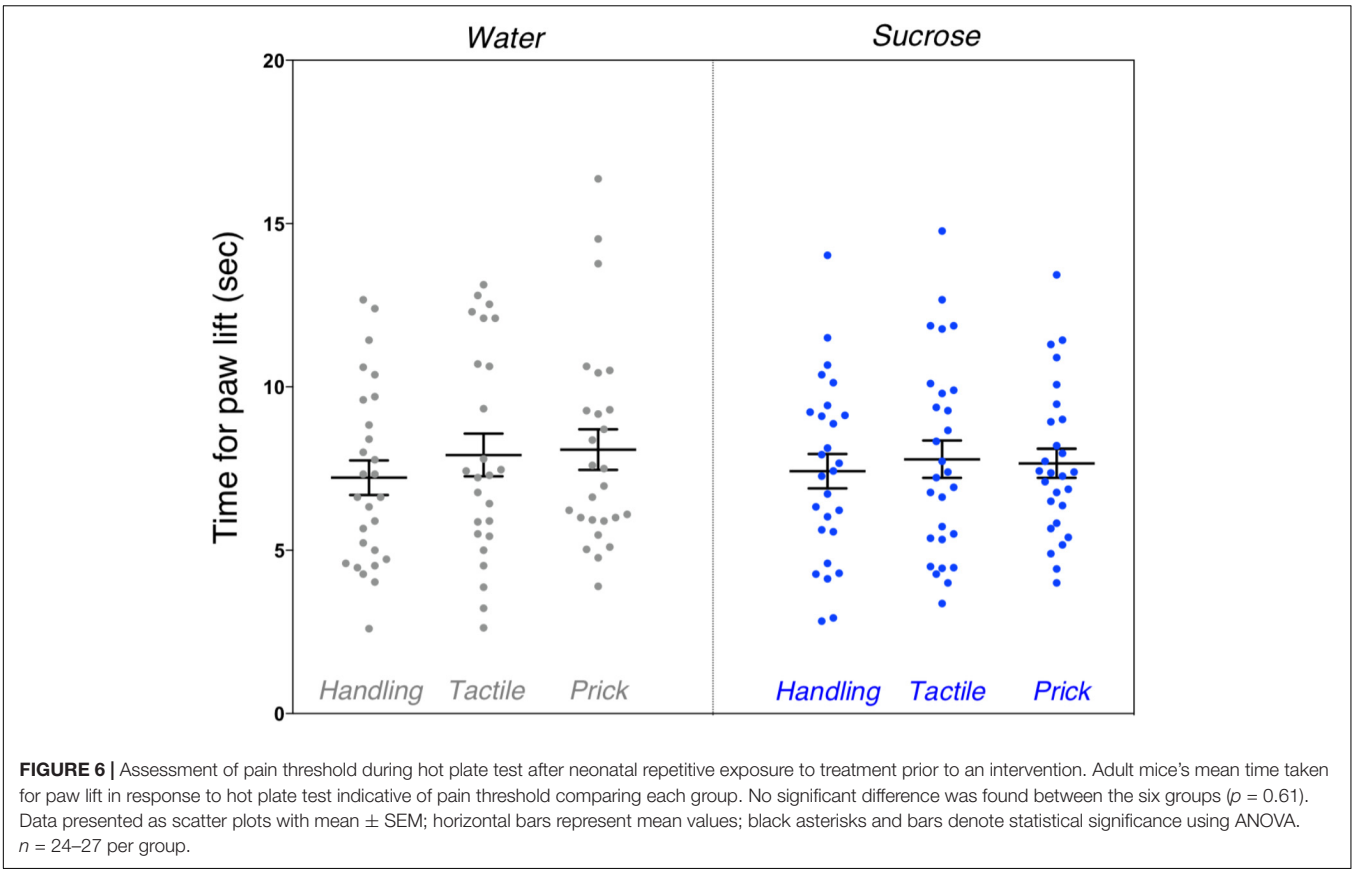

FIGURE 6 | Assessment of pain threshold during hot plate test after neonatal repetitive exposure to treatment prior to an intervention. Adult mice's mean time taken for paw lift in response to hot plate test indicative of pain threshold comparing each group. No significant difference was found between the six groups ($p = 0.61$). Data presented as scatter plots with mean ± SEM; horizontal bars represent mean values; black asterisks and bars denote statistical significance using ANOVA. $n = 24–27$ per group.

of early-life adversity (e.g., pups reared with limited bedding and nesting) is associated with reduced preference for palatable food (i.e., sugar) (Remus et al., 2015; Bolton et al., 2018). Early-life stress-induced behavioral disturbances, ranging from hippocampus-dependent memory deficits to problems with experiencing pleasure (anhedonia) have been well-documented (reviewed in Bolton et al., 2017). In our study, we aimed to mimic the use of sucrose as a treatment for procedural pain by repetitively exposing mouse pups to sucrose prior to an intervention. We examined if this early exposure to repetitive sugar would induce preference or aversion to sweet taste. Given the evidence of sugar addiction after excessive exposure intake (Avena et al., 2008), we were expecting to find that mice exposed to early sucrose, irrespective of the intervention, would consume more sugar water during the sugar preference test, which turned out not to be the case. As noted above, we interpreted our findings as more related to exposure to early stress (tactile or pain) in combination with sucrose, rather than the isolated effect of one of those stressors. If we interpret our current findings through the lense of an early-life stress exposure model, sucrose does not appear to be providing protection against the deleterious effects of early stress-related adversity (i.e., repetitve pain).

Assessing memory in rodents to reflect a cognitive process as complex as memory function in the human can be done by tapping into analogous brain regions. As such, in animal models, much of the research has focused on hippocampus-dependent memory, due to the availability of well-established standardized tests, such as the Morris water maze test which evaluates spatial navigation and memory (Rice and Barone, 2000), and well-characterized neural and molecular mechanisms (Bolton et al., 2017). We now have evidence that early repetitive exposure to both pain and sucrose has deleterious effects on both hippocampal volumes and hippocampus-dependent processes. Indeed, functionally, we showed in adulthood poorer short-term memory, as well as a reduced ability to experience pleasure (i.e., decreased consumption of sucrose water solution – anhedonia). Current understanding points to the involvement of posterior cerebellar regions (lobule VI, crus I, and crus II) in complex cognitive and memory operations in humans, such as working memory and spatial processing (Stoodley and Schmahmann, 2009). In our previous work, we found evidence of smaller volumes in posterior cerebellar subregions lobule VI and Crus I in adult mice previously exposed during the first week of life to sucrose before an intervention, which pertains to our current functional findings of poorer short-term memory (Tremblay et al., 2017b). The progressive stabilization of long-term memory after information acquisition is referred to as consolidation (Dudai, 2004). Two types of processes are commonly described: synaptic consolidation, which starts within a few minutes after the acquisition and lasts for hours in all memory systems and is hippocampal-dependent; and system consolidation, which takes longer to consolidate and will reorganize memories in a hippocampal-independent process (Dudai, 2004). The formation of short versus long-term memory relies on different neurological

pathways (Schimanski et al., 2002) and may explain why we did not find any significant effects of either early repetitive pain and/or sucrose exposure on long-term memory (i.e., system consolidation). In mice, intact hippocampal structures (especially hippocampal commissure) and corpus callosum are necessary in order to perform well on short-term memory tasks, which is not essential for long-term memory consolidation (Schimanski et al., 2002). Supporting this evidence is our previously reported findings of altered volumes in both hippocampal structures and corpus callosum in adult mice exposed to sucrose as pups (Tremblay et al., 2017b).

Contrary to our findings, Nuseir and colleagues reported recently beneficial effects of repetitive exposure to sucrose treatment before needle pricks (4 times/day from P1-14) compared to pain alone in newborn male rats on pain sensitivity and long-term memory (probe test at 24 h) outcomes (Nuseir et al., 2017). Previous work from this same group showed similar protective effects of sucrose pre-treatment on pain thresholds and long-term memory compared to exposure to pain only, following 8 weeks of daily pain and/or sucrose exposure (Nuseir et al., 2015). In this particular study treatments lasted far beyond period analogous to NICU intensive care of very preterm neonates. In contrast, we used a pain model that much more closely matches that of the human infant exposure in the NICU. Moreover, there are a number of important differences between their two study models and ours, which may explain our discrepant findings and make it difficult to compare the outcomes. They used much higher sucrose dosing (0.2 ml of sucrose 25%); whereas, we administered a dose 100 times lower adjusted to daily weights. They used a far more invasive painful stimulus (25G needle inserted through the paw while we used a superficial prick with a 30G needle). Finally, they did not use a vehicle control treatment (i.e., water solution) and used rats rather than mice.

In rodent models of neonatal pain, specific patterns of long-term behavioral effects from exposure to repetitive acute pain from needle-pricks or severe inflammatory pain from formalin injections in the first week of life of rat pups have been demonstrated, such as decreased locomotor activity (Bhutta et al., 2001), increased anxiety and defensive withdrawal behavior (Anand et al., 1999), and social hypervigilance (Anand et al., 1999). We expected that mice exposed to needle-pricks during the first week of life would have altered motor and anxiety-like behaviors, which was not the case. Again, the species (mice versus rat) and methodological differences may account for the differences in findings. That is, inflammatory pain or pain from injections may not have the same consequences as superficial needle-pricks. Our relatively innocuous needle-prick pain stimulus is an important difference, given that we used very thin needles (30G) and did not penetrate deeper layers, such as tendon or bone, in contrast to the more invasive procedures used in needle-prick pain in previous rat studies (Anand et al., 1999; Davis et al., 2018).

Differences in our experiemental design regarding the delivery of the skin-breaking stimulus also may influence findings. We undertook interventions 10 times daily for 6 days, based on the reported median exposure to painful procedures during NICU care (Roofthooft et al., 2014). Parallel to our findings on long-term memory impairment of early pain exposure, Henderson and colleagues showed that even a single injection of formalin in the hindpaw of infant rats at birth altered hippocampal-dependent memory in adulthood (Henderson et al., 2015). A study in rat pups exposed to four daily hindpaw needle-pricks (24G) or slight touch during the first 7 days of life showed effects on fear conditioning (auditory freezing only) at post-weaning, adolescence, and adulthood ages (i.e., Ps 24, 45, 66) (Davis et al., 2018). Similar to our findings, they failed to show an effect on long-term sensory thresholds unless rats were exposed to fear conditioning prior to testing. Indeed, re-exposing rats to a stressful condition seems to be required to induce a mechanical hypersensitivity at P27 only (not at P48 or 69) and this in both touched and pricked rats. Thus, since we did not perform a pain re-exposure in adulthood, these latter findings may explain why we did not find any significant differences in pain thresholds at adulthood between groups in our study.

Our recent findings of adverse effects of pain and sucrose on adult regional brain volumes (Tremblay et al., 2017b) and behavioral outcomes in adult mice reported here may be related to specific neurotransmitter system alterations. Sucrose, with its opioid-like effect (Spangler et al., 2004), may be acting through the mesolimbic system (i.e., reward system in the brain). It has been established that dopamine neurons respond to aversive and/or rewarding stimuli, consequentally some neurons may be releasing dopamine in response to both punishing (e.g., pain) and rewarding (e.g., sucrose) stimulants (Bromberg-Martin et al., 2010). In this context, dopamine released in the mesolimbic system can modulate the salience of pain stimuli (Taylor et al., 2016), that is, tuning into the associated reward of pain relief. It is possible that if sucrose and pain are repetitively given in combination during a critical period of brain development, this "double-hit" of nearly constant dopamine release could eventually have detrimental effects on the brain regions involved and consequently affect motivation/reward-like behaviors. Given that sucrose is currently being administered to thousands of very preterm infants for procedural pain managent in NICUs worldwide, it is imperative to conduct further research to investigate the implications of these exposures on the mesolimbic dopaminergic and reward/motivation systems by using animal models.

A potentially important difference in our studies was the addition of a non-nursing ICR female mouse to the litter to support the dam and improve survival of mouse pups. The ICR female likely added a buffering effect on pain through increased grooming and nurturing of the mouse pups during this early-life stress exposure. Maternal behavior (grooming and licking) has been shown to modulate effects of early pain exposure in rats (Walker et al., 2003; de Medeiros et al., 2009), where increased maternal behaviors reduced inflammation in response to neonatal formalin injections during the first 2 weeks of life of rat pups and thermal sensitivity in adulthood. Strong evidence in both pre-clinical and human studies emphasizes the importance of early life sensory stimulation embedded in mother-infant nurturing interactions for shaping neurodevelopment (Myers et al., 2015; Welch et al., 2015, 2017; reviewed in Curley and Champagne, 2016). Importantly here, we did not find any

protective effect of sucrose on pain since adult mice that received early repetitive painful stimuli irrespective of receiving water or sucrose as pre-treatment had similar long-term behavioral outcomes. The additional "maternal care" from the ICR non-nursing mouse may have been more powerful than sucrose at not only helping survival of our pups but also at alleviating the pain-related stress. Similarly, naturally occurring variations in maternal rearing behaviors in rodents impact neurobehavioral development, the stress-response system and gene expression (as reviewed in Curley and Champagne, 2016; Bedrosian et al., 2018); low compared to high licking/grooming mothers during the postnatal period results in poorer neurodevelopment in the offspring. Communal nesting, whereby several lactating females care for a pooled group of offspring, which is similar to our current model, has demonstrated comparable beneficial developmental effects on pups (as reviewed in Curley and Champagne, 2016). The focus of our current study was not to examine maternal rearing. Given that we did not monitor maternal behaviors of dam and ICR mouse, we can only speculate on the possible buffering effect of our communal nurturing set-up. Additonal research is needed to examine these interactions and possible mitigating effects of maternal care.

CONCLUSION

Based on our current and previous findings using the same mouse model of early repetitive pain and sucrose exposure, we have shown that sucrose was not protective for long-term adverse effects of procedural pain on both brain development and behavioral outcomes such as memory function. Most importantly, in the absence of pain, neonatal sucrose seems to negatively impact brain volumes and adversely affect adult memory. Animal research needs to be interpreted with caution and cannot be directly applied to humans. Nonetheless, given the world-wide use of sucrose for procedural pain treatment in the preterm population and the growing evidence in regards to possible detrimental effects of repetitve sucrose exposure during a period of developmental vulnerability of preterm infants, cautious use of this standard of care procedural pain management strategy in this fragile population is advocated. More clinical longitudinal studies assessing the long-term effects of early exposure to sucrose are urgently needed. Current studies show that human touch-based treatments (e.g., skin-to-skin, facilitated tucking, maternal touch) appear to protect brain development (Milgrom et al., 2010; Myers et al., 2015; Olsson et al., 2016; Maitre et al., 2017), therefore may be better alternatives for treatment of procedural pain in preterm infants.

AUTHOR CONTRIBUTIONS

MR, ST, LH, RG, and DG participated in conception, design of research, and interpreted results of experiments. MR and ST performed the experiments, prepared the figures and tables, and drafted the manuscript. MR, ST, and CC analyzed the data. All authors edited and revised the manuscript and approved the final version of manuscript.

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Conflict of Interest Statement: LH is a lead inventor of a medical device for pain management for preterm infants, for which she could receive remunerations in the future.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Psychology

ORIGINAL RESEARCH
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Persistence of Effects of VLBW/PT Birth Status and Maternal Emotional Availability (EA) on Child EA Trajectories

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Few studies have examined the longitudinal impact of birth status on the infant–mother relationship and on children's socio-emotional development. In the present study we investigated developmental patterns of such relationships [using the Emotional Availability (EA) Scales] in fullterm and VLBW/PT infants from infancy to emerging school age. Our objectives were to: (a) model the developmental trajectories of EA dimensions (maternal sensitivity, structuring, non-hostility; child responsiveness, involvement) in a VLBW/PT and fullterm sample, (b) identify potential effects of VLBW/PT status on these trajectories, and (c) determine whether the effects of VLBW/PT status on children's socio-emotional development (child EA) remained after accounting for the effect of maternal EA. Child–mother dyads (n = 109) were observed in home-based interactions (face-to-face and free play) when children were 6, 12, 18, and 57-months-old in fullterm (37–41 weeks, >2500 g; n = 48) and healthy VLBW/PT (26–32 weeks gestation, birth weight 800–1500 g, corrected for gestational age; n = 61) children. Developmental trajectories of maternal and child EA were assessed using multilevel growth modeling in Mplus. Results indicated that, even after controlling for maternal EA, there was a persistent negative effect of VLBW/PT birth status on child EA trajectories. Both initially and over time, VLBW/PT infants lagged behind their fullterm counterparts on levels of responsiveness and involvement with mothers. There was also a persistent positive effect of maternal EA (sensitivity and structuring) on child EA trajectories. Higher average levels of maternal sensitivity and structuring across time were also associated with higher and persistent levels of child responsiveness and involvement of their mothers. Importantly, results held after modeling both effects together, and after controlling for maternal education and child gender. Our results have implications for VLBW/PT children's development, the parent–child relationship, and integrating family level factors and relationship dimensions in early prevention and intervention programs.

Keywords: very low birth weight (VLBW) preterm (PT), mother–child relationship, developmental patterns over time, socio-emotional development, longitudinal, adversity

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INTRODUCTION

Biological birth status, and in particular premature birth, has long been considered of great consequence by developmental and health researchers and demonstrated to be a consequential risk factor for healthy development. Those children not only born preterm but very preterm (32 weeks' gestation and less) and/or with a very low birth weight (VLBW; less than 1500 g) are considered at even higher risk for adverse and multiple short and long term developmental and behavioral outcomes (Tessier and Nadeau, 2007; Delonis et al., 2017; Zelkowitz, 2017; Scott et al., 2018). Improvements in medical technology and perinatal and intensive neonatal care have resulted in a growing number of children born very preterm and/or VLBW. As a result, critical questions have arisen related to the quality, stability, and patterns of developmental outcomes in these new biologically at-risk survivors. It is commonly accepted that most non-disabled survivors tend to experience motor and cognitive delays (Brydges et al., 2018), language delays (Zimmerman, 2018), and more "subtle" problems such as deficits in mathematics, reading, and spelling, attention and behavioral problems (e.g., Breeman et al., 2016; Scott et al., 2018), and deficits in executive functions (e.g., Brydges et al., 2018), which persist throughout childhood (e.g., Aarnoudse-Moens et al., 2009; Chan et al., 2016). In addition, these children continue to lag behind their peers as they transition into adulthood (e.g., Aarnoudse-Moens et al., 2009). The present study addresses an important gap in this literature by examining the early mother–child relationship and socio-emotional development of VLBW preterm child–mother dyads.

While abundant research attention has been devoted to cognitive-related processes and intellectual outcomes, less is known about VLBW preterm (VLBW/PT) children's social and emotional development. Yet it appears that these children have difficulties in social adjustment and interactions with others and are generally less socially competent (Spittle et al., 2009; Zmyj et al., 2017). For example, they have been shown to have difficulties self-regulating and communicating (Nadeau et al., 2018). In addition, a few studies have examined emotion regulation strategies in VLBW/PT infant–mother dyads at 4 (Yaari et al., 2018), 6 (Jean and Stack, 2012), and up to 18 months (Atkinson et al., 2018), and results suggest subtle effects. There is also research that has examined neurodevelopmental vulnerabilities and parenting (e.g., sensitivity) as they relate to regulatory problems from birth to 18 months (Bilgin and Wolke, 2017). Despite a few studies having used short-term longitudinal designs and showed such effects as lower dyadic interaction quality (Delonis et al., 2017), little is known about the longitudinal socio-emotional processes and longer-term outcomes of these children; in particular, the impact of being VLBW/PT on socio-emotional development, how the mother–child relationship may influence such impact, the nature of the relationship patterns over time, and the persistence of any effects.

Yet establishing close relationships and connections with others promotes individual well-being (Emde and Spicer, 2000; Stack et al., 2012) while failure to do so can result in emotional and physical distress (Conger et al., 2000). Mother–child relationships form the foundation for children's socio-emotional development and their future relationships. These positive relationships often foster resiliency and protect against adversities throughout children's development (Musick et al., 1987; Luthar, 2006). However, a multitude of diverse conditions, including birth status (VLBW/PT), can threaten patterns of normative socio-emotional development and undermine the achievement of healthy outcomes later in life. For such at-risk children, the quintessential protective factor is a positive parent–child relationship, often with the child's mother (Luthar, 2006; Barbot et al., 2014), although father–child relationships are also clearly important. Supportive relationships, positive parenting, and avoidance of the use of specific parenting behaviors that are dysfunctional have also been underscored as factors that enhance children's adaptation (Luthar and Eisenberg, 2017). As a child develops, positive and reciprocal forms of emotional sharing are critical to the establishment and maintenance of healthy parent–child relationships (Biringen and Robinson, 1991; Aviezer et al., 1999; Bretherton, 2000; Lovas, 2005). In line with the Bioecological model (Bronfenbrenner and Morris, 2006), a consideration of contextual factors, including those at the family-level (in the present study, the relationship) are important to study and represent one layer of the larger system. Through this lens the importance of considering these relationship factors when studying the VLBW/PT child is highlighted, as these children may be more vulnerable to influences of family, demographics and environment (Giovannetti et al., 2013; Towers, 2018).

The mother–child relationship during development is therefore essential to consider in understanding the development of VLBW/PT children's growth and how relationship dimensions (child and mother) may mitigate and protect against maladaptive development and behavior, socio-emotional and academic outcomes, and promote and build social competence and academic achievement within this at-risk population. VLBW/PT infants (and their mothers) may be at risk-for relationship problems, due in part to their vulnerability and fragility, length of hospitalizations, and specific behaviors. During social interactions, VLBW/PT infants are known to be less alert, more excitable, harder to soothe, and have poorer self-regulation (Jean and Stack, 2012; Provenzi et al., 2017) compared to fullterm infants. Thus, they are poor social partners, and often demonstrate fewer relationship building behaviors, including co-regulation (Doiron and Stack, 2017), making it potentially more difficult for mothers to engage with their infants optimally. Yet, the mother (and father) can be integral in mediating and fostering their social development (e.g., Montagna and Nosarti, 2016; Zmyj et al., 2017) through the frequent interactions that take place as their relationship develops. Because few studies have examined the longitudinal impact of birth status on the infant–mother relationship and because of the crucial value of this information for understanding developmental and adaptive functioning and targets for intervention, we investigated developmental patterns of such relationships and socio-emotional development [using the Emotional Availability

(EA) Scales; Biringen et al., 2014] in fullterm and VLBW/PT children. We were particularly interested in the influence of VLBW/PT status on developmental trajectories in EA from infancy to emerging school age.

In considering relationships and relationship dimensions, a number of researchers have underscored maternal sensitivity as a protective factor against difficulties in the development of preterm infants (e.g., Faure et al., 2017; Neri et al., 2017; Provenzi et al., 2017; Zmyj et al., 2017). As a result, sensitivity is a variable that many believe should be measured as well as then linked to measure associations with children's socio-emotional development. Parental structuring and/or scaffolding, as well as directive and non-directive guidance are also considered to be important parenting practices (Vygotsky, 1978; Blandon and Volling, 2008; Briscoe et al., 2017) linked to children's socio-emotional outcomes. Similarly, the child's responsiveness to the mother and the inherent reciprocity in healthy social exchanges and emotional development is another integral factor to consider (e.g., Biringen and Robinson, 1991; Aviezer et al., 1999; Bretherton, 2000; Lovas, 2005). Together with the aforementioned studies on social interaction and dyadic quality, findings highlight the crucial way with which the parent–child relationship is implicated in VLBW/PT infants' social and emotional outcomes. However, no prior studies to our knowledge have examined developmental patterns of change in the mother–child relationship in fullterm and VLBW/PT child–mother dyads from infancy to emerging school age using a four-wave design, and none have examined the effects of being VLBW/PT on these trajectories over time.

The EA Scales are well-suited to capturing critical aspects (i.e., sensitivity) of the relationships between parents and their children, both early in a child life, and as they grow older (Biringen and Easterbrooks, 2012). Put simply, EA is a relational construct that encapsulates mothers' and children's ability to well-regulate their interactions (Emde, 1980; Emde and Spicer, 2000), while taking into account both partners' behaviors (Biringen, 2000). By using a multidimensional framework, the EA scales measure (via dyadic observational codes) parent-specific (parent EA), and child-specific (child EA) interactive behaviors that are widely regarded as important indicators of socio-emotional development. A growing body of evidence shows that the EA Scales reflect key indicators of the quality of the parent–child relationship and the child's socio-emotional development (child EA) (see 2012 special issue in Developmental and Psychopathology; for reviews see Biringen, 2000; Biringen and Easterbrooks, 2012; Biringen et al., 2014). In the present study, we focused on all dimensions of the EA Scales: young children's EA as captured by two dimensions (i.e., responsiveness and involvement) and mothers' EA as captured by three dimensions (i.e., sensitivity, structuring, and non-hostility) that are evaluated and coded observationally by the EA Scales (Biringen et al., 1998). Four of these five EA dimensions were measured at four occasions and one dimension (child involvement) at three occasions, from infancy to emerging school age in fullterm and VLBW/PT child–mother dyads; this enabled an examination of growth trajectories in the relationship and in socio-emotional development (child EA) over time in a group considered at-risk and born under adversity.

This examination of growth trajectories is both crucial and timely. A central goal in developmental research is to identify intra-individual and inter-individual developmental patterns and predictors of human development. Most of the studies that have examined this issue rely upon cross-sectional or two-wave designs that do not provide a sufficient basis for studying developmental patterns (Willett et al., 1998). Indeed, a limited number of studies have followed VLBW/PT children over time using a prospective longitudinal design with repeated measures. As such, due to this dearth of research, there is a limited understanding of the developmental trajectories of these children across time and the course and persistence of socio-emotional problems in this population. Considering the increasing rates of VLBW and PT births around the world, more multi-wave longitudinal studies examining the developmental trajectories of VLBW/PT children are needed. In the present longitudinal study with four measurement occasions embedded within a developmental framework, we examined intra- and inter-individual changes of an important aspect of the mother–child relationship and children's socio-emotional development in fullterm and VLBW/PT child–mother dyads, by using a multilevel growth modeling approach (Hedeker, 2004; Burchinal et al., 2006).

Consistent with Cicchetti and colleagues' developmental psychopathology framework (e.g., Barnett et al., 1993; Cicchetti, 2006; Cicchetti and Toth, 2009), to best understand the underlying mechanisms driving the appearance and maintenance of maladaptive and disordered behavior, and to identify ways to circumvent them, it is important to investigate all pathways to adaptive and maladaptive outcomes throughout development. To identify such pathways, developmental researchers are encouraged to examine risk and protective factors, often by analyzing associations across multiple levels so as to identify potential avenues for prevention and intervention for those most at risk for developing later disorders. Examining EA and the developmental trajectories in a sample including both fullterm and VLBW/PT child–mother dyads allows us to study a population varying in risk and provides an important means of understanding the pathways to adaptive and maladaptive outcomes. Both the Bioecological and the developmental psychopathology models framed the present study.

The primary objective of this study was to explore birth status as a risk factor for developmental patterns of change in the mother–child relationship and in child EA in a sample of typically developing fullterm and VLBW/PT children from infancy to emerging school age. The specific objectives of this study were threefold. As a needed intermediate step in investigating the effect of VLBW/PT status on EA, the first objective was to model and describe the developmental trajectories of five components of EA: maternal sensitivity, structuring and non-hostility, and child responsiveness and involvement, from infancy through emerging school age in a sample including both VLBW/PT

and fullterm children. The second objective was to identify potential effects of being VLBW/PT on these trajectories. We anticipated that VLBW/PT status would have a negative effect on these trajectories, particularly on child EA trajectories, if effects were revealed. The third objective was to determine whether the effect of VLBW/PT status on children's socio-emotional development (child EA) remained after accounting for a potential protective effect of maternal EA (a known predictor of children's socio-emotional development; Matte-Gagné et al., 2018). The persistence of effects has rarely been examined, and never with child EA and VLBW/PT birth status.

A longitudinal design with four time points (three for child involvement) was used. The trajectories were explored using multilevel growth modeling. This growth modeling technique provides strong statistical methods that are useful in describing individual developmental patterns and determining their predictors (Burchinal et al., 2006). Critically, this technique also allows for the partitioning of mother–child associations into within-dyad and between-dyad components, thereby isolating each mother's contribution to their specific child's socio-emotional development, while also estimating the influence of birth status and other important characteristics (e.g., child gender, maternal education). Finally, we conducted this with a group of VLBW/PT infants who were intensively screened, all serious complicating medical conditions were ruled out (see section "Materials and Methods"), and who were corrected for gestational age. Consequently, results can be largely attributed to early birth and VLBW and not to complicating medical factors common in this population.

MATERIALS AND METHODS

Participants

A total of 109 child–mother dyads were observed in interactions in their homes when children were approximately 6, 12, 18, and 57-months old. Following ethics review and approval (Hospital and University), 48 fullterm and 61 healthy VLBW/PT infants were recruited from a major community teaching hospital (Montréal, QC) at the same time and by the same research team for the present study in collaboration with the VLBW follow-up clinic and the chief Neonatologist. When VLBW/PT infants were between 3 and 4 months of age, nurses pre-screened the infants for medical issues and performed assessments of various health-related variables. Only those VLBW/PT infants who were healthy, were between 800 and 1500 g, and were living with their biological mothers, were included in the current study. We excluded infants who suffered from major illnesses or medical complications (e.g., retinopathy, hydrocephalus, neurological impairments, hearing problems, Grade IV intra-ventricular hemorrhage, etc.); infants with congenital abnormalities; infants who were frequently hospitalized since the neonatal period; infants of teenage (<18 years) or diabetic mothers; and infants with mothers who experienced a history of drug-abuse, mental illness, sexual assault, or inadequate prenatal care.

Mothers of VLBW/PT infants who were retained in the current study were informed of the current study's purpose. If they were interested in participating, they were contacted by telephone and were asked to voluntarily participate. Fullterm dyads were recruited using birth records from the same hospital, and were limited to those dyads with infants of normal birth weight (>2500 g) who were born between 37 and 41 weeks of gestation and had medical histories with no major health complications. Qualifying dyads received a letter requesting their participation and were contacted in the same manner as the VLBW/PT dyads.

Data in the current study were collected at four different time points, based on the ages of the children. All VLBW/PT infants were corrected for gestational age. VLBW/PT infants were 5.75 months old at T1 (SD = 0.52), 12.59 months old at T2 (SD = 0.51), 18.55 months old at T3 (SD = 0.55), and 59.13 months old at T4 (SD = 8.24). Fullterm infants were 5.42 months old at T1 (SD = 0.24), 12.44 months old at T2 (SD = 0.48), 18.53 months old at T3 (SD = 0.56), and 56.45 months old at T4 (SD = 5.79). **Table 1** provides information on demographic and medical variables for families with VLBW/PT and fullterm infants. At T1, t-tests revealed that mothers of fullterm children, when compared to mothers of VLBW/PT children, were somewhat younger [$t(109) = 2.29$, $p = 0.024$, $d = 0.65$; $M_{\text{fullterm}} = 30.23$ years; $M_{\text{VLBW/PT}} = 32.64$ years], and more educated [$t(109) = 3.44$, $p = 0.001$, $d = 0.43$; $M_{\text{fullterm}} = 14.52$ years; $M_{\text{VLBW/PT}} = 13.11$ years].

A small to moderate proportion of dyads did not participate at specific time points. For mother-VLBW/PT child dyads, 13 (21.3%) dropped out after T1, 4 (6.6%) dropped out after T2, 7 (11.5%) dropped out after T3. An additional 13 (21.3%) mother-VLBW/PT child dyads did not participate at one or two earlier time points, but had rejoined the study by T4. For

TABLE 1 | Demographic and medical characteristics for fullterm and VLBW/PT infants.

	Fullterm (n = 48)		VLBW/PT (n = 61)	
	M	***SD***	***M***	***SD***
Maternal age (years)*	30.22	5.01	32.63	5.58
Maternal education at birth**	14.52	2.06	13.11	2.11
Infant birth weight (g)***	3504	0.43	1097	0.27
Infant gestational age (weeks)***	39.54	1.11	28.54	2.31
Emergency C-section (%)***	35.00	0.63	0.80	0.51
1 min APGAR***	8.63	0.97	5.80	2.24
5 min APGAR***	9.19	0.54	7.80	1.43
Length of hospital stay (days)***	3.69	4.08	64.15	30.50
Infant length at birth (cm)***	50.67	4.42	37.37	3.43
Infant head circumference (cm)***	35.00	1.56	26.51	2.39
Infant weight at 6 months (g)	6904	994	6662	1072
Infant height at 6 months (cm)*	64.26	4.26	62.56	4.03
Infant age at 6 months (months and days)***	5.42	0.24	5.74	0.51

*Mean differences between groups were evaluated using t-tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed).*

mother-fullterm child dyads, 7 (14.6%) dropped out after T1, 4 (8.3%) dropped out after T2, 9 (18.8%) dropped out after T3. An additional 2 (4.2%) mother-fullterm child dyads did not participate at one or two earlier time points, but had rejoined the study by T4. In total, 26 (54.2%) mother-VLBW/PT child dyads and 24 (39.3%) mother-fullterm child dyads participated at all time points. An ANOVA did not reveal any significant differences on child EA at T1 depending on the number of waves of participation, or on its interaction with infants' birth status.

Procedure

As part of a larger study, the current study included a series of questionnaires, interviews, and naturalistic observations taking place in participants' homes. The home visits were approximately 90 min and occurred only at times when the mother believed they were the best timing for their child (e.g., when the child was well-rested, well-fed, and was alert). At each time point in the current study, researchers visited participating families and explained the overall procedure to mothers, asked mothers to sign an informed consent form, and set up the camera and study materials. Next, researchers requested that mothers take part in a short interview and complete a series of questionnaires assessing family socio-demographics. Upon their completion (roughly 20–30 min later), if mothers and children felt comfortable, they were asked to interact and play with each other as they normally would.

At T1 (when infants were roughly 6 months old), mother–infant dyads participated in two separate 2-min videotaped sessions where each member interacted with each other face-to-face. Infants were placed in an infant seat in front of mothers. Each of the 2-min periods came before and after a 2-min still face period where mothers were intrusted to hold a 'still face' (Still-Face procedure; Tronick et al., 1978). For the current study, we rely on coded EA data observed during the first "Normal" face-to-face period. At T2 (when children were roughly 12 months old), T3 (18 months old), and T4 (57 months old), mother–child dyads were instructed to engage in a 15-min free-play task utilizing standardized (i.e., child age-appropriate) toys while being videotaped in a well-lit room with minimal distractions. These toys included puzzles, doll, building blocks, books, and a tea set. Neither experimenters nor other family members were allowed in the room while interactions were taking place.

Measures

Demographic Information Questionnaire (DIQ)

Socio-demographic information was collected using the DIQ at T1 and included the following variables among others: mothers' current age, mothers' occupational status, mothers' education, children's age, and children's gender. Mothers' education was calculated by taking the maximum number of years of education for each participant, with values ranging from the completion of elementary school to the completion of college or university. Child gender was coded as 1 = *male*, 2 = *female*. The DIQ measure has proven effective in collecting participant demographics, and has been used in past studies (e.g., Serbin et al., 1998; De Genna et al., 2006; Briscoe et al., 2017).

EA Scales

To assess the quality of mother–child interactions, we coded dyadic interactions via the EA Scales (2nd Ed; Biringen et al., 1988, 1993). At the onset of the study (T1), only the 2nd edition of this measure was available. In order to maintain consistency in the manner EA was assessed, we retained this measure over all following time points (T2–T4). We assessed both mother and child dimensions of EA because both have been identified as important indicators of socio-emotional development. As such, the following dimensions were coded by observers during interaction sessions at each time point:

(1) *maternal sensitivity* to children's emotional needs and cues (e.g., maternal behaviors which both reflect a clear understanding of the child's emotions and provide an emotionally sensitive and developmentally appropriate response).
(2) *maternal structuring* of dyadic interactions as a function of the child's emotional needs (e.g., maternal behaviors which establish and reinforce limits while following the child's initiations).
(3) *maternal non-hostility* (e.g., maternal behaviors that are consistent with pleasantness, non-criticalness, patience, and that are non-rejecting or antagonistic).
(4) *child emotional and social responsiveness* to the mother (e.g., child behaviors that convey a willingness to engage with mothers, and expressions of clear enjoyment while doing so).
(5) *child involvement* (e.g., child behaviors which attend to, initiate, and are involving of mothers' interactions in play).

Codings were on 5- or 9-point scales. For the current study, we inverted the maternal non-hostility scores and termed the scale "hostility" (as in Stack et al., 2012). The upper-end of these five scales respectively represent optimal levels of maternal sensitivity and structuring, overtly hostile behaviors, and optimal levels of child responsiveness and involvement. All EA Scales except child involvement were coded at all four time points. Child involvement was only first coded at T2 (12 months) consistent with the measure. We provide further detail on how this difference affects the current study's design in the analytical plan below.

Past studies have demonstrated the validity of the EA Scales in measuring the EA of parents and children at different ages (Bornstein et al., 2012). While it is ideal to allow for longer periods of observation (Biringen et al., 2005), extensive research has reliably assessed EA in parent–child interactions using the EA scales in relatively short time periods (e.g., 5- to 15-min) across a wide range of contexts (for a review, see Biringen et al., 2014). Many studies have established the predictive and convergent validity of the EA scales; for example, EA scales have been found to associate with maternal depression (Easterbrooks et al., 2012), child attachment (Easterbrooks et al., 2012), adult attachment representations (Coppola et al., 2006), child emotion understanding (Garvin et al., 2012), family SES (Chaudhuri et al., 2009), child goal encoding (Licata et al., 2014), infant emotion regulation (Little and Carter, 2005), and infant sleep patterns (Scher, 2001). The EA construct and its scales

have received abundant research attention with studies ranging across age (0–14 years) and in normative, clinical and high-risk populations (e.g., feeding disorders; intellectual disabilities; high-risk community sample; disadvantaged; drug exposed and depressed mothers; see Biringen et al. (2014). For a more detailed description of the EA Scales, see Biringen and Easterbrooks, 2012; Biringen et al., 2014).

After completing a 3-day training course on-site, an original set of coders was certified on the proper and reliable coding of the Biringen tapes. Subsequent training continued to be provided by our trained team. In coding the present data the same coders coded the EA of both mothers and children at all time points in order to limit the introduction of rater error to the intra-individual variability in EA across time. As a further safeguard, the coders were instructed to complete five coding passes for each video record – one for each of the five EA dimensions. At each time point, at least a quarter of the sample was randomly selected to be double-coded; reliability coefficients (ICCs; intra-class correlation coefficients) were acceptable, ranging between 0.82 and 0.99).

Analytic Strategy

We conducted multilevel growth modeling analyses using *MPlus* (version 8.1) (Muthén and Muthén, 1998-2017) with maximum likelihood with robust estimators (MLR) to describe and predict intra-individual patterns of trajectories in mother and child EA. MLM was chosen given its ability to easily handle repeated measures of the same outcomes where time points moderately vary across participants (Singer and Willett, 2003; Burchinal et al., 2006). Multilevel growth modeling estimates inter-individual variability in intra-individual patterns of change over time by decomposing change over time into two levels: a Level 1 component which represents change across time in outcomes within the individual, and a Level 2 component which represents how such patterns of change differ between individuals in a sample. As an extension, this accommodates the inclusion of both time-invariant and time-variant predictors that might account for influences on initial levels and change across time. For each EA scale, we provide intra-class correlations coefficients (ICCs) that indicate the proportions of variances in EA that vary within individuals (across time) from total variances. These estimates are equivalent to within-person stabilities of EA.

Modeling Change in Maternal and Child EA Across Time

We followed a multistep procedure for estimating change in EA across time (Singer and Willett, 2003). Consistent with the first objective, we modeled intercepts (i.e., initial levels) and slopes (i.e., yearly rates of change – or trajectories) in the five dimensions of EA. We estimated three potential models: (a) one that estimated no over time change, (b) one that estimated linear change (defined individually in years), and (c) one that estimated both linear and non-linear change. To increase parsimony, only the best fitting models were selected. Non-significant ($p > 0.05$) parameters were then trimmed for the unconditional mean models (Model A). As previously noted, in contrast to the other four EA scales, which were assessed at all four time points (6 to 57 months), child involvement was assessed only over the latter three time points (12 to 57 months) consistent with the EA measure and how it is coded. While three data points still accommodate the estimation of linear growth curves, it should be noted that the interpretations regarding the intercepts and slopes for child involvement should be limited to the 12–57 months timespan.

VLBW/PT Status as a Predictor of EA Trajectories

Next, consistent with the second objective, once the overall trajectories were established, we investigated the extent to which VLBW/PT status explained the initial levels (intercepts) and trajectories (slopes) of EA. In order to reduce the likelihood that associations found in our model were due to confounding demographic variables, we controlled for child sex and maternal education, each taken from the DIQ measure. We selected these controls because education (as part of SES) and child gender have both been associated with family interaction processes (Conger and Donnellan, 2007; Zahn-Waxler et al., 2008), and there are more preterm births among boys (Zeitlin et al., 2002) and in low-SES families (Morgen et al., 2008). Three models were estimated. In the first model (Model B), VLBW/PT status, child sex, and maternal education were entered as predictors of the EA's intercepts. In the second model (Model C), non-significant ($p > 0.05$) effects were trimmed from the prior model, and then the same variables were entered as predictors of EA's slopes. In the third model (Model D), any remaining non-significant ($p > 0.05$) fixed effects were trimmed.

Maternal EA as Predictors of Child EA

Consistent with the third objective, we then entered maternal EA as predictors of the final child EA trajectories (previously presented in Model D). To this end, as recommended by Curran and Bauer (2011) we distinguished maternal EA's between-dyad effects on child EA from their within-dyad effects. To account for between-dyad effects, we aggregated maternal sensitivity and structuring across all assessment waves, and entered them as mean-centered time-invariant predictors at Level 2. To account for within-dyad effects, we entered maternal sensitivity and structuring as person-mean centered time-variant predictors at Level 1. Maternal hostility was not entered as a predictor due to low variance (see below). Models were separately estimated for child responsiveness and child involvement. Only maternal EA data from T2, T3, and T4 were entered for child involvement because the latter was not assessed at T1. Model E describes the initial results, and Model F describes the results after non-significant fixed effects ($p > 0.05$) had been trimmed.

Handling of Missing Data

Missingness in the current study came from two-sources: attrition and wave-level missingness. Roughly 41% of the sample did not participate at one or more points in the study. A description of missingness at each wave for both groups can be found in Section "Participants." To account for missing data, a total of 20 imputed datasets were generated from a Markov chain Monte Carlo simulation in *Mplus*. Final model estimates were derived from a meta-analysis of results from each dataset. Little's

test was not statistically significant, $\chi^2(109) = 112.61$, $p = 0.39$, therefore we treated the data as missing completely at random.

RESULTS

Descriptive Statistics

Table 2 presents means, standard deviations, ranges, and correlations of maternal and child EA over all four time points of the study. Mothers demonstrated consistently high levels of sensitivity and structuring and consistently low levels of hostility over the course of the study. Child responsiveness and involvement were consistently moderately high over the course of the study. In terms of mean level changes over time, we found a minor increase in child responsiveness and involvement between the earliest time point (T1 or T2) and T4 ($t = -4.34$, $p < 0.001$ and $t = -3.09$, $p = 0.003$, respectively).

A series of chi-square difference tests investigated whether the means of each EA scale significantly differed between mother-VLBW/PT child and mother-fullterm child dyads. Estimates were pooled across the 20 imputed datasets. Results indicated that mothers of VLBW/PT children demonstrated significantly less sensitivity at T1 only, $\chi^2(1) = 3.93$, $p = 0.047$, and less structuring at T4 only, $\chi^2(1) = 8.28$, $p = 0.004$. There were no differences between the two groups on maternal hostility at any time points. VLBW/PT children were observed to be significantly lower on responsiveness at T2, T3, and T4, $\chi^2(1) = 4.26$–11.52, $p = 0.001$–0.039, lower on involvement at T2, $\chi^2(1) = 11.35$, $p = 0.001$, and marginally lower on involvement at T3, $\chi^2(1) = 3.50$, $p = 0.061$. Taken together, these analyses provided preliminary evidence of initial, and in some cases, persistent and ongoing gaps in EA between mother-VLBW/PT child and mother-fullterm child dyads. However, growth curve analyses are better suited for detecting ongoing and stable differences between groups on maternal and child EA. These are described below.

Growth Curves in Maternal and Child EA

Consistent with the first study objective, multilevel modeling (MLM) was conducted to estimate the developmental trajectories of the five components of EA. First, we estimated whether there was enough evidence of variation in trajectories of EA to justify more complex analyses.

Results of the unconditional mean models (see Model A in **Tables 3** through **6**) revealed sufficient ($p < 0.10$) within-person variation, or change over time, on four out of the five EA scales; we did not find significant variation for maternal hostility, thereby excluding the scale from further analyses. Notably, we also found evidence of moderate stability in EA, as indicated by intra-class correlations coefficients (ICCs; proportions of within-person variation to total variation) ranging from 0.16 to 0.34.

We also estimated the intercepts and slopes of mean-levels of EA. Results are presented below.

Maternal EA

As seen in Model A in **Tables 3**, **4** the means of the intercepts of maternal sensitivity and structuring ($\gamma_{00} = 7.60$ and 4.28) and their variances ($\sigma_0^2 = 0.32$ and 0.13) were statistically significant. In terms of slopes, sensitivity non-linearly decreased over time ($\gamma_{10} = -0.36$ and $\gamma_{20} = 0.06$), and structuring decreased linearly over

TABLE 2 | Descriptive statistics and correlations across child age (relative stability) for the Emotional Availability (EA) Scales.

		Fullterm		VLBW/PT			Correlations across age (stability)		
EA dimensions	**Age**	***M***	***SD***	***M***	***SD***	**Range**	**12**	**18**	**57**
Sensitivity	6	7.90	0.86	7.53	1.00	3.00–9.00	0.17	0.42**	0.36**
	12	7.47	0.99	7.26	1.14	3.00–9.00	–	0.23^t	0.25*
	18	7.32	1.12	7.06	1.17	1.00–9.00	–	–	0.37**
	57	7.44	0.91	7.01	1.03	5.00–9.00	–	–	–
Structuring	6	4.44	0.56	4.32	0.69	2.00–5.00	0.19	0.28**	0.21^t
	12	4.37	0.60	4.13	0.77	3.00–5.00	–	0.29**	0.34**
	18	4.19	0.67	4.02	0.77	2.00–5.00	–	–	0.24*
	57	4.33	0.69	3.87	0.81	2.00–5.00	–	–	–
Hostility	6	1.02	0.14	1.10	0.42	2.00–5.00	0.38*	0.39**	0.33^t
	12	1.12	0.32	1.18	0.60	1.00–5.00	–	0.68**	0.41*
	18	1.08	0.23	1.20	0.63	1.00–5.00	–	–	0.47**
	57	1.16	0.42	1.15	0.41	1.00–3.00	–	–	–
Child responsiveness	6	4.90	1.65	4.90	1.38	1.00–7.00	0.11	0.09	0.11
	12	5.81	0.95	4.99	1.36	2.00–7.00	–	0.34**	0.27*
	18	5.59	1.08	5.06	1.12	2.00–7.50	–	–	0.21^t
	57	6.13	0.98	5.59	1.14	3.00–7.00	–	–	–
Child involvement	12	5.42	1.12	4.50	1.48	2.00–7.00	–	0.33**	0.23^t
	18	5.26	1.32	4.61	1.45	1.00–7.00	–	–	0.25*
	57	5.83	1.26	5.34	1.53	3.00–7.00	–	–	–

*N = 48 fullterm and 61 VLBW/PT children. Ages are in months. $^t p < 0.10$, *$p < 0.05$, **$p < 0.01$ (two-tailed).*

TABLE 3 | The growth models of maternal sensitivity between 6 and 57 months.

ICC = 0.29			Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{00}	7.60*** (0.09)	7.60*** (0.08)	7.61*** (0.09)	7.60*** (0.09)
	Maternal education	γ_{01}		0.11** (0.03)	0.11** (0.04)	0.12*** (0.03)
	Sex (being a girl)	γ_{02}		0.12 (0.14)		
	VLBW/PT status	γ_{03}		−0.15 (0.14)		
Rate of change	Intercept	γ_{10}	−0.36** (0.12)	−0.37** (0.12)	−0.40** (0.13)	−0.36** (0.12)
	Maternal education	γ_{11}			0.02 (0.06)	
	Sex (being a girl)	γ_{12}			−0.02 (0.21)	
	VLBW/PT status	γ_{13}			0.09 (0.24)	
Quadratic rate of change	Intercept	γ_{20}	0.06* (0.03)	0.06* (0.03)	0.07** (0.03)	0.06* (0.03)
	Maternal education	γ_{21}			0.00 (0.01)	
	Sex (being a girl)	γ_{22}			0.01 (0.05)	
	VLBW/PT status	γ_{23}			−0.04 (0.06)	
Variance/residual variance components						
Level 1	Within-person	σ^2_E	0.79*** (0.13)	0.79*** (0.13)	0.78*** (0.13)	0.79*** (0.13)
Level 2	In initial status	σ^2_0	0.32** (0.09)	0.24** (0.08)	0.25** (0.08)	0.25** (0.08)
Goodness-of-fit	LL		−621.30	−611.92	−610.31	−613.21
	AIC		1252.60	1239.84	1244.61	1238.43
	BIC		1272.99	1272.46	1293.55	1262.89

*N = 109. Standard errors are within parentheses. Rate of change is estimated in years. VLBW/PT, very low birth weight/preterm; LL, log likelihood; AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion. *p < 0.05, **p < 0.01, ***p < 0.001.*

TABLE 4 | The growth models of maternal structuring between 6 and 57 months.

ICC = 0.26			Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{00}	4.28*** (0.05)	4.28*** (0.05)	4.28*** (0.05)	4.28*** (0.05)
	Maternal education	γ_{01}		0.08** (0.03)	0.09** (0.03)	0.08** (0.02)
	Sex (being a girl)	γ_{02}		0.07 (0.09)		
	VLBW/PT status	γ_{03}		−0.12 (0.09)		
Rate of change	Intercept	γ_{10}	−0.06* (0.02)	−0.06* (0.02)	−0.05* (0.02)	−0.05* (0.02)
	Maternal education	γ_{11}			−0.01 (0.01)	
	Sex (being a girl)	γ_{12}			0.01 (0.04)	
	VLBW/PT status	γ_{13}			−0.08* (0.04)	−0.08* (0.04)
Variance/residual variance components						
Level 1	Within-person	σ^2_E	0.39*** (0.04)	0.39*** (0.04)	0.39*** (0.04)	0.39*** (0.04)
Level 2	In initial status	σ^2_0	0.13** (0.04)	0.09** (0.03)	0.09** (0.03)	0.09** (0.03)
Goodness-of-fit	LL		−461.81	−449.90	−447.63	−448.31
	AIC		931.61	913.80	911.26	908.61
	BIC		947.92	942.35	943.88	933.08

*N = 109. Standard errors are within parentheses. Rate of change is estimated in years. VLBW/PT, very low birth weight/preterm; LL, log likelihood; AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion. *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed).*

time (γ_{10} = −0.06). All variances in the rates of change for sensitivity and structuring, and their covariances with their respective intercepts, were non-significant and were trimmed.

Child EA

As seen in Model A in **Tables 5**, **6** the means of the intercepts of child responsiveness and child involvement (γ_{00} = 5.08 and 4.76) and their variances (σ^2_0 = 0.27 and 0.55) were statistically significant. In terms of slopes, both responsiveness and involvement linearly increased over time (γ_{10} = 0.18 for both). All variances in the rates of change for child responsiveness and involvement, and their covariances with their respective intercepts, were non-significant and were trimmed.

Associations Between VLBW/PT Status and EA

Consistent with the second study objective, we estimated the effect of VLBW/PT status on the developmental trajectories (the intercepts and the slopes) of EA (see Model D in **Tables 3** through **6**).

TABLE 5 | The growth models of child responsiveness between 6 and 57 months.

ICC = 0.16			Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{00}	5.08*** (0.10)	5.08*** (0.10)	5.08*** (0.10)	5.08*** (0.10)
	Maternal education	γ_{01}		0.07 (0.04)		
	Sex (being a girl)	γ_{02}		0.14 (0.16)		
	VLBW/PT status	γ_{03}		−0.37* (0.16)	−0.39* (0.19)	−0.48** (0.15)
Rate of change	Intercept	γ_{10}	0.18*** (0.04)	0.18*** (0.04)	0.18*** (0.04)	0.18*** (0.04)
	Maternal education	γ_{11}			0.02 (0.02)	
	Sex (being a girl)	γ_{12}			−0.01 (0.06)	
	VLBW/PT status	γ_{13}			−0.03 (0.08)	
Variance/residual variance components						
Level 1	Within-person	σ^2_E	1.35*** (0.14)	1.35*** (0.14)	1.34*** (0.14)	1.35*** (0.14)
Level 2	In initial status	σ^2_0	0.27* (0.13)	0.19^t (0.10)	0.20^t (0.11)	0.21^t (0.12)
Goodness-of-fit	LL		−715.70	−707.43	−707.92	−710.21
	AIC		1439.41	1428.86	1431.85	1430.41
	BIC		1455.72	1457.41	1464.47	1450.80

*N = 109. Standard errors are within parentheses. Rate of change is estimated in years. VLBW/PT, very low birth weight/preterm; LL, log likelihood; AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion. $^t p < 0.10$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed).*

TABLE 6 | The growth models of child involvement between 6 and 57 months.

ICC = 0.34			Model A	Model B	Model C	Model D
Fixed effects						
Initial status	Intercept	γ_{00}	4.76*** (0.15)	4.76*** (0.15)	4.76*** (0.15)	4.76*** (0.15)
	Maternal education	γ_{01}		0.09 (0.06)		
	Sex (being a girl)	γ_{02}		0.11 (0.22)		
	VLBW/PT status	γ_{03}		−0.56* (0.23)	−0.86** (0.29)	−0.70** (0.22)
Rate of change	Intercept	γ_{10}	0.18** (0.06)	0.18** (0.06)	0.18** (0.06)	0.18** (0.06)
	Maternal education	γ_{11}			0.02 (0.02)	
	Sex (being a girl)	γ_{12}			0.03 (0.07)	
	VLBW/PT status	γ_{13}			0.11 (0.11)	
Variance/residual variance components						
Level 1	Within-person	σ^2_E	1.50*** (0.21)	1.50*** (0.21)	1.50*** (0.20)	1.50*** (0.21)
Level 2	In initial status	σ^2_0	0.55** (0.19)	0.38* (0.17)	0.39* (0.17)	0.42* (0.18)
Goodness-of-fit	LL		−569.73	−560.01	−560.67	−562.76
	AIC		1147.47	1134.02	1137.33	1135.52
	BIC		1162.63	1160.55	1167.65	1154.47

*N = 109. Standard errors are within parentheses. Rate of change is estimated in years. VLBW/PT, very low birth weight/preterm; LL, log likelihood; AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed).*

Results indicated that VLBW/PT status was significantly associated with the intercepts, but not the slopes, of child responsiveness and involvement ($\gamma_{03} = -0.48$ and -0.70, $p < 0.01$). VLBW/PT children were observed to have lower stable levels of responsiveness and involvement when compared to their fullterm counterparts. Given that there were no effects of VLBW/PT status on the slopes of child responsiveness and involvement, both VLBW/PT and fullterm children linearly increased in child responsiveness and involvement at similar rates. As such, we find clear evidence that VLBW/PT children do not overcome their earlier initial with fullterm children.

Even after controlling for positive associations between maternal education and maternal EA ($\gamma_{01} = -0.08$, $p < 0.05$ for both sensitivity and structuring), VLBW/PT status was negatively associated with the linear slope of maternal structuring ($\gamma_{13} = -0.08$, $p < 0.05$). Follow up analyses revealed that only mothers of VLBW/PT children significantly decreased in structuring over time (slope = −0.08, $p = 0.002$ vs. slope = −0.01, $p = 0.69$ for fullterms).

Maternal EA as Predictors of Child EA

Consistent with the third study objective, we fit a model that allowed for the estimation of associations between maternal and child EA, then we determined if effects of VLBW/PT status remained after controlling for these associations (see Model F in **Table 7**).

As expected, results revealed the presence of significant positive associations between maternal and child EA at the

TABLE 7 | Final models for child responsiveness and child involvement.

			Child Responsiveness		Child Involvement	
			Model G	**Model H**	**Model G**	**Model H**
Fixed effects						
Initial status	Intercept	γ_{00}	5.16*** (0.09)	5.16*** (0.10)	5.07*** (0.13)	5.07*** (0.13)
	VLBW/PT status	γ_{03}	−0.23* (0.12)	−0.25* (0.12)	−0.40* (0.18)	−0.40* (0.18)
	Sensitivity mean	γ_{04}	0.61*** (0.16)	0.77*** (0.11)	0.40* (0.17)	0.40* (0.17)
	Sensitivity variation	γ_{20}	0.29** (0.09)	0.29** (0.09)	0.38*** (0.10)	0.38*** (0.10)
	Structuring mean	γ_{05}	0.21 (0.18)		0.50** (0.17)	0.50** (0.17)
	Structuring variation	γ_{30}	0.33*** (0.08)	0.33*** (0.08)	0.41*** (0.12)	0.41*** (0.12)
Rate of change	Intercept	γ_{10}	0.21*** (0.03)	0.22*** (0.04)	0.19*** (0.04)	0.19*** (0.04)
Residual variance components						
Level 1	Within-person	σ^2_E	1.13*** (0.12)	1.14*** (0.12)	1.18*** (0.16)	1.18*** (0.16)
Level 2	In initial status	σ^2_0	0.01 (0.05)	0.02 (0.05)	0.18 (0.11)	0.18 (0.11)
Goodness-of-fit	LL		−1630.86	−1632.45	−1207.37	−1207.37
	AIC		3287.72	3288.90	2444.74	2444.74
	BIC		3340.73	3337.83	2501.59	2501.59

*N = 109. Associations between the maternal EA (sensitivity and structuring) and the rate of change of child responsiveness and involvement were non-significant and were trimmed. Standard errors are within parentheses. Rate of change is estimated in years. VLBW/PT, very low birth weight/preterm; LL, log likelihood; AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion. *p < 0.05, **p < 0.01, ***p < 0.001 (two-tailed).*

within-dyad and between-dyad levels. At the within-dyad level, higher levels of maternal sensitivity and structuring at one time point predicted higher levels of child responsiveness ($\gamma_{20} = 0.29$ and $\gamma_{30} = 0.33$, $p < 0.01$) and involvement ($\gamma_{20} = 0.38$ and $\gamma_{30} = 0.41$, $p < 0.001$) at the same time point. At the between-dyad level, the intercepts of maternal sensitivity were positively associated with the intercepts of child responsiveness ($\gamma_{04} = 0.77$, $p < 0.001$) and involvement ($\gamma_{04} = 0.40$, $p < 0.05$). The intercepts of maternal structuring were only positively associated with the intercepts of child involvement ($\gamma_{05} = 0.50$, $p < 0.01$).

Importantly, independent of positive associations between maternal and child EA, VLBW/PT status continued to be negatively associated with the intercepts of child responsiveness ($\gamma_{03} = -0.25$, $p = 0.04$), and child involvement ($\gamma_{03} = -0.40$, $p = 0.03$). Taken together, even after accounting for positive within-dyad and between-dyad relationships between maternal and child EA, VLBW/PT children demonstrate lower estimated mean trajectories of child responsiveness and involvement when compared to fullterm children. Because there were no differences in the rates of change between the two groups, VLBW/PT children never overcame their initial gaps with fullterm children. Together, this pattern of results indicates that children who experienced consistently higher levels of maternal structuring and sensitivity over the course of the study, and children who were born fullterm, were both more responsive and involving of their mothers (and this effect persisted over time) compared to children who consistently experienced consistently lower levels of sensitivity and structuring or being VLBW/PT.

DISCUSSION

The effects of VLBW/PT status on the quality of the child–mother relationship and children's socio-emotional development were assessed for the first time using an advanced growth modeling approach. This was an original direction as no studies to our knowledge have examined these effects of VLBW/PT children using a four-wave design from infancy to emerging school age. The findings have important implications for researchers, clinicians and health practitioners, and parents of preterm infants, as well as for prevention and intervention programs.

The principal results from our study's growth curve analyses indicate that, even after controlling for maternal EA, there was a persistent negative effect of VLBW/PT birth status on child EA trajectories, i.e., responsiveness and involvement. After 6 months of age, VLBW/PT infants lagged behind their fullterm counterparts on their levels of responsiveness and involvement with their mothers. These findings remained even after accounting for the effects of maternal sensitivity and structuring over time. When we tested for mean level changes there was evidence for linear increases in means of responsiveness and involvement for both fullterm and VLBW/PT children, but the VLBW/PT children still continued to lag behind the fullterm children. Due to the fact that the effects persisted when maternal variables were controlled for in the models, we can be fairly confident that maternal EA are not responsible for this lagged difference in the two groups.

These results are certainly consistent with other studies that show effects of prematurity in cognitive, motor, emotion regulation, executive functioning, and other domains that continue in these children (e.g., Aarnoudse-Moens et al., 2009; Chan et al., 2016; also refer to introduction). However, studies investigating the persistent effect of preterm birth require repeated measures of the same variables and these are not common. In fact, there is a noticeable absence of long-term follow-up studies of preterm infants, particularly healthy VLBW/PT and specifically studies that address relationship and socio-emotional processes. This is the case in both clinical

settings and in empirical research. Typically, follow-up studies are conducted on the groups of infants that are not healthy (or mixed groups) and are largely focused on physical development and cognitive outcomes (as opposed to psychological or developmental follow-ups). These generally take place in medical settings and not in the home environments. Thus, less is known about the psychological and socio-emotional development of healthy preterm infants and their relationships with their parents at home and little is known about developmental trajectories over time.

Our primary study variables were the mother–child relationship, and in particular two child EA dimensions as indicators of socio-emotional development (responsiveness and involvement), suggesting that it may be social interaction behavior that is at the core of our findings. Briefly, emotional and social *responsiveness* to the parent is measured through observing a willingness to engage and active engagement and positive response and enjoyment in the interaction. *Involvement* includes paying attention to mothers, and encouraging their involvement in play sessions (for a more detailed description of the EA Scales, see Biringen and Easterbrooks, 2012; Biringen et al., 2014). Both dimensions are important indicators of socio-emotional development and both embrace an active participation and initiative on the part of the child. Our results indicate that low child EA, reflected in responsiveness and involvement of their mothers may be a risk factor for VLBW/PT children. Our findings are consistent with those of others that indicate that VLBW/PT children have difficulties in socio-emotional domains relative to their fullterm peers (e.g., Doiron and Stack, 2017; Zmyj et al., 2017; Nadeau et al., 2018). They have been demonstrated to have more limited regulatory skills (e.g., Jean and Stack, 2012; Yaari et al., 2018), show more distancing and social monitoring (Montirosso et al., 2010), and rely more on their mothers in a reunion period following the still-face (Jean and Stack, 2012). These results suggest that preterm infants rely less on their own self-regulatory abilities and more on their mothers than their fullterm counterparts. However, these latter studies were not repeated measures designs over four waves and did not examine persistence of these effects. In a short-term longitudinal study focusing on the second year of life and play at 18, 24, and 30 months, Salvatori et al. (2016) found that responsiveness and involvement improved over time and global EA was lower in the preterm groups. However, they did not find differences between ELBW, VLBW, and fullterm on any of the dimensions of EA; notably these groups were tested individually and their sample had participated in a parenting intervention prior. Similarly, Matte-Gagné et al., 2018 showed an increase in child EA across infancy, however their sample was not preterm or VLBW. Finally, in a longitudinal study from 7 to 11 years Nadeau et al., 2018 showed increasing levels of victimization and social isolation in preterm children at 11 years leading to their argument for social marginalization, and underscoring that there are longer term problems in social functioning in children born premature that warrant further investigation.

Emotional and social competencies are considered central to school readiness, early school success, academic behaviors and achievement, and are also associated with attitudes toward learning and positive adjustment (Denham et al., 2016; Pekrun et al., 2017). Social interaction and interpersonal skills are a key component of these competencies; thus our results raise cause for concern. Given that parents and caregivers are central socializing agents they are pivotal in impacting these skills through their relationship with their child, sensitivity to cues and parenting strategies, and such means as play, social exchanges, modeling and demonstrations, as well as feedback and validation. Targeting them in interventions and public health initiatives, as well as pre-natal classes and post-natal well baby clinics are important directions toward assuring timely dissemination, awareness, and ultimately for enhancing these skills in VLBW/PT children.

Complementing our study's contribution is that these results were demonstrated in a healthy VLBW/PT group and by measuring developmental patterns in relationship quality and two indicators of child socio-emotional development for the first time over four waves. That is, the conservative nature of our VLBW/PT group and the correction for gestational age largely suggest that our results are attributable to early birth and VLBW and not confounded by other medical or perinatal status variables.

Beyond the principal findings associated with the central focus of our paper, that of the effect of VLBW/PT status, there were several additional findings. Decreases in sensitivity and structuring were demonstrated however there was also a persistent positive effect of maternal EA (sensitivity and structuring) on child EA trajectories. That is, both within-dyad and between-dyad effects for maternal sensitivity and structuring were found. Regarding the former, higher levels of each at a given time point predicted higher levels of concurrent child responsiveness and involvement with mothers. Regarding the latter, across time the consistent mean levels of maternal sensitivity and maternal structuring predicted higher consistent levels of child responsiveness and child involvement with mothers. That is, children of mothers who expressed a higher average level of maternal sensitivity and structuring more often expressed a higher average level of responsiveness and were consistently more involving of their mothers across the preschool years, relative to those receiving lower maternal EA. Importantly, results held after modeling both effects together, and after controlling for maternal education and child gender. We also found that, for VLBW/PT children, maternal structuring linearly decreased over time whereas it remained stable for fullterm children. Such decreases may suggest that parenting stress associated with raising a VLBW/PT infant may result in disengagement in structuring behaviors over time. Alternatively, the decreases in structuring may suggest that because VLBW/PT infants are less engaged and involved in their interactions, mothers become less involved in the interactions over time, or less implicated in the play interaction.

Maternal sensitivity has long been considered and demonstrated to be a critical parenting dimension and has been linked to children's socio-emotional outcomes (Bohr et al., 2018). Similarly, structuring (or scaffolding; Vygotsky, 1978) is also considered a key parenting dimension, has a long history, and is integrated in many parenting studies including those with a teaching, cognitive stimulation, or learning component (e.g.,

Saltaris et al., 2004; Briscoe et al., 2017). Recently in a sample of fullterm and preterm infants, Gueron-Sela et al. (2015) showed that preterm infants had poorer cognitive outcomes at 12 months when their parents used lower levels of co-parental structuring at 6 months. However, a moderating effect of temperamental reactivity was also revealed. Together with the present findings, there is evidence to support parenting and relationship factors as positive, and in some cases protective, in that they aid in contravening adversity (Fritz et al., 2018).

Finally, across groups, mothers with more education were observed to be consistently higher in sensitivity and structuring. This is in line with past findings concerning parenting, and is suggestive of higher-level and greater breadth of knowledge bases on parenting, development and developmental expectations. In a study of long-term cognitive outcomes of ELBW children, low maternal education was associated with poorer outcomes (Voss et al., 2012 as cited in Pelc and Gajewska, 2018). However, it is important to underscore that those with lower levels of education in our current study sample were not poor on these EA dimensions. Our findings suggest that VLBW/PT children have a specific vulnerability for socio-emotional difficulties and may require increased monitoring regarding their socio-emotional development even if they are healthy and growing up in a highly educated and sensitive family environment.

Consistent with the developmental psychopathology and bioecological models, our findings underscore the importance of examining early adverse experiences (VLBW and prematurity) in at-risk populations in prospective, longitudinal designs that include multiple levels over time in order that we may more deeply understand adversity, development, and those factors that may mitigate such adversity. Early care experiences and the quality of the mother–child relationship are known to be positive and these can potentially enable and foster change and adaptive outcomes in VLBW/PT children's socio-emotional competencies where effects persist, such as responsiveness and involvement, if directly targeted. Promoting a nurturing, supportive, and caring environment early on is generally indicated (e.g., Dilworth-Bart et al., 2018).

The contributions of the present study are best evaluated within the context of several limitations and the need for further research. The nature of the four-wave prospective longitudinal design and the analysis of EA trajectories were strengths. Expanding the longitudinal design into middle and later childhood would enrich our understanding. Integrating additional variables that may mediate, or contextual factors that may contribute to the trajectories would also be an important step. Future studies should link relationship and socio-emotional variables over time to child outcomes in later childhood and adolescence and examine additional individual-level and family variables such as parental stress, mental health, and psychopathology. While studies in the broad cognitive domain have shown that deficits persist, even here the developmental pathways to adult outcomes are not clear and a number of variables likely play important roles in influencing these pathways and outcomes; these include genetic susceptibility, and environmental sources such as family and social support as moderators (Taylor, 2017). Mother, child, and contextual factors are likely to create and explain variations in the over time trajectories of maternal EA (Stack et al., 2012; Matte-Gagné et al., 2018). Worth noting is that mothers were the participants in the present study. Fathers are also an integral part of families and their role in building the relationship and influencing children's trajectories is warranted. Finally, in our sample maternal education was positively associated with maternal EA, but also differed between the two birth status groups. As such, we cannot rule out the possibility that positive associations between birth status and maternal EA may be a function of maternal education, or related factors (e.g., conscientiousness, SES, stress, etc.). For example, women who are less diligent or organized may attain less education and may be more likely to struggle when raising a VLBW/PT child. However, future studies are warranted to further examine such possibilities using different methodologies to disentangle the effects of maternal education and birth status.

Taken together our results have implications for integrating the parent–child relationship and early interactions, as well as dimensions of the child's socio-emotional competencies, in interventions and intervention programs. Importantly, given the persistence of the effects, this should take place early. While there have been some studies showing intervention effects with prematurity on various domains (Spittle et al., 2015; Evans et al., 2017; Faure et al., 2017; Fritz et al., 2018) most have focused on cognitive and motor outcomes (see also Spittle et al., 2015, for a systematic review) and few on domains relating to socio-emotional processes and competencies (Towers, 2018). A few exceptions exist (e.g., Wu et al., 2016 with a family centered RCT on emotion regulation; Fitzgerald's, 2017 commentary on the same study). Notably it is also difficult to compare intervention programs and their effects given the wide variation in program elements, foci and gestational age (Spittle et al., 2015). However, what does seem to reliably surface are parenting and family-level factors that may contravene adversity (e.g., Zmyj et al., 2017; Fritz et al., 2018). Additional research is essential in order that targeting the key variables in prevention and intervention can be furthered.

CONCLUSION

In a longitudinal design that measured relationship variables beginning in infancy through early childhood, VLBW/PT birth status and low child EA were risk factors for children's socio-emotional development. As underscored, our results convey important implications for the design of preventive efforts and interventions, and for ensuring the integration of key child and parent variables into existing interventions. Moreover, there are implications for pre- and post-natal follow-ups, parenting interventions, and health and social policy. Building positive relationships and enhancing social and interpersonal skill sets for at-risk infants are central to enriching less adaptive relationships and to fostering resilient families. Ultimately, these are integral to supporting better futures for our children.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Concordia University Human Research Ethics Committee and the IRB/HREC of the major teaching hospital. All parents provided written informed consent for themselves and their children in accordance with the Declaration of Helsinki. The protocol was approved by the Human Research Ethics Committees at Concordia University and the Hospital.

AUTHOR CONTRIBUTIONS

DS contributed to the conception and design of the study, interpreted its findings, and wrote multiple drafts of the manuscript. CM-G and DD contributed to the statistical analysis, the interpretation of the study's findings, and wrote sections of the manuscript. All authors read and approved the submitted version.

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Severe Perinatal Hypoxic-Ischemic Brain Injury Induces Long-Term Sensorimotor Deficits, Anxiety-Like Behaviors and Cognitive Impairment in a Sex-, Age- and Task-Selective Manner in C57BL/6 Mice but Can Be Modulated by Neonatal Handling

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Perinatal brain injury (PBI) leads to neurological disabilities throughout life, from motor deficits, cognitive limitations to severe cerebral palsy. Yet, perinatal brain damage has limited therapeutic outcomes. Besides, the immature brain of premature children is at increased risk of hypoxic/ischemic (HI) injury, with males being more susceptible to it and less responsive to protective/therapeutical interventions. Here, we model in male and female C57BL/6 mice, the impact of neonatal HI and the protective effects of neonatal handling (NH), an early life tactile and proprioceptive sensory stimulation. From postnatal day 1 (PND1, modeling pre-term) to PND21 randomized litters received either NH or left undisturbed. HI brain damage occurred by permanent left carotid occlusion followed by hypoxia at PND7 (modeling full-term) in half of the animals. The behavioral and functional screening of the pups at weaning (PND23) and their long-term outcomes (adulthood, PND70) were evaluated in a longitudinal study, as follows: somatic development (weight), sensorimotor functions (reflexes, rods and hanger tests), exploration [activity (ACT) and open-field (OF) test], emotional and anxiety-like behaviors [corner, open-field and dark-light box (DLB) tests], learning and memory [T-maze (TM) and Morris Water-Maze (MWM)]. HI induced similar brain damage in both sexes but affected motor development, sensorimotor functions, induced hyperactivity at weaning, and anxiety-like behaviors and cognitive deficits at adulthood, in a sex- and age-dependent manner. Thus, during ontogeny, HI affected equilibrium especially in females and prehensility in males, but only reflexes at adulthood. Hyperactivity of HI males was normalized at adulthood. HI increased neophobia and other anxiety-like behaviors in males but emotionality in females. Both sexes showed worse short/long-term learning, but memory was more affected in males. Striking neuroprotective effects of NH were found, with significantly lower injury scores, mostly in HI males. At the functional level, NH reversed the impaired reflex responses and improved

memory performances in hippocampal-dependent spatial-learning tasks, especially in males. Finally, neuropathological correlates referred to atrophy, neuronal densities and cellularity in the affected areas [hippocampal-CA, caudate/putamen, thalamus, neocortex and corpus callosum (CC)] point out distinct neuronal substrates underlying the sex- and age- functional impacts of these risk/protection interventions on sensorimotor, behavioral and cognitive outcomes from ontogeny to adulthood.

Keywords: neonatal hypoxic ischemic injury, neonatal handling, sensory stimulation, animal model, sex, gender medicine, behavior, cognition

INTRODUCTION

Perinatal brain injury (PBI) due to hypoxia-ischemia (HI) is such a devastating early insult that it is considered to be a major contributor to perinatal morbidity and mortality. The prevalence of neonatal HI encephalopathy (HIE) due to an oxygen and glucose deprivation during birth is 1.5–3 and up to 6 per 1,000 livebirths in developed and developing countries, respectively (Kurinczuk et al., 2010). The perinatal brain is highly susceptible to damage due to the prevailing development processes. The most vulnerable regions to injury are the ones with greatest metabolic demands (sensorimotor cortex and basal ganglia, thalamus, cerebellum and brainstem; Thorngren-Jerneck et al., 2001). Consequently, PBI can lead to long-term neurologic disability in both children and adults, including cognitive limitations, learning difficulties, attention or motor deficits (van Handel et al., 2007) and even cerebral palsy, and seizures (Platt et al., 2007). Despite the improvements in neonatal care, brain damage in term newborn infants still remains a clinical problem, with research constrained by obvious ethical limitations. Most importantly, the immature brain of premature children is at increased risk of hypoxic ischemic injury (Vannucci and Hagberg, 2004) with males born prematurely being reported as most susceptible to it and with worse developmental and adult outcomes (Elsmén et al., 2004; Peacock et al., 2012; Månsson et al., 2015).

It is considered that the experimental model of HI-induced neonatal injury initially described by Vannucci and Vannucci (1997) for the rat (Rice et al., 1981), and also adapted to the mouse in several laboratories (Sheldon et al., 1998; Hagberg et al., 2002; Northington, 2006) is a useful translational technique to better understand the effects of HI injury in human brain. Moreover, this technique at postnatal day (PND) 7–10 is equivalent to a term human infant (Semple et al., 2013; Mallard and Vexler, 2015). Although many experimental studies, mostly in rats, report morphological, biophysical and biochemical changes following HI brain insult (Towfighi et al., 1991; Huang and Castillo, 2008; Shrivastava et al., 2012), there is a scarcity of data to understand the consequential behavioral changes. However, some reports do suggest that injured animals exhibited sensorimotor deficits (i.e., Jansen and Low, 1996; Bona et al., 1997) and suffered certain learning disabilities (i.e., Young et al., 1986; Balduini et al., 2000; Ikeda et al., 2001; McAuliffe et al., 2006). Sex differences regarding the final outcome after an adult stroke or after neonatal HI had been documented (Bona et al., 1998; Hagberg et al., 2004; Hurn et al., 2005; Smith et al., 2014; Netto et al., 2017). These differences are justified by the presence of sex-specific hormones that may influence the consequences of early HI brain injury (Hill and Fitch, 2012) but also to socio-economic and neonatal variables (Månsson et al., 2015). In spite of these sex differences, most studies involving HI still demonstrate usage of male and female animals indistinctly (i.e., Chou et al., 2001; Ten et al., 2003; Lubics et al., 2005; Spandou et al., 2005; Ikeda et al., 2006; Pazos et al., 2012).

On the other hand, developmental psychobiology and neuroscience have pointed out ontogeny as a singular window of brain vulnerability but also plasticity, where early-life paradigms involving exposure to distinct stimuli during the 1st weeks of life are shown to be critical for short- and long-lasting modeling of the brain structure and function (Levine, 1957; Levine and Broadhurst, 1963; Levine et al., 1967). In rodents, early postnatal stimulation [neonatal handling (NH) in its most frequent form] consisting of brief maternal separation with/without tactile stimulation, prompts profound and long-lasting effects of anxiety and stress responses, novelty-seeking, learning and memory through different epigenetic neurobiological mechanisms (revised by Fernández-Teruel et al., 2002). More importantly, compelling effects of this intervention also include the rescue of perinatal brain insults due to stress, malnutrition or alcohol exposure (revised by Raineki et al., 2014). In the case of brain injury induced by HI, prevention of hippocampal damage (Rodrigues et al., 2004) and improvement of learning (Chou et al., 2001) were elicited in rats by maternal separation and tactile stimulation starting at PND8. Beneficial effects of tactile sensory stimulation have been attributed to the fact that neural pathways from skin to the CNS mature before other sensory systems (Montagu, 1953).

Therefore, the aim of the current research work was to do a longitudinal study of the behavioral and functional impact of HI brain injury in male and female mice, at weaning and at adulthood. We also aimed to assess the effects of NH used as a protective sensory intervention. For this purpose, we evaluated the short- and long-term effects of a neonatal HI insult in the behavioral profile of gold-standard C57BL/6 mice strain at PND7, an age modeling full-term babies. At the same time, a group of animals was used to assess the potential preventive effects of NH administered during the ontogenic development of the pups, from PND1 to

weaning (PND21), mimicking an intervention started in pre-term babies and lasting all through their childhood. For the behavioral and functional screening of the pups at weaning (PND23) and their long-term outcomes (adulthood, PND70) we used a series of tests to evaluate four dimensional areas: somatic development and sensorimotor integration, exploratory behavior, emotionality and anxiety-like behaviors, and cognition. Finally brain pathology was measured by means of brain injury score to evaluate the neurologic impact of the risk/protection events in different neuroanatomical regions at 90 days of age (PND90). This will allow us to find the functional correlates.

MATERIALS AND METHODS

Animals

Sixty-six C57BL/6 mice were bred and maintained in macrolon cages (35 cm × 35 cm × 25 cm) under standard laboratory conditions (food and water *ad libitum*, 22°C ± 2°C, 50%–70% relative humidity, 12 h light:dark cycle, lights on at 08:00 h).

Experimental Design

A longitudinal study with factorial design HI × NH × S was made to evaluate the functional impact of HI, the effects of NH in sham and HI animals, and the factors sex and age. Age factor was studied to evaluate the long-term effects, from weaning at PND23 to adulthood (PND70), of the risk/protective interventions studied. The total 66 mice came from 12 L with an average of 5.50 + 0.45 pups. Litters were randomly distributed by treatments and gender in 8 different experimental groups (n = 7–11, **Figure 1A**). Two animals (1 male/sham, 1 female/hypoxia) died from PND23 to PND90, and were therefore excluded of the statistical analysis.

Hypoxia/Ischemia

HI brain damage occurred at PND7 by permanent left carotid occlusion and exposure to hypoxia as previously described (Sheldon et al., 2001). Briefly, a midline ventral skin incision was made under isoflurane anesthesia (4.5% v/v for induction and 2.5% v/v for maintenance, and 0.6 L/min of O_2); the left carotid artery was exposed and sutured with a 8/0 silk surgical suture. After surgery, pups were returned to their dam for at least 1.5 h to recover. Later, litters were placed for 55 min in a hypoxic chamber containing 8% of oxygen balanced with nitrogen, with controlled humidity and temperature maintained at 37°C.

Handling

NH was administered from PNDs 1 to 21 (Fernández-Teruel et al., 1991). The first daily session, administered in the morning (9:30 a.m.), consisted of first removing the mother from the litter, and then weighing the pups and placing them gently and individually in plastic cages (35 cm × 15 cm × 25 cm) lined with soft article towel. After 4 min in this situation, each pup was individually (and gently) handled and stroked thrice with the thumb on the dorsal surface (rostro-caudal direction) for 3–4 s and returned to the same cage for the remaining 4 min. At the end of the 8-min period, each pup was gently handled for another 3–4 s, stroked again and then returned to its home cage. When all the pups from 1 L were back in their home cage, the mother was returned to it. The same procedure (without weighing the animals) was conducted in the afternoon (2nd time; approximately at 4:30 p.m.). NH finished at PND 21. Weaning was done at PND 21, after finishing the last NH session. Non-handled groups were left undisturbed, except for regular cage cleaning once a week, until weaning.

Behavioral and Functional Assessments

Animals were assessed in a longitudinal design, at weaning (PND23) and adulthood (PND70 or 70-days-old) as summarized in the time line shown in **Figure 1**. A three-stage protocol (**Figure 1B**) for behavioral and functional phenotype assessment (Giménez-Llort et al., 2002) evaluated the somatic development (primary screening), sensorimotor functions and motor activity (secondary screening) as well as non-cognitive and cognitive functions (tertiary screening). The tests used were as follows: somatic development (weight), sensorimotor functions [visual and hind limb reflexes, rod test and hanger test, cylinder test (CYT)], locomotor [activity (ACT) and open-field (OF) test] as well as non-cognitive and cognitive functions-emotional and anxiety-like behaviors [corner, OF and dark-light box tests (DLB)]; learning and memory (TM test and MWM). All the apparatus were thoroughly cleaned, with 5% ethanol, and dried between trials/animals.

Body Weight (BW)

Body Weight (BW) was monitored at weaning (PND23), adulthood (PND70) and at the end point (PND90).

Sensorimotor Functions (SMT)

The physical condition of the mice was evaluated by their BW and performance in sensorimotor tasks. Visual reflex and hind limbs extension reflex were measured three times by holding the animal by its tail and slowly lowering it toward a black surface. Complete extension of the forelimbs towards the surface (visual reflex) or the extension of hind limbs were considered a positive response. Motor coordination and equilibrium were assessed twice (20-s trials) in two consecutive rod tasks of increasing difficulty. The distance covered and the latency to fall off a 1.3 cm wide wooden wire rod and a 1 cm diameter metal wire rod (both, 1 m long) were recorded. The hanger test was used to measure prehensility or grasping and motor coordination by the distance covered and the number of elements of support and the latency to fall. The animal was allowed to cling with its forepaws from the middle of a horizontal wire (2 mm diameter, 40 cm length, divided into eight 5 cm segments) for two trials of 5 s. A third trial of 60 s was used to complement these measures with that of muscle strength or resistance. All the apparatus were suspended 40 cm above a padded table.

Cylinder Test (CYT)

The CYT was used to assess forelimb asymmetry (Schallert et al., 2000). Animals were individually placed in a Plexiglas transparent cylinder (10 cm diameter, 12 cm height). Each animal was video-recorded for 5 min. Initial forepaw (left/right/both) placement of each weight-bearing full rear was recorded.

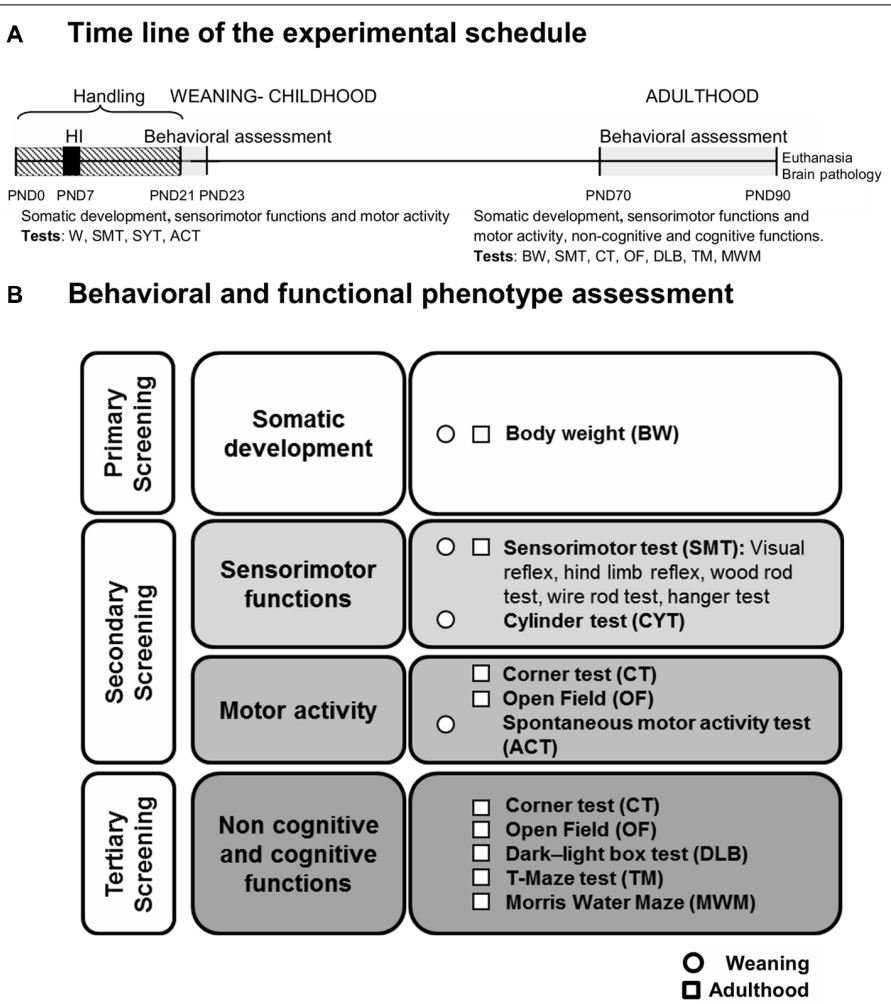

FIGURE 1 | (A) Time line of the experimental schedule. **(B)** Behavioral and functional phenotype assessment: a three-stage protocol for behavioral and functional phenotype assessment evaluated the somatic development (primary screening), sensorimotor functions and motor activity (secondary screening) as well as non-cognitive and cognitive functions (tertiary screening). The tests used were the following: somatic development (weight), sensorimotor functions (visual and hind limb reflexes, rod test and hanger test), locomotor [activity test (ACT) and open-field (OF) test] as well as non-cognitive and cognitive functions-emotional and anxiety-like behaviors [corner, OF and dark-light box (DLB) tests]; learning and memory [T-maze test (TM) and Morris Water Maze (MWM)]. The behavioral assessment was performed at weaning and/or adulthood.

The asymmetry score that reflected the preference of the unimpaired (left) limb was calculated according to the following formula:[(number of left contacts + $\frac{1}{2}$ both contacts)/number of (left + right + both contacts)]$\times$100; where 50% indicates an animal that explores symmetrically with both limbs, higher scores (>50%) indicate a greater reliance on the ipsilateral limb, and lower scores (<50%) indicate a greater reliance on the contralateral limb. We also measured the number and the total amount of grooming time, the latency of "hoppy" or "pop-corn behavior" described by Wahlsten (1974) as vigorous jumping shown in pups, and the number of full rears performed in the 5 min.

Spontaneous Motor Activity Test (ACT)

The mice were individually tested in a multicage activity meter system (four home cages −35 cm × 35 cm × 25 cm—simultaneously, Sensor Unit PANLAB 0603, Panlab, S.L., Barcelona, Spain) set to measure horizontal and vertical spontaneous motor activity during 30 min. Animals were individually tested in a standard (but novel and clean) home cage containing a small amount of clean sawdust on the floor.

Corner Test (CT) and Open Field Test (OF)

Neophobia was assessed in the corner test (CT) for 30 s. Animals were individually placed in the center of a clean standard home cage, filled with wood save bedding. Latency of the first rearing, number of corners visited and of vertical displacements (rearings) were recorded. Immediately after, exploratory and anxiety-like behaviors were measured during 5 min in a white open-field (homemade, wooden, 55 × 55 × 25 cm) under 20 lux light conditions. Mice were individually placed in the center of the arena. Horizontal (crossings, 10 × 10 cm) and vertical (rearings) activities were recorded for each minute of the test.

Dark–Light Box Test (DLB)

Anxiety and risk assessment were measured for 5 min after introducing the animals into the dark compartment of the DLB (Panlab, S.L., Barcelona, Spain). The apparatus consisted of two compartments (black, 27 cm × 18 cm × 27 cm, white, 27 cm × 27 cm × 27 cm illuminated by a red 20 W bulb) connected by an opening (7 cm × 7 cm). The experimental room was kept in darkness (without illumination). Mice were introduced into the black compartment and observed for 5 min. Total number of entries (all four paws), and time spent in the white-illuminated compartment were recorded. Latency to enter, time spent and number of entries into the lit compartment, the number of stretch attendances and self-groomings were recorded.

T-Maze Test (TM)

Exploratory activity, anxiety, working and short-term memory were assessed in an enclosed TM (woodwork; short arm: 40 × 15 × 30; goal arms: 30 × 15 × 30 cm), three trials 15 min apart, in a day. Each trial involved one forced and one free choice. Mice were placed inside the short arm of the maze and the latency to reach the intersection and the time elapsed until mice completed 20 s in the forced arm were recorded. Fifteen seconds later, mice were allowed to explore the maze in a free choice trial where both arms were accessible. The arm chosen was recorded and considered an error if it was not different of that in the forced choice.

Morris Water Maze (MWM)

Animals were tested for spatial learning and memory in three paradigms in the MWM test consisting of 1 day of cue learning and 4 days of place learning for spatial reference memory, followed by one probe trial. Mice were trained to locate a hidden platform (7 cm diameter, 1 cm below the water surface) in a circular pool for mice (Intex Recreation Corp., Long Beach, CA, USA; 91 cm diameter, 40 cm height, 25°C opaque water), located in a completely black painted 6 m^2 test room. Mice failing to find the platform were placed on it for 10 s, the same period as the successful animals. The protocol (Giménez-Llort et al., 2007) was used as follows: 1 day of cue learning, 4 days of place learning followed by a probe trial.

Cue Learning With a Visible Platform

On the first day, the animals were tested for the cue learning of a visual platform consisting of four trials in 1 day. In each trial, the mouse was gently released (facing the wall) from one randomly selected starting point (E or W) and allowed to swim until it escaped onto the platform, elevated 1 cm above the water level in the *N* position and indicated by a visible striped flag (5.3 × 8.3 × 15 cm). Extra maze cues were absent in the black painted walls of the room.

Place Learning With a Hidden Platform

On the following day, the place learning task consisted of three trial sessions per day for 4 days with trials spaced 30 min apart. The mouse was gently released (facing the wall) from one randomly selected starting point (E or W, as these are equidistant from the target) and allowed to swim until escaped onto the hidden platform, which was now located in the middle of the S quadrant. Mice that failed to find the platform within 60 s were placed on it for 10 s, the same period as was allowed for the successful animals. White geometric figures, one hung on each wall of the room, were used as external visual clues.

Removal

Two hours after the last trial of the place learning task, the platform was removed from the maze and the mice performed a probe trial of 60 s to evaluate their spatial memory for the platform position.

Analyses

Behavior was evaluated by both direct observation and analysis of videotape-recorded images. Variables of time (escape latency, quadrant preference), distance covered, and swimming speed were analyzed in all the trials of the tasks. The escape latency was readily measured with a stopwatch by an observer who was unaware of the experimental group, and was confirmed during the subsequent video-tracking analysis. A video camera placed above the water maze recorded the animal's behavior and thereafter an automated system (Smart, Panlab S.L., Barcelona, Spain) enabled computerized measurement of the distance traveled by the animal during the trials. The swimming speed (cm/s) of the mice during each trial was calculated. In the probe trial, the time spent in each of the four quadrants, the distance traveled along them, and the number of crossings over the removed platform position (annulus crossings) were also measured retrospectively by means of the automated video-tracking analysis.

Neuropathological Analysis

Brain damage was analyzed by histological analysis at PND90 (Shrivastava et al., 2012). Mice were i.p. anesthetized (ketamine and xylazine 80/10 mg/Kg) and perfused using 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). Subsequently, brains were postfixed for 4 h in the same fixative, cryoprotected in 30% sucrose, frozen with dry CO_2, and finally stored at −80°C until use. Brains were serially cut in a cryostat (Leica CM3050 S) in 30 µm thick sections and stored in −20°C mounted on Flex IHC slides (Dako). To determine the injury score, slides were processed for Nissl staining. One series of parallel sections from each animal (6–10 mice/survival time) was air dried at room temperature for an hour, rinsed and incubated with Nissl solution (0.1% toluidine blue in walpole buffer 0.2 M and pH 4.5) at room temperature for 3 min and washed with distilled water. Sections were dehydrated, cleared in xylene, and coverslipped with DPX.

Statistics

Results are expressed as means ± SEM. Repeated measures ANOVA (RMA) with a 2 × 2 × 2 HI × NH × S factorial analysis with HI, NH and S (sex) as main factors and within subjects A (Age) or T (Time course) analysis was used followed by *post hoc* tests. Two independent measures were analyzed with Student's *t*-test, while those obtained for the same animals at PND23 and

PND70 were analyzed with paired *t*-test. Behavioral correlations with brain damage were analyzed with Pearson's correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Short and Long Effects of HI and Their Counteraction by Handling

Neuropathological Analysis

HI and NH effects and HI × NH interaction were found in the neuropathological analysis (**Figure 2**). No sex effects were detected at the level of brain injury induced, as shown by the injury scores and histological appearance (only males shown). HI animals presented high injury scores as compared to null values in the two control groups, sham and handled. HI + NH animals were not exempt of injury (vs. sham or handling, all $F_{(1,65)} > 7.9463$, $P < 0.009$), but the total score and that at the different neuroanatomical areas was significantly decreased as compared to HI animals. When we measured the injury scores in the different brain regions, we observed that HI + NH males were different from HI in most regions; however, in females, the significant differences only appeared when we measured neocortex and the corpus callosum (CC) atrophy (Student's *t*-test, all $P < 0.04$).

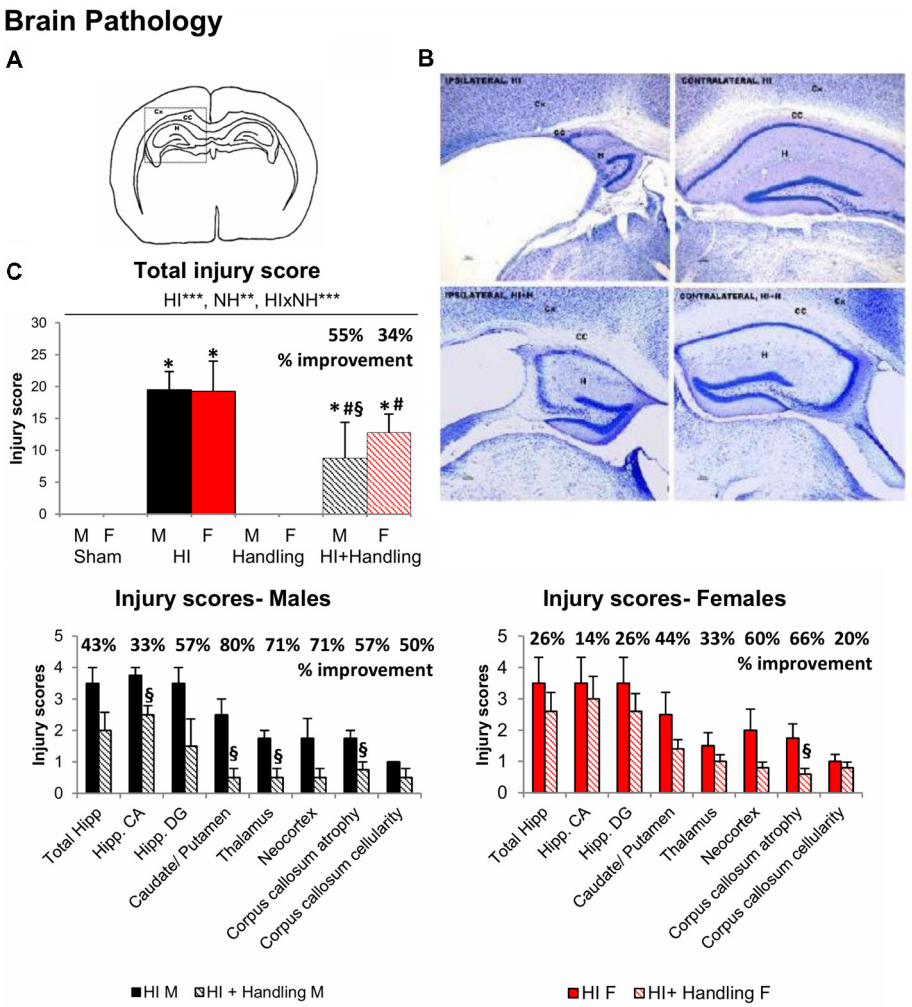

FIGURE 2 | Neuropathological analysis after hypoxic/ischemic (HI). **(A)** Drawing modified in Adobe Photoshop CS showing brain areas analyzed for quantification of brain damage in the ipsilateral side. CX, cortex; CC, corpus callosum; H, hippocampus. **(B)** Nissl staining showing HI effects on the cortex, hippocampus and CC of the contralateral (right side of the panel) and ipsilateral (left side of the panel) hemisphere 90 days after hypoxia. **(C)** Graphs show the changes in the total injury score along with the injury score in different regions analyzed. HI males and females presented higher injury scores than sham and HI + NH mice when we analyzed total injury score, the last ones were also different from sham. When we measured the injury scores in the different brain regions, we observed that HI + NH males were different from HI in most regions; however, in females, the significant differences only appeared when we measured the CC atrophy. Results are presented as mean ± SEM. Statistics: repeated measures analyses of variance (RMA), two-way ANOVA: "HI" Hypoxia effect; "NH" handling effect; "S" sex effect; "A" age effect $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Student *t*-test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}P < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex.

Somatic Development (Primary Screening)

Body Weight (BW) PND23–PND70–PND90

HI resulted in slower weight gain (**Figure 3A**) that was sex-dependent and worsened with age (both $P < 0.001$). Less weight gain was apparent in males since PND70, while in female the differences were observed later, at PND90 (S × A, $P < 0.001$). Handling was not able to reverse this HI effect.

Sensorimotor Functions and Motor Activity (Secondary Screening)

Sensorimotor Functions (SMT)-PND23-PND70

At the sensorimotor level (**Figure 3B**), HI females showed impairment of visual and hind limb extension reflexes, even at PND23 and worsening at PND70, which was the same for males of this age (all, $P < 0.05$). Prehensility, equilibrium and coordination measured in the wire rod and hanger tests were also found to be impaired and were in this order of severity ($P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively). Handling was able to modulate the HI-induced impairment in reflex responses and HI × NH interaction effects were observed in the coordination and equilibrium. However prehensility (elements of supports when holding from a hanger) was not improved by handling.

Cylinder Test (CYT)—PND23

In the CYT (**Figure 4**) HI increased the incidence of unimpaired paw preference in both males and females, ($P < 0.001$) and handling was able to reserve this effect, but only in males ($P < 0.05$). However, the detailed analysis of the use of the paws (used alone, alternatively, both at a time and the total number of vertical rearings) indicated that NH exerted beneficial effects also in females, as they showed an increased number of rearings using the impaired right arm, and were able to alternate both arms ($F's_{(1,65)} > 3.168$, $P < 0.05$). Latency of first self-grooming behavior showed a clear sex-dependent effect, with females being faster in the expression of this emotivity-related behavior. All the other groups, showed fast elicitation of it, too. However, in the male sex, the total number of episodes made a difference between HI-animals and those with NH or HI+NH. It is also important to note the appearance of

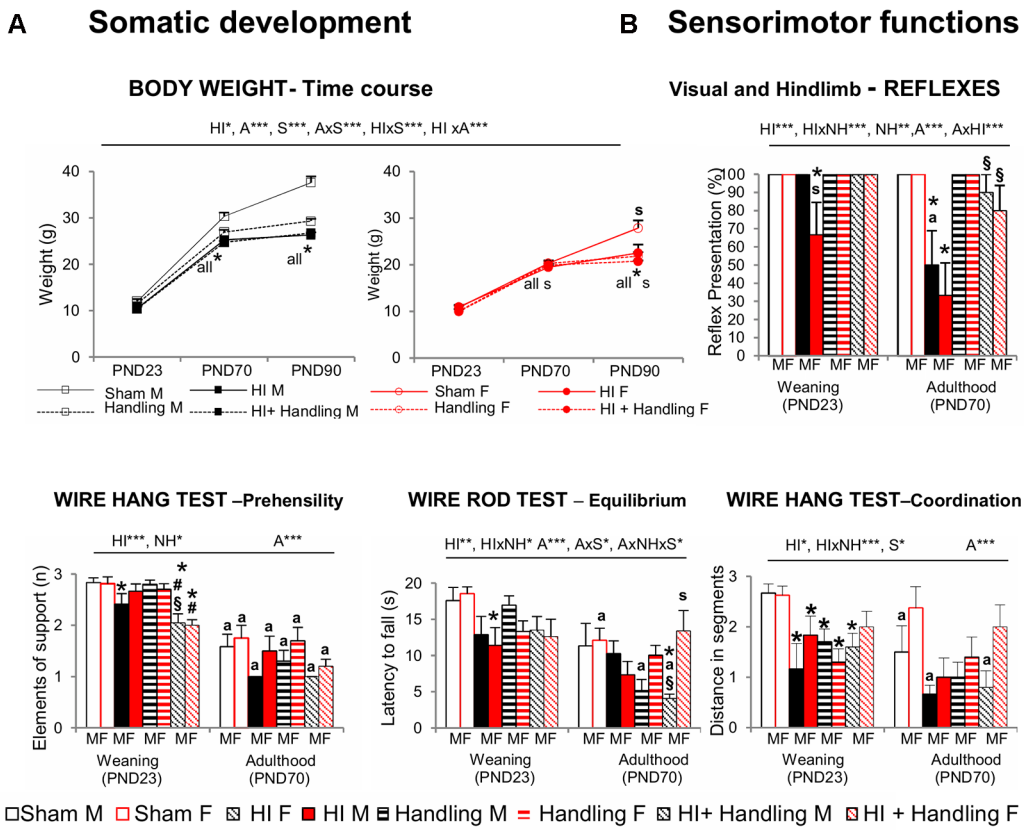

FIGURE 3 | (A) Somatic development [postnatal day (PND)23–PND90] and **(B)** sensorimotor functions (PND23–PND70). Short- and long-effects of HI in males and females C57BL6 and effects of neonatal handling (NH). Results are presented as mean ± SEM. HI animals presented slower weight gain, with male being more sensitive than female; handling could not reverse the hypoxia's effect. At sensorimotor level, HI impaired visual and hindlimb reflexes, which were first shown in females (PND23). Equilibrium, prehensility and coordination were also affected. Handling modulated the impaired reflex responses and an interaction with HI was also seen in other sensorimotor functions. Statistics: RMA, two-way ANOVA: "HI" Hypoxia effect; "NH" handling effect; "S" sex effect; "A" age effect $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, followed by Duncan's *post hoc* test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}P < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex; $^{s}P < 0.05$ female vs. male of the same treatment. Paired *t*-test comparison day 23 vs. day 70, $^{a}P < 0.05$.

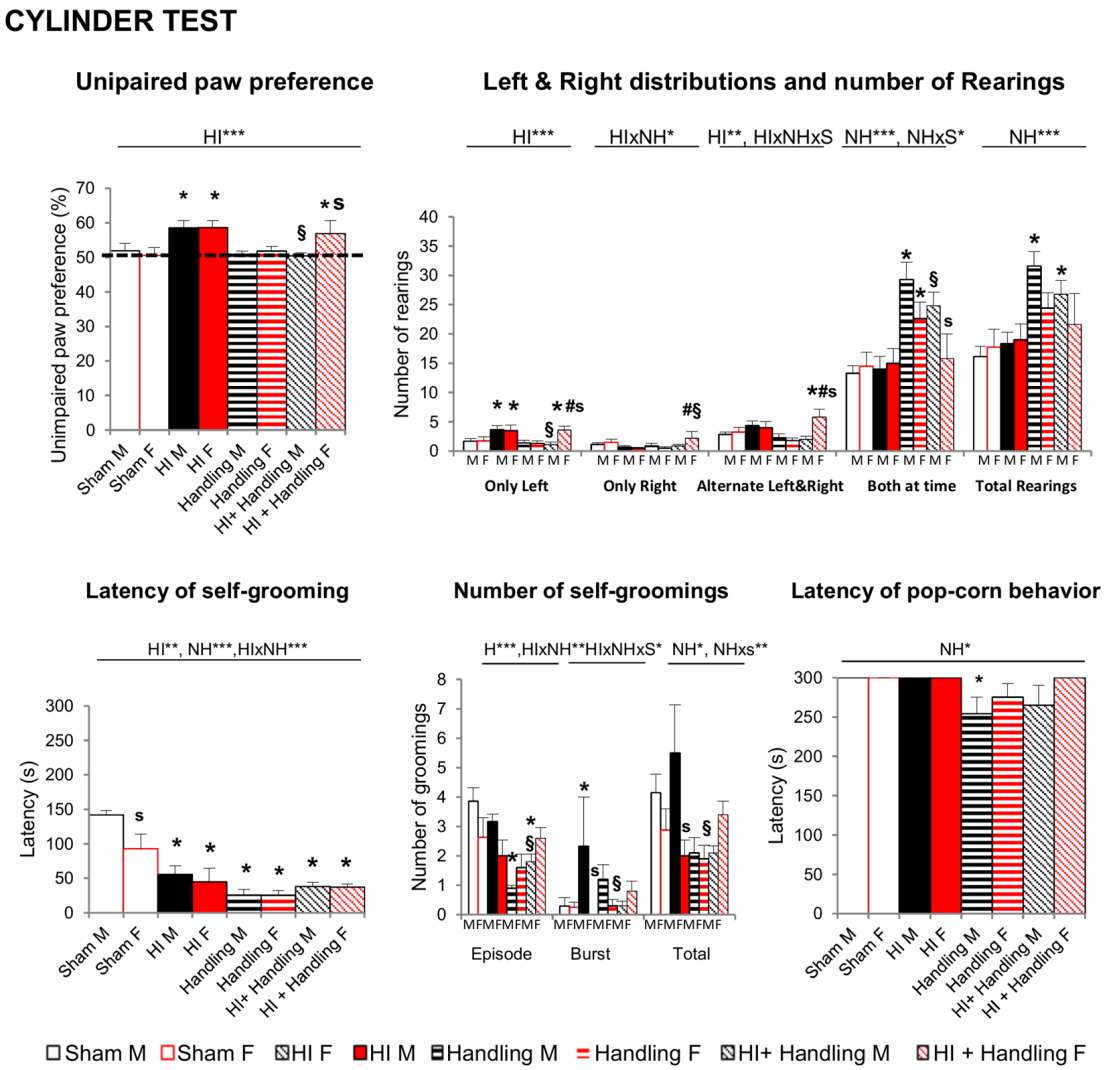

FIGURE 4 | Cylinder Test (CYT): short- and long-effects of HI in males and females C57BL6 and effects of NH. HI of both genders and HI + NH females presented higher paw preference scores (>50%) which indicate a greater reliance on the ipsilesional limb. Moreover, these groups presented a greater number of left rearings and H + NH female also presented more alternate left and right contacts. When we measured the total rearings performed, handling and HI + NH males presented the higher scores. Latency of first self-grooming behavior showed a clear sex-dependent effect, with females being faster in the expression of this emotivity-related behavior. All the other groups, showed fast elicitation of it, too. However, in the male sex, the total number of episodes made a difference between HI-animals and those with handling or HI+NH. Latency of pop-corn behavior was shorter in groups with handling, reaching statistical significance in handled males. Statistics: RMA, two-way ANOVA: "HI" Hypoxia effect; "NH" handling effect; "S" sex effect; "A" age effect $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, followed by Duncan's *post hoc* test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}p < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex; $^{s}P < 0.05$ female vs. male of the same treatment.

burst, attempts to initiate the self-grooming, as a characteristic of HI-males, that was restored to normal levels in HI+NH (F's$_{(1,65)} > 2.37$, $P < 0.05$). Latency of pop-corn behavior was shorter in groups with NH, reaching statistical significance in NH males ($P < 0.05$).

Spontaneous Motor Activity Test (ACT)–PND23

At PND23 in the spontaneous motor ACT, the time course and total motor activity counts recorded in a 30 min period indicate that HI increased the horizontal component of activity in males (vs. sham, $P < 0.05$) and that NH counteracted this effect (**Figure 5**). However, HI+NH females presented an increased activity. Like in the CYT, NH males presented a significant increase in rearings in comparison to sham, HI female and HI+NH male ($P < 0.05$).

Non-Cognitive and Cognitive Functions (Tertiary Screening)–PND70–PND90

Corner Test (CT) and Open Field (OF)

Significant effects of HI, NH and S were found in the CT (**Figure 6A**). Horizontal activity measured by numbers of

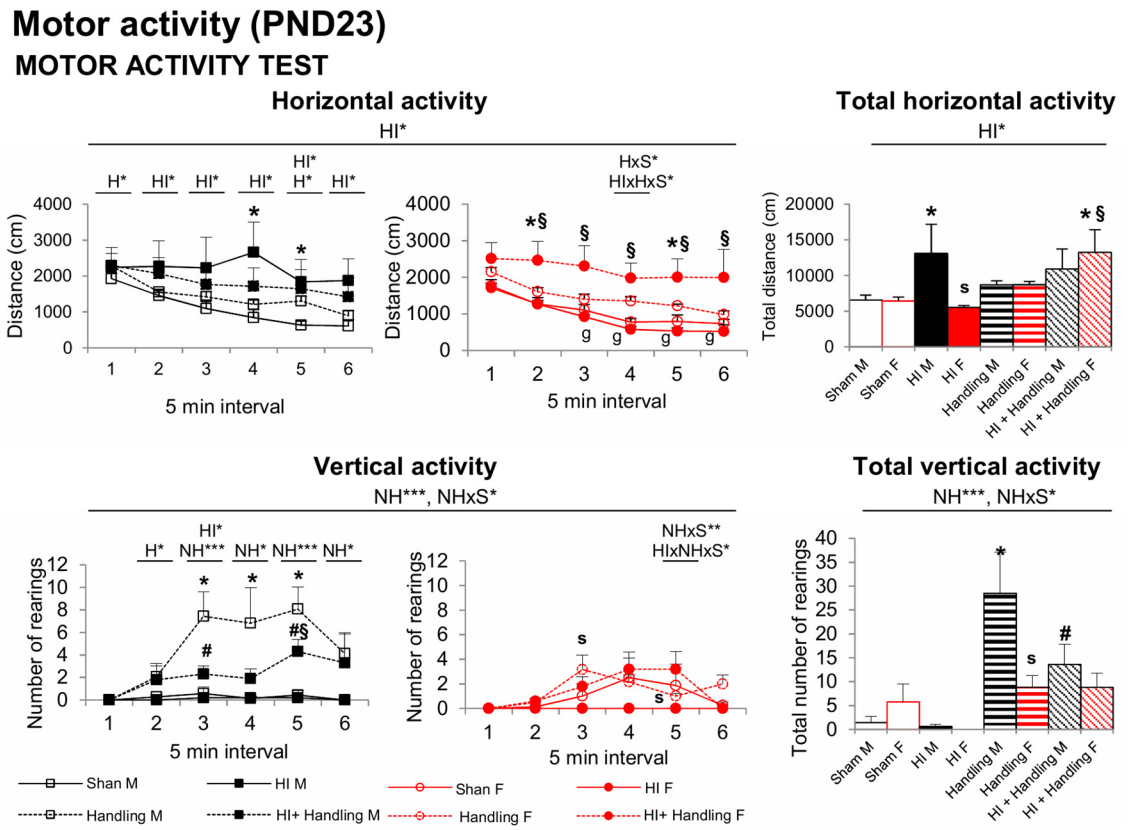

FIGURE 5 | Spontaneous motor ACT: short- and long-effects of HI in males and females C57BL6 and effects of NH. Motor activity (PND23). Horizontal activity in ACT was increased in HI males and HI + NH females; however, the vertical activity was higher in handling males. Statistics: RMA, two-way ANOVA: "HI" Hypoxia effect; "NH" handling effect; "S" sex effect; "A" age effect $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, followed by Duncan's *post hoc* test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}P < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex; $^{s}P < 0.05$ female vs. male of the same treatment.

corners visited was reduced in HI and HI + NH mice. Vertical activity was also influenced by hypoxia and handling, with these animals showing higher latencies to perform a first rearing and a reduction in the total number of rearings (all F's$_{(1,65)} > 5.457$, $P < 0.05$). HI males were more neophobic than sham and HI females; NH and HI+NH animals of both genders were also more neophobic than their respective shams.

In the OF Test, the ethogram (sequence of behavioral events) was faster in sham, handled males and females and only in HI and HI+NH females reaching the statistical significance in most of the latencies studied (all $P < 0.05$). Accordingly, the 1st minute of test was the most sensitive showing HI, NH and G differences with HI × NH, HI × S and NH × S interactions (F's$_{(1,65)} > 5.038$, $P < 0.05$), especially in vertical activity. Regarding the locomotor activity, HI effects with NH × S and HI × NH × S interactions were showed when we study the total vertical activity (F's$_{(1,65)} > 7.401$, $P < 0.05$). HI males performed less rearings than sham, HI+NH males and HI females while HI+NH females performed lesser rearings than sham, HI and NH females ($P < 0.05$). Moreover, in total horizontal activity, HI males performed lower number of crossing than HI females ($P < 0.05$) No differences in self-grooming or defecation were recorded (**Figure 6B**).

Dark-Light Box Test (DLB)

HI effect was observed when we measured the number of entrances into the lit area (**Figure 6C**), where HI males and HI+NH females exhibited reduced number of entries. Stretch attendance activity reflected HIxS and HIxNHxS interactions (all F's$_{(1,65)} > 4.154$, $P < 0.05$). HI males also presented a reduced number of stretch attendance in comparison with sham and HI females. No more significant anxiety changes were detected in the other variables.

T-Maze Test (TM)

No changes in working memory were apparent in the TM. Differences in the first latency to reach the intersection (**Figure 6D**) were found with significant HI effect and NHxS interactions (all F's$_{(1,65)} = 5.326$, $P < 0.05$). The highest latency to reach the intersection point of the TM was observed in HI+NH females as compared to other counterparts. Moreover, HI males presented a higher first T-latency in comparison with sham, although there is no significant difference. HI male also spent more time reaching the intersection.

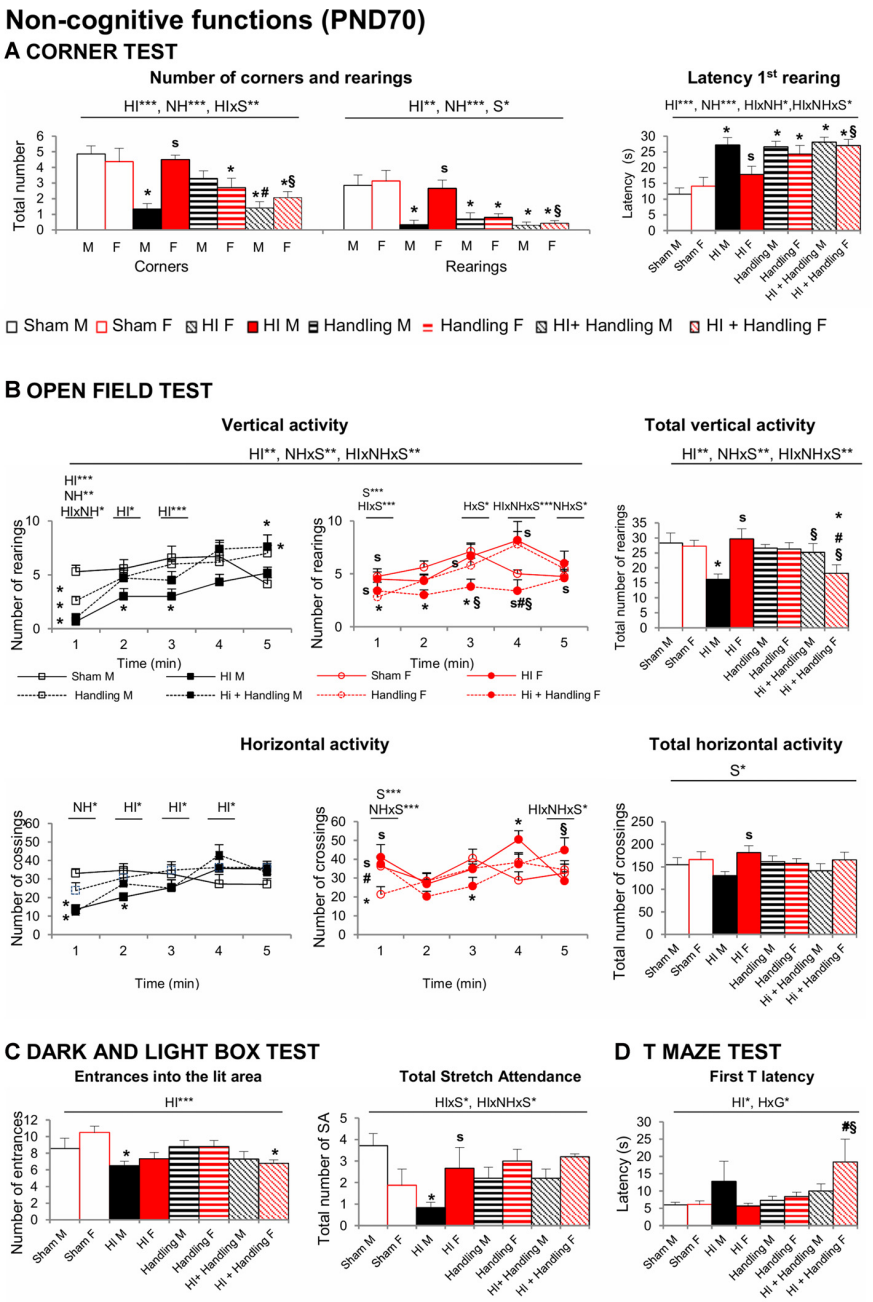

FIGURE 6 | Corner test (CT). OF. DLB. TM: short- and long-effects of HI in males and females C57BL6 and effects of NH. Non-cognitive functions were evaluated during PND70–PND90. Results are presented as mean ± SEM. **(A)** The neophobia in the CT was increased in hypoxia males and in both handling and HI + NH males and females. HI induced anxiety-like behavior in the OF **(B)** that lead to reduced exploratory activity with males being mostly affected. The anxious profile could be also observed in several variables measured by DL **(C)** were HI males and HI + NH females performed fewer entrances into the lit area. Moreover, HI males presented also fewer number of stretch attendance. Finally, in the TM **(D)** the highest values in the first T-latency were spent by HI + NH female, although there's no significant difference, HI male also spent more time to reach the intersection. Statistics: RMA, two-way ANOVA: "HI" Hypoxia effect; "NH" handling effect; "S" sex effect; "A" age effect $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, followed by Duncan's *post hoc* test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}P < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex; $^{s}P < 0.05$ female vs. male of the same treatment.

Morris Water Maze (MWM)

Figure 7A illustrates the "day-by-day" (left panel) and "trial-by-trial" (right panel) acquisition curves. All days presented the temporal effect (all F's$_{(1,65)} = 9.319$, $P < 0.001$), especially in place task learning, when the cue was removed and the platform was hidden, animals exhibited different acquisition curve. The HI and HI + NH animals found the hidden platform slower along the 4 days of the test as showed by a longer distance covered

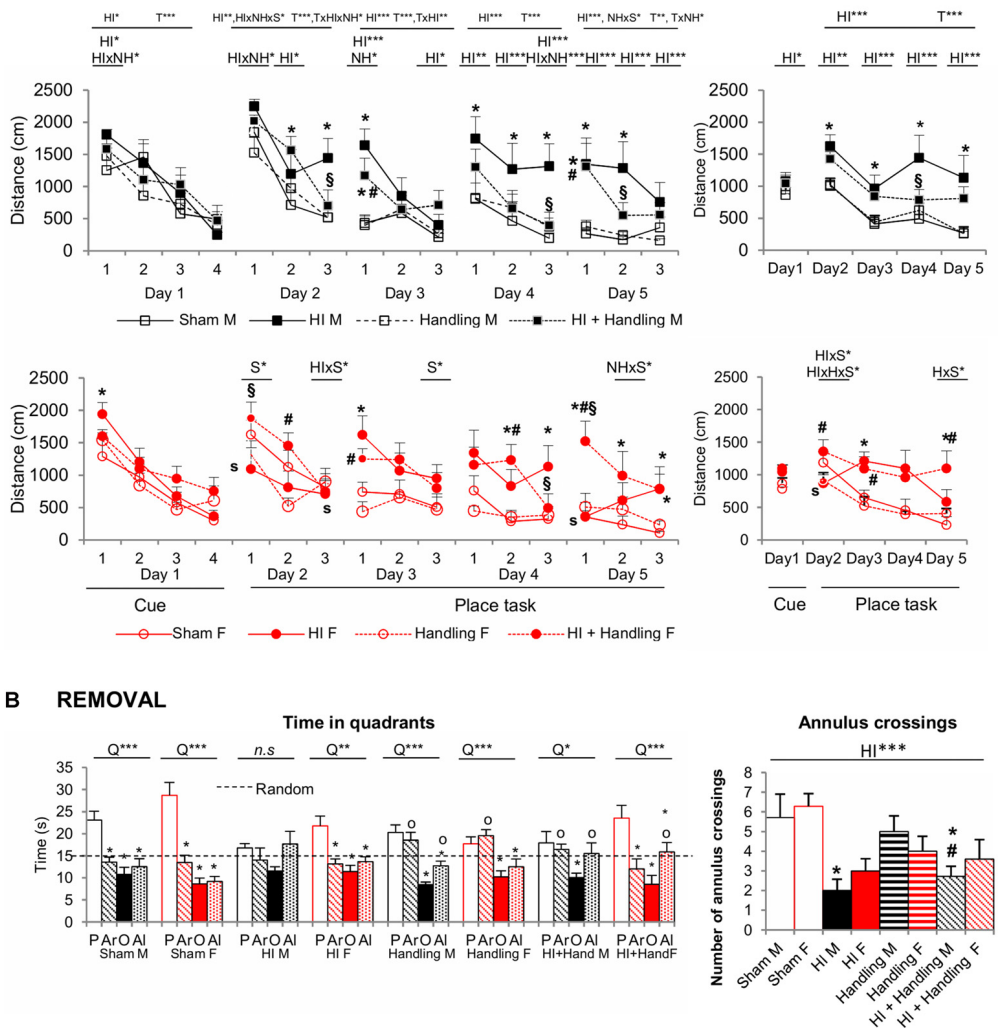

FIGURE 7 | MWM: short- and long-effects of HI in males and females C57BL/6 and effects of NH. Cognitive functions were evaluated during PND70–PND90. Results are presented as mean ± SEM. Mean distance to reach the platform in the cue and place tasks for spatial learning. All days presented time effect. **(A)** Cognitive deficits were observed in the MWM in both sexes with reduced total learning capacities (acquisition of the task) and worse short and long-term learning. In HI mice a reduction of the mean distance covered to find the platform could be recorded in both "trial-by-trial" and "day-by-day", especially in place tasks learning and were mainly important in HI males. Memory in HI males was more clearly affected than females in the MWM, however it was modulated by handling **(B)** In the probe trial for short-term memory, hypoxia males did not distinguish between the trained quadrant (P) and the adjacent (Ar, Al) or the opposed one (O) presenting a random swimming while handling (male and female) and HI + NH male presented a scanning swimming. Finally sham (male and female), HI and HI + NH female presented a focal searching swimming due they distinguish between the trained quadrant and the rest of the quadrants. The number of annulus crossings was also fewer in HI and HI + NH males. Statistics: Duncan's *post hoc* test. $^{*}P < 0.05$ vs. sham of the same sex; $^{\#}P < 0.05$ vs. handling of the same sex; $^{\S}P < 0.05$ vs. hypoxia of the same sex; $^{\$}P < 0.05$ female vs. male of the same treatment. Preference for the trained quadrant (P) as compared to adjacent right (Ar) and left (Al) or Opposite (O) quadrants in the probe trial. ANOVA, "Q" preference for trained quadrant. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; followed by Duncan's *post hoc* test $^{*}P < 0.05$ vs. P quadrant, $^{o}P < 0.05$ vs. O quadrant. n.s. non-significant statistical differencs is found.

to find the platform in comparison to sham mice (HI and S effects; HI × NH, HI × S and HI × NH × S interactions, $F_{(1,65)} > 3.908$, $P < 0.05$). Memory in HI males was more clearly affected than females in the probe trial of the MWM (**Figure 7B**) for short-term memory. HI males did not distinguish between the trained quadrant (P) and the adjacent (Ar, Al) or the opposed ones (O) presenting a random swimming. In contrast handled (male and females) and HI+NH males presented a scanning swimming. Finally, sham (male and female), HI and HI+NH females presented a focal searching swimming as they distinguished between the trained quadrant and the rest of the quadrants (Lang et al., 2003; all $P < 0.05$). Although we observed

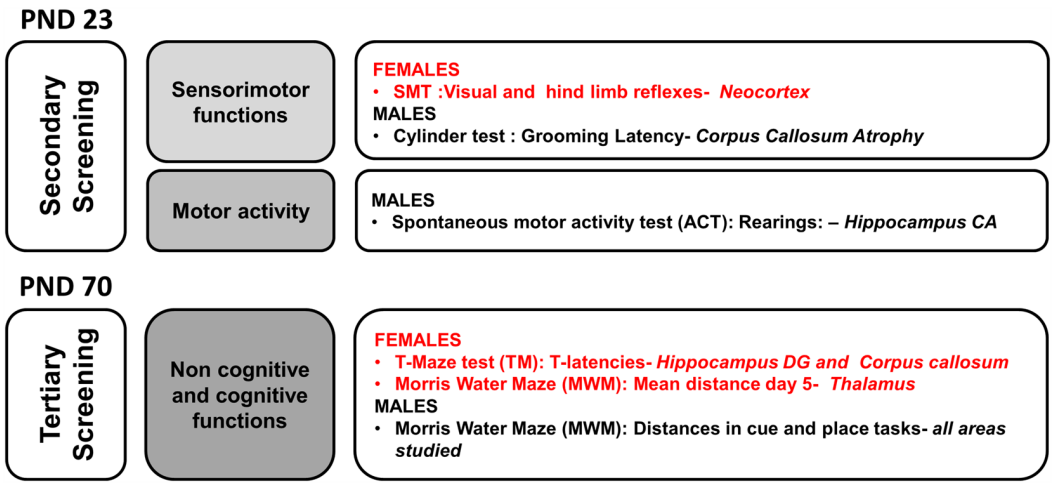

FIGURE 8 | Neuroanatomical correlates of short- and long-term behavioral effects of HI in males and females C57BL/6 and those of NH. The graphical abstract summarizes the strongest neuroanatomical-behavioral correlations ($P < 0.01$) found in males (55 variables) and females (17 variables). This distribution, indicates that in spite of similar injury score, the NH was more effective restoring functions in males and that they were those related to the cognitive domains. In contrast, the domain of non-cognitive function was the more benefited in HI + NH females.

BEHAVIORAL DOMAINS	BEHAVIORAL TESTS	Sex effect				Age effect	Handling effect
		Male PND23	Male PND70	Female PND23	Female PND70		
SENSORIMOTOR							
Reflexes	Reflex test		✓		✓	✓	✓
Equilibrium	Wire rod test			✓		✓	
Prehensility	Hanger test	✓				✓	
Paw preference	Cylinder test	✓		✓			✓
LOCOMOTOR							
Locomotor activity	Motor activity test	✓				✓	✓
Vertical activity	Open field		✓			✓	✓
NEUROPSHYCHIATRIC- LIKE							
Anxiety	Open field, Dark-light		✓				✓
Neophobia	Corner test		✓				✓
Emotionality	T-maze				✓		
COGNITIVE							
Working memory	T-maze						
Memory	Morris water maze		✓		✓		✓
Acquisition tasks	Morris water maze		✓		✓		✓

FIGURE 9 | Graphical abstract of sex- age- and task-dependent behavior impact of HI brain injury and its modulation by handling.

that this memory effect in males was modulated by handling, HI and HI + NH males presented a worse performance when we analyzed the annulus crossing in comparison to sham (HI effect $F_{(1,65)} = 17.047$, $P < 0.001$, *post hoc* Duncan's test, all $P < 0.05$).

Correlations Analysis

In a matrix of 175 × 9 (behavioral × injuries score) variables studied, we performed a meaningful correlation analysis (see **Figure 8**) between those variables where HI showed a deleterious effect and/or handling an effect (see **Figure 9**). Neonatal handled and sham animals were excluded from this analysis, since injury score was null. As detailed in **Figure 8**, the strongest correlations ($P < 0.01$) were found in 55 cases in male variables as compared to 17 cases in females. This distribution indicates that in spite of a similar injury score, the NH was more effective in restoring functions in males and within those

related to the cognitive domains. In contrast, the domain of non-cognitive function was improved more in HI + NH females.

DISCUSSION

The present work addresses the short and long-term behavioral and functional impact of neonatal HI brain injury in a term (PND7) mice model using a longitudinal approach and taking into consideration sexual dimorphism. It also provides evidence of a remarkable neuropathological protection elicited by NH. This tactile and proprioceptive sensory stimulation was administered to mice from PND1, a temporal frame modeling premature children, when the human immature brain is at increased risk of HI injury, mostly in males. NH not only ameliorated the behavioral outcomes and functional capacities but also showed differences in a sex- age- and task-selective manner. Several levels of study were considered from behavior to neuropathology, including key areas of clinical interest such as BW, sensorimotor function, physical/motor activity, emotionality, cognitive function and finally brain pathology in different neuroanatomical regions. The evaluation of these risk/protective events studied at weaning and adulthood indicates that benefits observed in the developmental outcome also result in long-term sustained effects, which contributes to their translational interest. Besides, the results indicate that even with similar HI-induced brain injury scores, interaction effects with sex are important to be taken into consideration when assessing the outcome of preventive and/or therapeutic strategies.

In contrast to most studies describing detailed neuropathological changes in the rat model, here we provide the neuropathological tissue damage at 90 days, and the neuroanatomical distribution in both male and female C57BL/6 mice strains. In agreement with a devastating early PBI insult, the histopathological analysis of HI brains provided evidence of the severity of the experimentally induced brain injury, and the histopathological protection conferred by NH intervention. PBI is considered as one of the major contributors to perinatal morbidity and mortality. Here, the mean index of postnatal mouse mortality due to surgery or hypoxia was 19.31%, with 18.46% for males and 20.00% for females, showing no statistical differences between sexes.

High variability in size and severity of the infarct between animals is a significant drawback of the HI experimental model (Vannucci and Vannucci, 2005; Millar et al., 2017). It seems that sex, severity, time of injury or even the brain lateralization of the lesion can significantly affect the outcome of Rice-Vannuci model (Lubics et al., 2005; Sanches et al., 2013b, 2015). In this regard, it is noticeable that, in the present work, the HI-procedure induced homogeneous total injury scores and neuroanatomical distribution in both sexes. Most importantly, this allowed us to highlight the capacity of NH to reduce the injury score in most of the areas, albeit not all of them reached statistical significance.

In contrast to other early life interventions studied in the literature, we unveil for the first time a relevant sex-dependent improvement of NH on HI, with HI + NH males being more responsive than females. This is important to note since sex differences have been considered in recent years. It has been reported that male infants are more vulnerable to perinatal insult than female infants, and they also suffer more long-term cognitive deficits. Males showed increased risk of development disorders, including speech and language, autism, learning disabilities and cerebral palsy compared to females (Donders and Hoffman, 2002; Rutter et al., 2003; Marlow et al., 2005; Tioseco et al., 2006; Hill and Fitch, 2012). Some authors also refer that being "male" has been identified as a universal risk factor for the incidence of neonatal stroke as well as developmental delays (Elsmén et al., 2004; Peacock et al., 2012; Månsson et al., 2015). Moreover, after pediatric traumatic brain injury, girls demonstrate a significantly better outcome in tests of learning and memory (Donders and Hoffman, 2002; Hurn et al., 2005). In our present work, HI males showed a worse performance in the memory test as compared to HI females and the improvement induced by NH in HI males was supported by better levels of hippocampal preservation.

Behavioral and functional phenotype assessment was performed using a three-stage protocol (Giménez-Llort et al., 2002). The somatic development was evaluated as primary screening, and BW was used for that. Feeding dysfunction and nutritional problems has been associated with poor growth and health status in children with cerebral palsy and neurological impairment (i.e., Reilly et al., 1996; Sullivan et al., 2000; Fung et al., 2002). Experimentally, the few number of studies addressing this issue has shown lower daily weights in HI rats (Andiné et al., 1990; Balduini et al., 2001; Lubics et al., 2005; Girard et al., 2012). Here we found differences in growth rate of HI mice that worsened with age and were long-lasting. The results also indicate that in males sex BW was more sensitive to the impact of HI than in females, who exhibited lower weight later in adulthood. This was in agreement with the sex-dependent vulnerability that we found in the behavioral outcome.

The secondary screening evaluated the sensorimotor functions and motor activity. Strength, motor coordination and several reflex responses (righting, geotaxis, gait and cliff aversion) were reported as impaired after HI insult in rats and mice (Ten et al., 2003; Fan et al., 2005; Lubics et al., 2005; Karalis et al., 2011). Here, other sensorimotor tasks such as the visual and extension reflexes, prehensility or grasping, equilibrium and coordination were found also impaired by HI. Again, for the first time, we show the relevance of "sex and age" factorial interaction as well as the comparative degree of functional severity. Spontaneous functional recovery in motor coordination and righting, geotaxis and gait reflexes were found in some of those previous works (Lubics et al., 2005; Karalis et al., 2011). In our case, in spite of the high maturation of motor systems in adults, these tasks were more demanding for them, due to increased body size vs. the rods widths. This could explain that functional motor recovery was only seen for HI-induced hyperactivity in males. Thus, HI-males

spontaneously returned to the normal values shown by sham animals, but deficient reflexes were still evident at 70 days of age.

On the other hand, this persistence of sensorimotor impairments until adulthood makes the beneficial effects induced by NH more remarkable, mostly in the reflex responses. This early life tactile and proprioceptive sensory stimulation reversed not only the impairment in reflexes but also exerted beneficial effects on coordination and equilibrium. Senses of touch, balance and proprioception are the first of the seven sensory systems developed during ontogeny. Thus, in the present work, the improvement of the vestibular system can be considered a notorious effect of NH, as compared to sensory outputs achieved in other studies (Paolucci et al., 2015). It is likely that the handling protocol, that involves the animals being held by the experimenter and the pups individually resting in the cage, may constitute a scenario where the vestibular system is challenged, trained and reinforced. In fact, early tactile and vestibular stimulations were postulated as crucial for motor behavior development (i.e., Labarba et al., 1974; Clark et al., 1977) with the maturation rate of inhibitory systems (Oakley and Plotkin, 1975) as a hypothesis to explain hyperexcitability stages, such as the pop-corn behavior shown by mice (Wahlsten, 1974) and as also observed here, in terms of emotional behaviors, which can be modulated by sensorimotor training (Caston and Lateurte, 1997). In agreement with the pyramid of learning postulated by Taylor and Trott, 1991 (as cited in Williams and Shellenberger, 1994), an improvement in the sensory integration dysfunction induced by HI, mostly in males, should also facilitate the improvement in learning capacities, as shown by our results. In the histological analysis, the injury score of the underlying neuroanatomical areas was reduced, but further experiments with detailed evaluation of these areas will provide clues about this sensory integration hypothesis.

Regarding the motor development, although some authors have described no impairment in the CYT (Sanches et al., 2013a), most of them reported that HI causes a preference to use the unimpaired forepaw (Grow et al., 2003; Chang et al., 2005; Jones et al., 2008; Kim et al., 2008; Lee et al., 2010; van Velthoven et al., 2010; van der Kooij et al., 2010; Pazos et al., 2012), as was also shown in the present work. In agreement with literature (Fan et al., 2011, 2013), no differences between male and female were observed. The poorer motor skills in children with neonatal encephalopathy compared to control could be related with the size of the CC (Van Kooij et al., 2008). This is important to note, since in the present work the CC is the area that shows, in both sexes, a statistical significant reduction of injury score in mice receiving NH.

The tertiary screening assessed locomotor activity and non-cognitive functions. It is well established that hippocampal areas are highly vulnerable to HI, and that hippocampal injury leads to hyperactivity (Shen et al., 1991). Most of the studies report that HI mice present hyperactivity in spontaneous ACTs or in the OF test (Balduini et al., 2000, 2001; Ten et al., 2004; McAuliffe et al., 2006; Arteni et al., 2010; Schlager et al., 2011; Rojas et al., 2013). Lubics et al. (2005) also detected that although HI were more active; when locomotion requires a higher level of coordination, mice can be hypoactive. Short test duration (Chou et al., 2001) and assessment in the light period (Antier et al., 1998) can also elicit reduced activity in HI rodents, probably because under these conditions they reflect an anxiogenic response. Thus, in the present work we show that the expression of neophobia and anxiety-like behaviors depend on the anxiogenic conditions of the test. In mild anxiogenic conditions the animals were found hyperactive, exhibiting a hyperexcitability stage, while with higher illumination they showed reduced exploratory activity. This was consistent with the behavioral responses shown in the other tests (the DLB and the performance in the long arm of the TM) and is also in agreement with other works (Girard et al., 2009, 2012; Carletti et al., 2012; Sanches et al., 2013a,b; Soares et al., 2013).

Academic performance and intellectual abilities are important aspects in children with neonatal encephalopathy (Robertson and Finer, 1988, 1993; Moster et al., 2002; van Handel et al., 2007). At a translational level, cognitive impairment has been reported many times, especially in the MWM. Learning impairments (Young et al., 1986; Ikeda et al., 2001; Ten et al., 2003; de Paula et al., 2009; Arteni et al., 2010) related to a longer time to escape in ischemic group (Balduini et al., 2001; Chou et al., 2001; Ten et al., 2004; Ikeda et al., 2006; Huang et al., 2009) and memory dysfunction in the probe trial (Ten et al., 2003, 2004; Huang et al., 2009). Like us, no impairments in swimming ability or speed have been observed in injured animals (Ikeda et al., 2001, 2006; Arteni et al., 2003). Arteni et al. (2010) described lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral HI in the rat. In other works (Ikeda et al., 2001; Arteni et al., 2003) only animals that suffered a right HI injury performed worse in the working memory tasks. This lateralized effect could explain why, in our case, working memory is not affected in HI animals.

To the best of our knowledge, there are no reports regarding sex differences in the functional recovery following HI in neonatal handled animals. Nesting environment (Mason et al., 2018), rehabilitative training (Tsuji et al., 2010) or early-life interventions based on environmental rearing conditions (Pereira et al., 2007, 2008; Fan et al., 2011; Rojas et al., 2013, 2015; Nie et al., 2016; Schuch et al., 2016) that share mechanisms of action with NH (Fernández-Teruel et al., 2002), also show sex-specific neuroprotection patterns. In these works, the sex differences analyzed in the recovery of HI after environmental enrichment or rehabilitative training in rats, described partial recovery in working memory in adolescent rats (Pereira et al., 2008) and improved swimming time and length in females but not in males after rehabilitative training (Tsuji et al., 2010). Thus, it is remarkable to note that the above mentioned studies showed sex-specific neuroprotection patterns, but with female sex as the most resilient, while males seemed to be less responsive to the interventions. On the other hand, different protocols for maternal separation lead to distinct behavioral outputs, from behavioral protection without morphological changes (Chou et al., 2001) or reduction of hippocampal CA volume (Lehmann et al., 2002) to worsening

of the effects of neonatal HI (Tata et al., 2015). In other experimental models of aging, neurological and psychiatric diseases, NH has also demonstrated positive effects in behavior, such as a reduction of anxiety-like behavior or an improvement in learning and memory (Levine and Otis, 1958; Alasmi et al., 1997; Gschanes et al., 1998; Raineki et al., 2014). Also, we have previously shown that NH has long-term effects on reducing the impact of N-Methyl-D-aspartate (NMDA) excitotoxicity, reducing the incidence of seizures, their number and severity in rats psychogenetically selected for high- and low-avoidance (Fernández-Teruel et al., 2002). Furthermore, we have proved that the behavioral outcome in brain damage related to Alzheimer's disease can be modulated by NH, in both males and females at adulthood (Cañete et al., 2015) and even at very advanced stages of disease in 17-month-old triple-transgenic mice (Torres-Lista and Giménez-Llort, 2015).

Many studies reported morphological, biophysical and biochemical changes following HI brain insult, especially in ipsilateral cerebral cortex, hippocampus, striatum and thalamus, after arterial occlusion (Towfighi et al., 1991; Huang and Castillo, 2008 ; and our own precedent work Shrivastava et al., 2012), but there is scarcity of data to understand the consequential behavioral changes. Similarly, although morphological neuroprotective action in the hippocampus was reported after tactile stimulation (Rodrigues et al., 2004), no behavioral outcomes were evaluated. Therefore, in the present study, we also aimed to estimate the translation of the injury score on function for both risk (HI) and protection (NH) interventions. We looked for meaningful correlations related to behavioral variables, showing the functional impact of damage due to brain injury and its protection by NH. On the one hand, the analysis of neuropathological correlates shows that the level of damage induced/restored, measured in terms of atrophy, neuronal densities or cellularity in the affected areas, can be functionally correlated with behavioral variables. On the other hand, the behavioral correlates referred to changes in the five main behavioral domains of the pyramid of learning (i.e., physical/motor, sensory, behavioral, emotional and cognitive). The analysis identified the hippocampus as the most affected area, which could explain why it was difficult to completely reverse all the cognitive deficits in females. Caudate/putamen, thalamus and CC showed the highest percentages of prevention that may underline the better behavioral outcome in tasks dependent on these areas.

Concerning the translation of the experimental NH administered to mice from PND1, it could model an early tactile and proprioceptive sensory stimulation implemented on preterm infants. Since the functional sensory response of preterm children is immature (Fitzgerald, 2005), the clinical benefits may apply more specifically to older preterm infants (>30 weeks gestational age) or HI infants post hypothermia treatment. The neuroprotective effect of early-life stimulation could also be important during the pregnancy or prenatal period, as considered by Netto et al. (2018) and Durán-Carabali et al. (2017). At the clinical level, the standard of care in cases of moderate to severe HIE is therapeutic hypothermia which has been demonstrated to increase long-term survival without disability (Tagin et al., 2012). Despite the efficiency of hypothermia, it is not enough to prevent all injury or neurological symptoms. Brain damage in term newborn infants therefore remains a clinical problem due to there being limited therapeutic outcomes and since research is constrained by obvious ethical limitations. The therapeutical approaches investigating how to prevent or minimize the consequences of the HI insult have reported efficacy of handling depending on the severity of the damage (Chou et al., 2001). In our study, we demonstrate for the first time that, under similar injury conditions, males are the sex with better responsiveness to this early life intervention, mostly at the neuropathological level, as shown by the injury scores and the different number of areas protected. To a lesser extent, this protective effect on the neuropathological consequences of HI insult also has a functional impact on the behavioral output. This was shown by the better performances in some tasks and the neuropathological correlates that point out distinct neuronal substrates underlying the sex- and age- related functional impacts of these risk/protection interventions on sensorimotor, behavioral and cognitive outcomes from ontogeny to adulthood.

In conclusion, HI brain damage affected motor development and sensorimotor functions, and induced hyperactivity at weaning; anxiety-like behaviors and cognitive deficits during the adulthood in a sex- and age-selective manner. At the functional level, handling reversed the impaired reflex responses and allowed improvement in memory performances in the hippocampal-dependent spatial learning test (MWM), in males. At an individual level, remarkable neurological protection elicited by NH correlated with improved functional capacities. Strong correlations were found between the sensorimotor, behavioral and cognitive outcomes and the injury scores based on atrophy, neuronal densities and cellularity in the different affected areas (hippocampus, caudate/putamen, thalamus, neocortex and CC). These neuropathological correlates point at distinct neuronal substrates underlying the functional capacity to meet task-dependent performance demands and neuroanatomical targets for recovery. Overall, the present results provide evidence on a therapeutical potential of early life interventions based on tactile and proprioceptive sensory stimulation in the newborns with brain injury. It supports those in the literature who defend the benefits of perinatal rearing conditions as being important to be considered as adjuvant to the current treatments. Moreover, it shows a sex-specificity that benefits male sex, who were more at risk and reported to be less responsible to most interventions.

ETHICS STATEMENT

All experimental procedures were approved by the Ethical Committee of Universitat Autònoma de Barcelona (CEEAH 811) in accordance with Spanish regulations and the European Communities Council Directives (2010/63/UE).

AUTHOR CONTRIBUTIONS

LG-L conceived and designed the experiments. KS and MR performed the risk/protection strategies. KS performed and

analyzed the neuropathological studies. AM performed and analyzed the behavioral studies. LG-L, KS and AM wrote the manuscript. All authors revised and approved the final version of the manuscript. AM and KS equally contributed to the present work.

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Postweaning Isolation Rearing Alters the Adult Social, Sexual Preference and Mating Behaviors of Male CD-1 Mice

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Objective: No study has comprehensively evaluated the effect of postweaning isolation on the social and sexual behaviors of a certain strain of rodents in ethology. The present study aims to explore how and to what extent isolation rearing after postweaning affects the social and sexual behaviors of male CD-1 mice in adulthood systematically.

Methods: Male CD-1 mice were randomly assigned to two groups: isolation reared (IS, one mouse per cage, $n = 30$) and group housed (GH, five mice per cage, $n = 15$). The mice underwent isolation rearing from postnatal day 23 to day 93. Then, social affiliation, recognition and memory were measured through selection task experiments. Social interaction under a home cage and novel environment were measured via resident–intruder and social interaction test, respectively. Furthermore, sexual preference, homosexual and heterosexual behaviors were measured.

Results: Our study found that postweaning isolation increased the social affiliation for conspecifics ($p = 0.001$), reduced social recognition ($p = 0.042$) and impaired social memory. As for social interaction, isolated mice showed a remarkably increased aggression toward the intruder male in a home cage or novelty environment. For instance, isolated mice presented a short attack latency ($p < 0.001$), high attack frequency ($p < 0.001$) and long attack duration ($p < 0.001$). In addition, isolated mice exhibited further social avoidance. Contrastingly, isolated mice displayed a reduced sexual preference for female (IS: 61.47 ± 13.80%, GH: 70.33 ± 10.06%, $p = 0.038$). As for heterosexual behavior, isolated mice have a short mating duration ($p = 0.002$), long mounting latency ($p = 0.002$), and long intromission latency ($p = 0.015$). However, no association was observed between postweaning isolation and homosexual behavior in male CD-1 mouse.

Conclusion: Postweaning isolation increased the social affiliation, impaired the social cognition and considerably increased the aggression in social interaction of adult male CD-1 mice. Postweaning isolation induced a decreased sexual preference for female in adulthood. Postweaning isolation extended the latency to mate, thereby reducing mating behavior. No association was observed between isolation and homosexual behavior.

Keywords: postweaning, isolation rearing, social behavior, sexual preference, mating behavior, male CD-1 mice

INTRODUCTION

Early life stress can produce far-reaching deleterious effects on behavior in adult life (Lupien et al., 2009). Postweaning isolation in rodents leads to severe behavioral problems in adult life (Fone and Porkess, 2008). Studies on aberrant behavior changes in rodents resulting from stress have implications for further understanding of human behavior and psychiatric disorders. For rat and mouse, weaning can be as early as 18 days after birth. However, postnatal day (PND) 21 is often used as the beginning of postweaning.

Postweaning social isolation in rat and mouse induces behavioral changes that provide a set of models for abnormal social behaviors. Social behavior is critical for establishing and maintaining social structures in rat and mouse. In ethology, behavioral paradigms that assess social motivation/affiliation, cognition and interaction are used to elucidate the social behavior of rodents (Bicks et al., 2015). Social affiliation is often assessed via social preference tests that evaluate the time spent with a novel social target compared with the time spent on exploring a novel object or simply an empty cage (Winslow, 2003). Six weeks of postweaning isolation increase the preference of male Prairie Vole for the novel conspecific over the empty cage (Pan et al., 2009). Social cognition is a complicated concept and is difficult to measure accurately. In rodents, researchers often test social recognition or memory to reflect the social cognition function. Social recognition and memory are key aspects of normal social functioning and are considered requirements for forming long-term relationship, dominance, and other complex social behaviors in animals (Bicks et al., 2015). Social recognition and memory are measured in selection tasks with habituation/dishabituation sequences. Isolated mice from PND 30 to PND 60 exhibited a decline capability for social recognition and were less likely to discriminate between familiar and unfamiliar conspecifics (Kercmar et al., 2011). This finding indicated isolation affects the social cognition. Social interaction tests involve observing how two unfamiliar rats/mice placed together in a novel environment will explore the new cage and investigate each other (Crawley, 2007). Social interactions in rats and mice include approaching, following, sniffing, climbing onto and grooming each other (Winslow, 2003; Crawley, 2007). Isolated rats from PND 19 to PND 72 displayed marked elevations in the numbers of contacts and total time spent in contact during social interaction test (Varlinskaya and Spear, 2008; Hermes et al., 2011; Moller et al., 2013).

The strain and species generality of the effect of postweaning isolation on social behavior was studied. However, the finding was inconsistent in rodents. Six weeks of isolation increased the preference of male Prairie Vole for the chamber containing a novel male individual over the empty chamber in the social affiliation test (Pan et al., 2009). However, male Wistar rats isolated from PND 25 to PND 63 showed no difference in social affiliation compared with group-housed conspecifics (Lukasz et al., 2013). These findings indicated that the effect of isolation on social affiliation may be varied amongst strains of rodents. On the contrary, the isolated male C57BL/6J mouse from PND 30 to PND 60 presented a reduced capability for social recognition to an unfamiliar female mouse from a familiar conspecific (Kercmar et al., 2011). Male CD-1 mice isolated for 4 weeks showed reduction in social recognition toward conspecifics (Fujiwara et al., 2017). Male juvenile Swiss and C57BL/6J mice that undergone 7- and 30-day social isolation showed no impaired short-term social memory, but the long-term social memory was impaired (Gusmao et al., 2012; Monteiro et al., 2014). As for reciprocal social interaction, isolated Wistar rats, beginning at PND 22 and maintained for 2 weeks, displayed less approach and further avoidance behaviors (Hol et al., 1999). Similarly, after isolating for 3 weeks (beginning at PND21), male Sprague-Dawley (SD) rats displayed an increased latency to approach the unfamiliar conspecific and a decreased number and duration of social contacts (Lukkes et al., 2009). Male CD-1 mice isolated for 4 weeks presented a reduced social interaction, increased escape behavior and further aggressive behavior (Koike et al., 2009). However, male Mongolian gerbils isolated from PND 28 displayed marked increases in sniffing, aggression, wrestling, walking and digging in social interaction test (Shimozuru et al., 2008). These results indicated that different strains of rodents will exhibit various social behaviors after social isolation. Furthermore, postweaning isolation induced a consistent increase in aggression in rat and mouse. Isolation-reared rats exhibited further playful fights during social interaction (Han et al., 2011). Isolated mice, including C57BL/6J, NC900 and CD-1 strains, presented short attack latency, further aggressive behavior and increased biting and tail ratting times during the social interaction test (Ibi et al., 2008; Nehrenberg et al., 2010; Dang et al., 2015). In summary, the effect of postweaning isolation on social behavior may be different due to the used strain of rat or mouse.

The effect of social isolation on sexual preference has not been confirmed in rat or mouse. Sexual preference can be defined as

the inclination of a rat/mouse to spend more time to interact sexually with one conspecific than with another when given the selection between an oestrous female and a sexually active male (Bakker et al., 1995). Postweaning isolated rats showed higher preference for the oestrous female than the active male (Bakker et al., 1995). However, male rats housed alone for 60 days in adulthood spent less time examining the odors of females in the odors preference test (Brown, 1985). The effect of postweaning isolation on the sexual preference of CD-1 mice remains unclear.

Sexual behavior is an aspect of sociability with highly interactive characteristics, not only a natural capability but also having some element of nurture (Hull and Dominguez, 2007). Sexual behavior in rodents includes sexual motivation, sexual exploration, sexual pursuit, copulatory mounting, copulation, and postcopulatory grooming (Green, 1966). The basic sequence consists of mating behavior, which is described as sniffing, following, mounting, intromission and ejaculation in male and lordosis posture in female. Isolation has been proven to produce sexual behavior changes in rodents. Most studies on postweaning isolation have shown that isolated rat and mouse displayed sexual behavior deficits. For instance, postweaning isolated rat displayed fewer mounts and intromissions than socially housed males in adulthood (Bakker et al., 1995). In addition, after isolation for 10–12 weeks, male rats (PND 56) showed an extension of ejaculation latency in sexual behavior compared with the double-housed control (Wallace et al., 2009). For females, C57BL/6J mice individually housed from day 25 to day 60 less often displayed lordosis postures in sexual behavior (Kercmar et al., 2014). However, one study reported the facilitation of sexual activity by isolation in Swiss, C57BL/6J and DBA mice. Isolation rearing for 2 weeks induced further mounts, intromissions and ejaculation and short latencies to the first mount and intromission (de Catanzaro and Gorzalka, 1979).

The social and sexual behaviors of rat and mouse have been investigated for numerous purposes in laboratories, ranging from theoretically oriented investigations of the causes and mechanisms of sociability and reproduction to a search for drugs to treat social or sexual dysfunction. Nonetheless, the effect of isolation during the critical period of growth on social and sexual behaviors has not been thoroughly disclosed. We hypothesized that postweaning isolation can comprehensively alter social and sexual behaviors. However, no study has systematically examined the effect of postweaning isolation on social and sexual behaviors, especially concentrating on a certain strain of rodents. Therefore, we aimed to disclose the effect of postweaning isolation rearing in CD-1 mice, due to its extensive utility in experiments and studies. In addition, we only focused on males. As the behavioral performance amongst different genders was substantially varied, the contributions of females are considered equally important. In short, the aims of the present study are: (1) to evaluate the social affiliation, recognition and interaction of the male CD-1 strain of mouse systematically after postweaning isolation, (2) to determine the sexual preference of male CD-1 mice after isolation rearing and (3) to confirm the mating behavior changes, including homosexual and heterosexual behaviors, of male CD-1 mice after isolation rearing.

MATERIALS AND METHODS

Animals

Forty-five CD-1 mice (PND 20, Vital River, Beijing, China) were housed in black opaque polypropylene cages under standard conditions (21–25 °C, 40–60% humidity, food and water *ad libitum*, 12 h:12 h light/dark cycle). Animals were acclimatized for 3 days. Then, mice were randomly divided into two groups at 23 days of age on the basis of weight: isolation reared (one mouse per cage) and group housed (five mice per cage). The randomization process was implemented by the SAS9.2 software package. We started the experiments 10 weeks after isolation rearing. All experiments were performed in compliance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication No. 80-23, revised 1996) and under the approval and supervision of the Academy of Experimental Animal Centre of the Institute of Medicinal Plant Development (China).

Social Affiliation Test

Two types of selection task were adopted to assess social affiliation. The equipment was an open field box with black walls and floor. Two clear cylindrical cages (diameter and height of 8.5 and 11 cm, respectively) were placed in the left and right sides of the apparatus. In the first experiment, an unfamiliar male mouse was placed in a cage, whereas another cage remained empty (Winslow, 2003). Each mouse was placed in the center of the equipment and allowed to explore the arena freely for 5 min, which was recorded in a video. The total time spent near the two cylindrical cages was measured and analyzed by watching the video. The recognition index consisted of dividing the stranger exploration time by the total exploration time. In the second experiment, an unfamiliar male mouse was placed in a cage, whereas another cage contained an object. Each mouse was placed in the equipment and allowed to explore the stranger mouse and object freely for 5 min. The total time spent near the two cylindrical cages was analyzed. The recognition index also consisted of dividing the stranger exploration time by the total exploration time.

Social Recognition Test

Social preference paradigms with habituation/dishabituation sequences were adopted to assess social recognition (Kaidanovich-Beilin et al., 2011; Kentrop et al., 2018). The apparatus used the equipment of the social affiliation test. The experimental procedure consisted of three phases: habituation with environment, social interest and social discrimination. In the habituation phase, each mouse was placed in the middle compartment and allowed to explore the arena and two empty cages freely for 5 min. In the social interest phase, an unfamiliar male mouse was placed in a cage, whereas another cage remained empty. The test mouse was placed in the apparatus again for 5 min to ensure familiarization with the stimulus mouse. In the social discrimination phase, an unfamiliar mouse was placed in another empty cage. The test mouse was placed back and allowed to explore the familiar and unfamiliar stimulus mouse for 5 min, which was recorded in a video. The times spent near each cage

and near the two cages were analyzed. The discrimination index consisted of dividing the unfamiliar mouse exploration time by the total exploration time.

Social Memory Test

The capability to recognize familiar female conspecifics was tested via a social memory test using equipment consisting of one cage within an open field box (Bielsky et al., 2004). In the test, a female stimulus mouse was placed in the cage. Each test mouse was placed in the equipment, allowed to explore the cage for 60 s and then removed. The test mouse was placed in the equipment again after 9 min, and this process was repeated three times. After a final 9 min break, a new, unfamiliar female was placed in the cage for the fifth test. During each 1 min trial, the sniffing duration of the tested mice was recorded and analyzed after completing the experiment by watching the video.

Resident–Intruder Test

The resident–intruder test was adopted to measure the aggression of mice (Koolhaas et al., 2013). In the test, a group-reared CD-1 mouse of a similar age was placed in the home cage of each test mouse, and their behaviors were videotaped over a 10 min period. The following behaviors were analyzed: aggressive (biting, tail rattling, wrestling, and lateral threats) and non-aggressive (sniffing each other) social behaviors. The indicators were measured: attack latency, attack frequency and duration, upright posture and unaggressive social contact.

Social Interaction Test

The social interaction test was adopted to measure deficits in reciprocal interaction behavior (Berry et al., 2012). The test was conducted in the open-filed box of black Plexiglas with a transparent wall in front, under a novel environment for the test mouse. On the day of testing, test and stimulus mice were placed simultaneously into the apparatus for 10 min. The stimulus mouse was marked by a yellow, scentless and non-toxic paint before testing to discriminate the experimental subject from the stimulus conspecific during data collection. The frequency and duration of environmental exploration, social interaction (body, nose and anogenital sniffing and exploration by stimulus mouse), non-social behaviors (self-grooming, motionlessness, upright posture and rest) and aggressive behaviors (latency to attack and attack frequency and duration) were determined (Hamilton et al., 2014).

Sexual Preference Test

The social preference paradigm was adopted to measure the sexual preference in mice (Winslow, 2003). Isolation-reared male mice and control group were sexually inexperienced. A three-chambered box (each chamber: 40 cm × 40 cm × 40 cm) was used as the test apparatus (Zhang et al., 2013). A CD-1 sexually experienced and active male (aging 13 weeks) and an oestrous CD-1 female were placed on each side the chamber. A cylindrical wire mesh cage (diameter and height of 8.5 and 11 cm, respectively) was used to prevent the experimental animal from having physical contact with the stimulus mice. The testing period lasted for 15 min. The time spent for exploring female and male stimulus mice was recorded. The sexual preference index was calculated by dividing the time spent for exploring the female to the total time spent for exploring the female and male (Zinck and Lima, 2013).

Homosexual Behavior Test

Three days after the sexual preference test, isolation-reared mice and the control group, still sexually inexperienced, were tested for homosexual behavior. The day before the test, each mouse was placed singly into the test apparatus and allowed to habituate it for 30 min. During testing, the test mouse habituated the test box (40 cm × 40 cm × 40 cm) for 10 min, after which a sexually active and experienced male was placed in the box. The testing period lasted 30 min. Anogenital sniffing duration, latencies to first mount and intromission, total numbers of mounts, intromissions and ejaculations and lordosis were recorded (Haga et al., 2010). Lordosis was defined as a male exhibiting the sexual receptivity of a female with four paws grounded, the hind region elevated from the floor of the test chamber, with no evidence of attempt to escape or exhibit a defensive upright posture, and the back slightly arched (Kudwa et al., 2005).

Heterosexual Behavior Test

Three days after the homosexual behavior test, all mice were used in heterosexual behavior test (Siegel et al., 1981; Sisk and Meek, 2001). The test box used in the homosexual behavior test was utilized. An oestrous female was prepared by injecting 20 μg of oestradiol benzoate and 500 μg of progesterone into an ovariectomised female 48 and 4 h, respectively, prior to testing. During testing, the test mouse was placed in the test apparatus and allowed to habituate it for 10 min. Then, an oestrous female (postnatal 13 weeks) was placed in the test box. The testing period lasted 30 min. Latencies to first mount, intromission and ejaculation, refractory period and frequencies and durations of mounts, intromissions and ejaculations were recorded.

Statistical Analyses

Data were analyzed using the SPSS 22.0 software package. Comparisons of parametric data were performed using Student's *t*-test and the Chi-squared test for two groups. For non-parametric data, the Mann–Whitney *U*-test was used for two groups.

RESULTS

Social Affiliation

In the selection task involving a stimulus mouse and empty cage, isolation-reared mice spent more time exploring stimulus mice than did group-housed mice (IS: 7.43 ± 3.74, GH: 0.40 ± 4.02, $p = 0.019$). Isolation-reared mice spent less time exploring the empty cage than did group-housed mice (IS: 49.28 ± 14.54, GH: 37.81 ± 15.32, $p = 0.022$). The recognition index was higher in isolation-reared mice than in group-housed mice (IS: 87.85% ± 4.40%, GH: 79.25% ± 7.19%, $p = 0.001$) (**Figure 1A**), which indicates that postweaning isolation rearing increases the CD-1 mouse social affiliation for conspecifics.

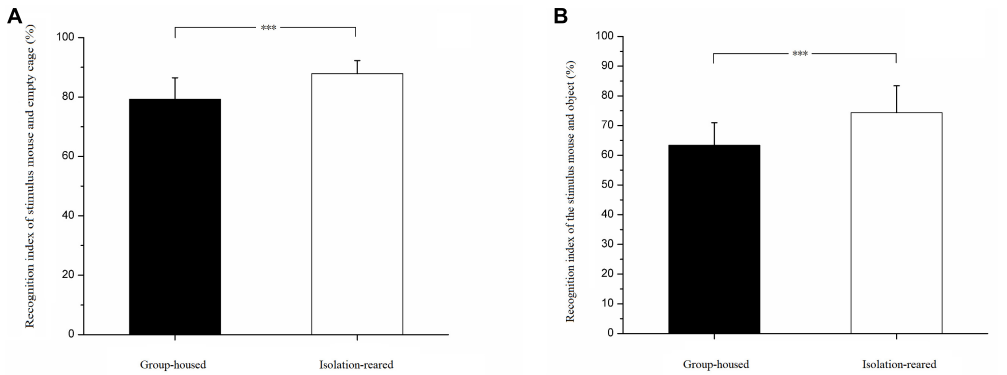

FIGURE 1 | Results of social affiliation test. **(A)** Recognition indexes of stimulus mouse and empty cage. **(B)** Recognition indexes of stimulus mouse and object. *** indicate $p < 0.001$. Bar represent mean. Error bar represent standard deviation (SD).

In the selection task involving a stimulus mouse and object, isolation-reared mice spent more time exploring stimulus mice than did group-housed mice (IS: 55.66 ± 18.13, GH: 33.46 ± 13.66, $p < 0.001$). However, isolation-reared and group-house mice spent similar times exploring objects (IS: 19.08 ± 7.83, GH: 18.15 ± 4.66, $p = 0.624$). The recognition index was higher in isolation-reared mice than in group-housed mice (IS: 74.34% ± 9.10%, GH: 63.36% ± 7.61%, $p < 0.001$) (**Figure 1B**), which indicates that postweaning isolation rearing increases the male CD-1 mouse social affiliation for conspecifics.

Social Recognition

In the habituation phase, both isolation-reared and group-housed mice displayed no difference in their exploration times of two unfamiliar male mice. In the testing phase, isolation-reared mice spent more time exploring familiar male mice than did group-housed mice (IS: 24.71 ± 10.74, GH: 12.67 ± 7.15, $p < 0.001$). In addition, isolation-reared mice spent more time exploring unfamiliar male mice than did group-housed mice (IS: 39.74 ± 12.41, GH: 23.93 ± 10.89, $p < 0.001$). However, the discrimination index was lower in isolation-reared mice than in group-housed mice (IS: 62.45 ± 9.77, GH: 70.06 ± 13.92, $p = 0.042$) (**Figure 2**), which indicates that postweaning isolation rearing reduces the ability of male CD-1 mice to recognize familiar male conspecifics.

Social Memory

All mice were exposed to the same female stimulus mouse thrice for 60 s each time as the first three tests and then introduced to a new female stimulus mouse as the fourth test. The time spent exploring declined from the first to the fourth tests ($p = 0.050$, 0.077, 0.030, and 0.025, respectively). The exploration time recovered in the fifth test (test with a new female, $p = 0.071$). Exploration times recovered to levels observed in the first test during the fifth test in group-housed mice but not in isolation-reared mice, which indicates that postweaning reduces the ability of male CD-1 mice to memorize familiar female conspecifics and recognize a new female (**Figure 3**).

Aggression

In the resident–intruder test, isolation-reared mice displayed a shorter latency to attack ($p < 0.001$) (**Figure 4A**), higher attack frequency (IS: 46, GH: 0, $p < 0.001$), longer attack duration ($p < 0.001$) and increased frequency of upright posture (IS: 1, GH: 0, $p = 0.003$) (**Figure 4B**) compared with group-housed mice. However, non-aggressive social contact times and durations were not different between the isolation-reared and group-housed mice (IS: 20, GH: 16, $p = 0.495$ and IS: 100.99 s, GH: 176.03 s, $p = 0.077$, respectively). These results indicate that postweaning increases aggression in male CD-1 mice.

Reciprocal Social Interaction

Compared with the control mice, isolated mice displayed significantly increased aggression and rejection of contact from the stimulus mouse in the social interaction test within a novel environment. However, no differences in terms of social contact with the stimulus mouse and self-grooming were

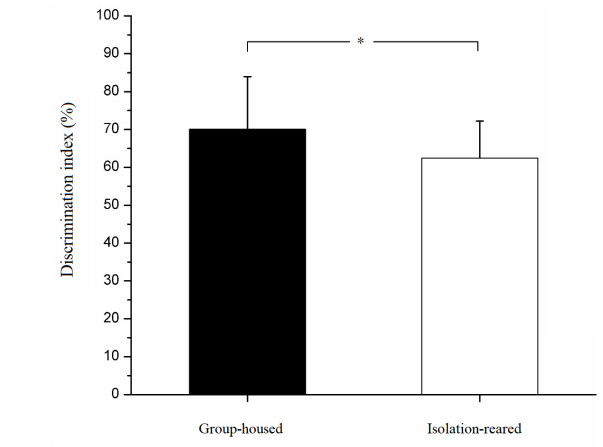

FIGURE 2 | Discrimination index of isolation-reared and group-housed mice in the social recognition tests. *Indicate $p < 0.05$. Bar represent mean. Error bar represent SD.

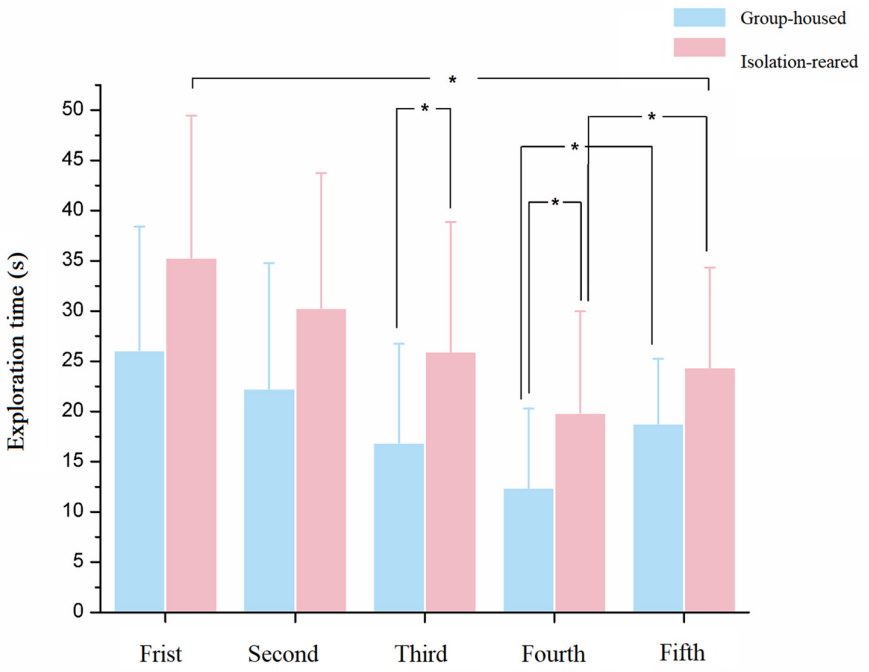

FIGURE 3 | The exploration time of isolation-reared and group-housed mice in the social memory tests. *Indicate $p < 0.05$. Bar represent mean. Error bar represent SD.

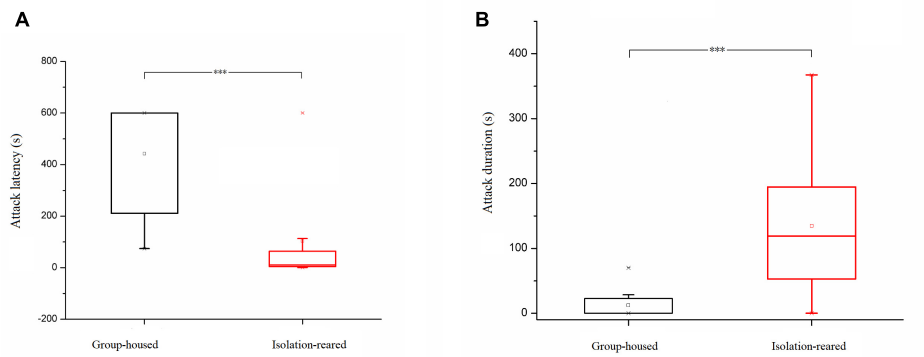

FIGURE 4 | Box plots of attack latency and duration in the resident–intruder tests. **(A)** Attack latency of isolation-reared and group-housed mice. **(B)** Attack duration of isolation-reared and group-housed mice. ***Indicate $p < 0.001$. Bar represent minimum or maximum. □Represent mean.

observed between the isolated and group-housed mice during environment exploration. Specifically, isolated mice displayed a higher incidence of attack (IS: 96.6%, GH: 7.1%, $p < 0.001$), shorter attack latency ($p < 0.001$), higher frequency of attack ($p < 0.001$), and longer attack duration ($p < 0.001$) than did group-housed mice. Isolated mice also showed a short duration of environment exploration ($p = 0.002$). No difference in the frequency of environment exploration was observed between groups ($p = 0.459$) (**Table 1**).

In the test of social contact with the stimulus mouse, no differences in the frequency and duration of exploration of the stimulus mouse ($p = 0.428$ and 0.856) were observed between the isolated and group-housed mice. In addition, isolated mice showed lower frequencies and durations of rejection during contact by the stimulus mouse (both $p < 0.001$) compared with group-housed mice.

Sexual Preference

Our results illustrate that isolated mice spent more time exploring male mice than did group-housed mice (IS: 78.70 ± 36.12 s, GH: 47.23 ± 19.65 s, $p = 0.003$). However, the time spent exploring the female mouse did not differ between the isolated and group-housed mice (IS: 132.79 ± 64.65 s, GH: 101.44 ± 47.82 s, $p = 0.114$) (**Figure 5A**). The sexual preference index was significantly lower in isolation-reared mice than in group-housed mice (IS: 61.47 ± 13.80, GH: 70.33 ± 10.06,

TABLE 1 | Social interaction of isolation-reared and group-housed mice.

	Isolation-reared	Group-housed	*Z*	*p*
Frequency of environment exploration	30 (12.5)	26.5 (3.0)	−0.74	0.459
Duration of environment exploration	347.18 (164.03)	452.11 (122.6)	−3.16	0.002
Frequency of stimulus mouse exploration	22 (20)	23 (9.25)	−0.79	0.428
Duration of stimulus mouse exploration	96.72(160.97)	98.00 (54.80)	−0.18	0.856
Attack latency	20.19 (40.72)	600.00 (0.00)	−5.08	< 0.001
Frequency of attack	45 (41.5)	0 (0)	−4.84	< 0.001
Duration of attack	122.94 (113.78)	0.00 (0.00)	−4.71	< 0.001
Frequency of self-grooming	2(1)	2(2)	−0.52	0.604
Duration of self-grooming	10.75 (5.86)	15.33 (19.08)	−0.96	0.337
Frequency of rest	0(1)	0(2)	−0.36	0.722
Duration of rest	0.00(4.59)	0.00(1.53)	−0.46	0.648
Frequency of upright	53 (33.5)	82.5 (32.25)	−3.51	< 0.001
Frequency of tail-rattling	1(2)	0(0)	−3.20	0.001
Duration of tail-rattling	1.10 (4.65)	0.00(0.00)	−3.20	0.001
Frequency of contacting from stimulus mouse	0.00 (0.00)	3.5(4.75)	−5.20	< 0.001
Duration of contacting from stimulus mouse	0.00 (0.00)	31.49(56.02)	−5.44	< 0.001

Median (Interquartile range, IQR).

p = 0.038) (**Figure 5B**), thereby implying that postweaning isolation may lead to decreased interest in females amongst male CD-1 mice.

Homosexual Behavior

No sexual behaviors, included mounting, intromission and ejaculation, were observed between the test mice and stimulus males. Isolated mice showed decreases in anogenital sniffing frequency and shorter anogenital sniffing durations (p = 0.003 and 0.008, respectively) compared with group-housed conspecific mice (**Table 2**).

TABLE 2 | Homosexual behavior of isolation-reared and group-housed mice.

	Isolation-reared	Group-housed	*Z*	*p*
Anogenital sniffing frequency	0 (2)	4 (4)	−2.98	0.003
Anogenital sniffing duration	0.00 (1.92)	5.39 (6.25)	−2.64	0.008

Median (IQR).

Heterosexual Behavior

The mating success rate tended to be lower in the isolation group than in the group-housed group (IS: 80.0%, GH: 86.7%), although no statistically significant difference between groups was observed (p = 0.458). The latency to first mount was longer in the isolation group than in the group-housed group (p = 0.002) (**Figure 6A**), which means the former required a longer time to enter into a sexual procedure than did the latter. The latency to first intromission was longer in the isolation group than in the group-housed group (p = 0.015) (**Figure 6B**), which indicates that the former required a longer time to attract female mice than did the latter group. No statistically significant difference between the two groups was observed in terms of latency to ejaculation and refractory period. The total mating duration was shorter in the isolation group (**Table 3**) than in the group-housed group, and differences observed were statistically significant (p = 0.002) (**Figure 6C**). No statistically significant differences between the two groups were observed in terms of frequency of mounting before ejaculation, total frequency of mounting, number of intromissions and total number of mating (**Table 4**).

DISCUSSION

Main Finding

Postweaning isolation comprehensively altered the adult social affiliation, social recognition and social interaction behaviors

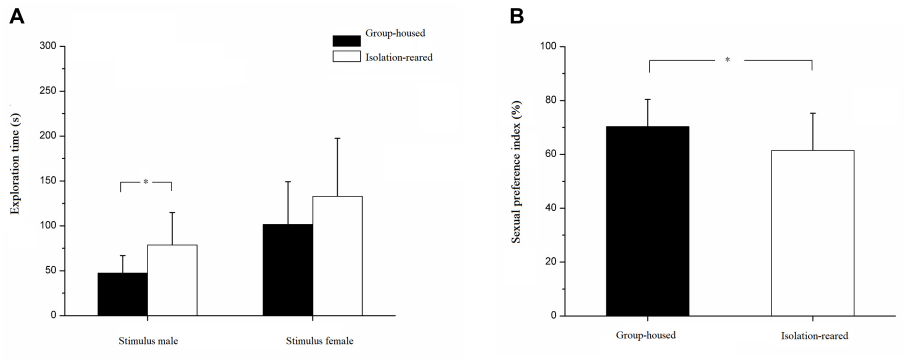

FIGURE 5 | Results of sexual preference test. **(A)** The exploration time of isolation-reared and group-housed mice toward stimulus male and female. **(B)** The sexual preference indexes of isolation-reared and group-housed mice. *Indicate $p < 0.05$. Bar represent mean. Error bar represent SD.

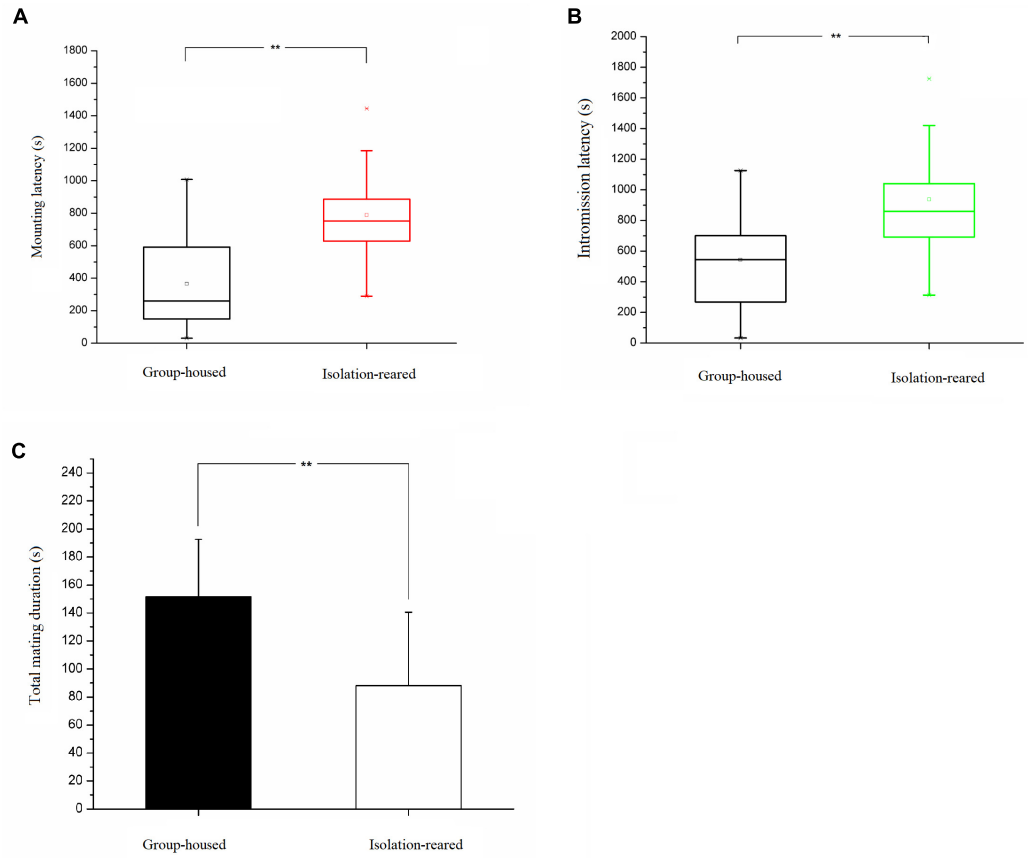

FIGURE 6 | Results of heterosexual behavior test. **(A)** Box plot of the mounting latency of isolation-reared and group-housed mice. **(B)** Box plot of the intromission latency of isolation-reared and group-housed mice. **(C)** The total mating duration of isolation-reared and group-housed mice. **Indicate $p < 0.01$.

TABLE 3 | Heterosexual behavior of isolation-reared and group-housed mice.

	Isolation-reared	Group-housed	*t/t'*	*p*
Mounting latency	788.70 ± 262.77	365.03 ± 288.65	−3.87	0.002
Intromission latency	937.30 ± 369.87	542.94 ± 352.40	−2.75	0.015
Ejaculation latency	16.58 ± 9.78	17.37 ± 13.03	−0.20	0.845
Refractory period	173.00 ± 89.84	192.87 ± 106.91	0.58	0.565
Total mating duration	88.27 ± 52.40	151.65 ± 40.87	3.44	0.002

Mean ± SD.

TABLE 4 | Results of heterosexual behavior test.

	Isolation-reared	Group-housed	*Z*	*p*
Number of mounting before ejaculation	2 (2)	2 (3)	−0.77	0.442
Number of intromissions	18 (19)	18 (21)	−0.28	0.779
Total number of mounting	11 (7.75)	18 (10.5)	−1.26	0.207
Total number of mating	7 (5.5)	10 (8)	−1.47	0.142

Median (IQR).

of male CD-1 mice. In the social affiliation test, isolated mice spent more time exploring conspecifics than the empty cage or object. In the social recognition and memory test, isolated mice displayed lower discrimination indices in exploring novel males and female, which indicates a reduced ability to discriminate a novelty mouse from a familiar one. In the resident–intruder test, isolated mice showed remarkably increased aggression toward the intruder male in their home cage. In the social interaction test, isolated mice also exhibited abnormal attack behaviors toward male conspecifics in a novel environment. In the sexual preference test, isolated mice displayed a reduced sexual preference for females. In the homosexual behavior test, no association was observed between postweaning isolation and homosexuality in male CD-1 mice. In the heterosexual behavior test, isolated mice revealed a shorter mating duration, longer latency to mount and longer latency to intromission than did group-housed mice.

Postweaning Isolation Altered Adult Social Affiliation, Social Cognition and Social Interaction in Male CD-1 Mice

Our results reveal that isolated mice display increased affiliation for a stimulus mouse in the social affiliation tests, decreased recognition for a novel male in the social recognition test, reduced memory for a familiar female in the social memory test and increased aggression in the social interaction test.

CD-1 mice are social animals, and their typical group social structure has been widely researched for many purposes. Several ethological experiments have been designed to measure social behavior in rodents. However, past studies usually used only one or two methods to detect one or two types of social behaviors, and no study systematically examining the effect of postweaning isolation on the social ability and behavior of a certain strain of rats or mice has yet been published.

Firstly, selection task paradigms have been adopted to measure social affiliations in rodents. One kind of experimental design involves observation of a test mouse choosing between an empty cage and a cage containing a stimulus conspecific. In some cases, the experimenter places an object in the empty cage and observes the rodent's choice making between an object and a conspecific to measure social affiliation. In the present study, isolated CD-1 mice spent more time near the cage containing a stimulus mouse than did group-housed mice, which is consistent with findings involving isolated male prairie voles (Pan et al., 2009). In addition, we found that isolated CD-1 mice demonstrate a higher affiliation for a conspecific than do group-housed mice in the choice between an object and a conspecific. This finding indicates that postweaning isolation increases the social affiliation for conspecifics in male CD-1 mouse.

Secondly, selection tasks with habituation/dishabituation sequences have been adopted to measure social recognition or memory in rodents. We found that postweaning isolation reduces the ability of male CD-1 mice to recognize a novel male, memorize familiar females, and recognize a new female. Our finding is consistent with Fujiwara's study on CD-1 mice (Fujiwara et al., 2017) and previous work on C57BL/6J mice but not Swiss mice (Kercmar et al., 2011; Gusmao et al., 2012). Nevertheless, all evidence indicates that postweaning isolation impairs social cognition in mice.

Thirdly, in the resident–intruder and novel environment social interaction tests, compared with group-housed mice, isolated male CD-1 mice showed significantly increased aggression toward male conspecifics with a shorter attack latency, more attack behaviors (e.g., biting, tail-rattling, upright) and extended attack duration. Most strains of rats and mice show increased aggression during social interaction after postweaning isolation (Pinna et al., 2004; Ibi et al., 2008; Toth et al., 2011). We also observed that, compared with group-housed mice, isolated mice show remarkably enhanced avoidance behaviors during social interaction by rejecting contact from the stimulus mice. Thus, postweaning isolation increases social affiliation with conspecifics, impairs social cognition, and completely destroys social function in male CD-1 mice.

Postweaning Isolation Reduced Sexual Preference in Male CD-1 Mice

Our study found that postweaning isolated mice display a lower sexual preference index compared with that of group-housed mouse. The sexual preference test utilizes animals' interest and natural exploration of individuals of the opposite sex and determines changes in an animal's sexual preference for the opposite sex by measuring the times they spend exploring conspecifics of the same and opposite sex (Zhang et al., 2013). In most experiments, a sexually active male rat/mouse and an oestrous female are placed on both sides of a three-chamber test box and covered with a metal wire cage to avoid running and physical contact with the target mouse (Winslow, 2003). The sexual preference index is then calculated to detect changes in animal sexual preference by measuring the times of non-contact exploration between the test animal and conspecifics of the same and opposite sex.

In this study, the sexual preference index of the isolated male CD-1 mice was lower than that of the group-housed mice, indicating a decrease in female exploration time in the former. In terms of exploration time, the average exploration time of isolation-reared mice was higher than that of the group-housed mice, although the difference observed between groups was not statistically significant. Isolation-reared mice spent more time exploring their male counterparts than did group-housed mice, and the difference found was statistically significant. Thus, the decreased sexual preference index of isolated mice is due to the increased time spent exploring males rather than the decreased time spent exploring females. The sexual preference index of isolated mice was higher than 50%, which indicates that these mice spent more time exploring females than males. Therefore, isolation rearing does not reduce the interest of male mice in exploring oestrous females but increases their interest in exploring their male counterparts, leading to a decrease in their sexual preference index.

Postweaning Isolation Did Not Induce Homosexual Behaviors in Male CD-1 Mice

The results of the sexual preference test suggest that the isolated mice are more interested in exploring males than are group-housed mice. Therefore, we used the homosexual behavior test to detect homosexual behavior in isolation-reared and group-housed mice. No unified method to detect the homosexual behavior of rodents has yet been reported. In the experiments, we replaced the oestrous female with a sexually experienced male at a sexually active age according to the test method of heterosexual behavior (Crawley, 2007). The interaction between the test mice and their sexually active male counterparts was used to observe whether the former exhibited homosexual behaviors. The observational indicators included male homosexual behaviors, such as anogenital sniffing, mounting, intromission, ejaculation, and assumption of the posture of female sexual receptivity, also known as lordosis. The results showed that neither of the groups exhibit any specific homosexual behavior, such as intromission or ejaculation. No mounting between the test mice and the stimulus mice or lordosis was observed. However, the isolation-reared mice showed shorter anogenital sniffing durations than did the control group, indicating decreased interest in mating with the male mice. Moreover, isolation-reared mice displayed strong aggression toward males and refused contact from other

male mice in the social interaction tests. This finding suggests that the observed increased exploration interest of the test mice for mice of the same sex may be due to hostility and aggression toward the same sex rather than sexual interest.

Postweaning Isolation Extended the Latency to Mate, Leading to Reduced Mating Behaviors in Male CD-1 Mice

Our study found that isolated mice tend to have a lower mating success rate compared with group-housed mice. In addition, the former had shorter mating durations than the latter. These results indicate that postweaning isolation reduces the heterosexual behaviors of male CD-1 mice. However, isolated mice demonstrated longer latencies for mounting and intromission. An increase in latency to mount means an increase in time from the first meeting to the first mount, whilst increased latency to intromission means a longer time interval from meeting to intromission. The findings reveal that isolated male mice need more time to identify, judge and perform the mating process compared with group-housed mice. No significant difference was observed between the two groups in terms of latency to ejaculation and refractory period, which may imply that the sexual physiological function of male mice is not affected by isolation. In addition, no statistically significant difference was observed between the two groups in terms of intromission frequency, latency to ejaculation, and refractory period, which also indicates that sexual function is not affected by isolation. However, sexual behavior is not only simply a physical activity; it is also a social activity closely related to social function. Isolation rearing induces extended latencies to mount and intromission. During this latency, the interaction between a male and a female influence when the mating process should begin. Whilst increases in mating latency seem to be caused by impaired social function in isolated mice, the related mechanism remains unclear and requires further study. Taken together, the results indicate that postweaning isolation extends the latency to mate and further leads to reduced mating behaviors in male CD-1 mice.

One advantage of this study is that we systematically evaluated the effect of postweaning isolation on adult social behaviors in terms of social affiliation, social recognition, social memory and social interaction in male CD-1 mice. Another advantage is that we evaluated the sexual preference and homosexual and heterosexual behaviors of male CD-1 mice in adulthood, all of which are closely related to social function. We believe our findings add some knowledge to the body of research on isolation-induced social and sexual behavior disorders in CD-1 mice.

This study presents some limitations that must be acknowledged. Firstly, we only tested the first sexual behavior of mice and did not observe dynamic changes in sex preference and sexual behavior continuously. Secondly, the results of the current study on the association between isolation rearing and sexual behavior changes in mice may not be generalized to include humans. Nevertheless, we believe our findings are of great significance to future explorations on etiology between loss of social relationship during crucial development periods and social and sexual behavior changes in adulthood.

CONCLUSION

Postweaning isolation increased the social affiliation of adult male CD-1 mice for conspecifics, impaired their social recognition and destroyed their social function during social interaction in both the home cage and a novel environment. Postweaning isolation also induced decreased sexual preference for females in the CD-1 mice and extended their latency to mate, leading to reduced mating behaviors. No association was observed between postweaning isolation and homosexual sex in male CD-1 mice.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

S-YX and X-ML contributed in the experimental design. X-PW contributed in the technical development. Z-WL, CL, and NJ performed the experiments. Z-WL analyzed the data and wrote the manuscript. YY helped perform the data analysis and worked over the first draft of the manuscript. All authors approved the final version of the manuscript.

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Effects of an Early Postnatal Music Intervention on Cognitive and Emotional Development in Preterm Children at 12 and 24 Months: Preliminary Findings

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Preterm birth is associated with a higher prevalence of neurodevelopmental deficits. Indeed, preterm children are at increased risk for cognitive, behavioral, and socio-emotional difficulties. There is currently an increasing interest in introducing music intervention in neonatal intensive care unit (NICU) care. Several studies have shown short-term beneficial effects. A recent study has shown that listening to a familiar music (heard daily during the NICU stay) enhanced preterm infants' functional connectivity between auditory cortices and subcortical brain regions at term-equivalent age. However, the long-term effects of music listening in the NICUs have never been explored. The aim of this study was to evaluate at 12 and 24 months the effects of music listening in the NICU on cognitive and emotional development in preterm children by comparing them to a preterm control group with no previous music exposure and to a full-term group. Participants were 44 children (17 full-term and 27 preterm). Preterm children were randomized to either music intervention or control condition (without music). The preterm-music group regularly listened to music from 33 weeks postconceptional age until hospital discharge or term-equivalent age. At 12 months, children were evaluated on the Bayley Scales of Infant and Toddler Development, Third Edition, then with 4 episodes of the Laboratory Temperament Assessment Battery (assessing expressions of joy, anger, and fear, and sustained attention). At 24 months, the children were evaluated with the same tests, and with 3 additional episodes of the Effortful Control Battery (assessing inhibition). Results showed that the scores of preterm children, music and control, differed from those of full-term children for fear reactivity at 12 months of age and for anger reactivity at 24 months of age. Interestingly, these significant differences were less important between the preterm-music and the full-term groups than between the preterm-control and the full-term groups. The present study provides preliminary, but promising, scientific findings on the beneficial long-term effects

of music listening in the NICU on neurodevelopmental outcomes in preterm children, and more specifically on emotion mechanisms at 12 and 24 months of age. Our findings bring new insights for supporting early music intervention in the NICU.

Keywords: preterm children, early intervention, music, anger reactivity, fear reactivity, emotion regulation

INTRODUCTION

Numerous studies reported a higher prevalence of neurodevelopmental deficits in children born prematurely compared to full-term children. More specifically, preterm survivors are at increased risk for cognitive (Bhutta, 2002; Brydges et al., 2018), behavioral (Bröring et al., 2018; Franz et al., 2018), and socio-emotional difficulties (Clark et al., 2008; Treyvaud et al., 2012; Lejeune et al., 2016; Montagna and Nosarti, 2016) which can negatively impact on their academic achievements (Akshoomoff et al., 2017; Twilhaar et al., 2018) and tend to persist into adolescence and adulthood (Hack et al., 2002; Cooke, 2004; Indredavik et al., 2005; Linsell et al., 2018). From an early age, emotional, attentional and inhibition impairments are frequently reported in preterm infants (Anderson et al., 2011; Aarnoudse-Moens et al., 2012). Interestingly, some researchers followed the developmental trajectory of these abilities longitudinally in a cohort of very preterm infants in comparison to their full-term peers. At 12 months, preterm infants exhibited greater reactivity to anger and lower reactivity to fear than full-term infants (Langerock et al., 2013). At 24 months, they were described by their parents as having a higher level of negative affect (Lejeune et al., 2015). At 42 months, they had higher scores of frustration and fear levels, and were less accurate when naming emotional facial expressions, including happiness, sadness, fear, anger and disgust (Witt et al., 2014). In addition, 12- and 24-month-children infants showed distinct attentional patterns compared to full-term children (Langerock et al., 2013; Lejeune et al., 2015). Preterm children also exhibited early inhibition difficulties compared to their full-term peers at 24 and 42 months (Witt et al., 2014; Lejeune et al., 2015). These studies highlight the necessity of implementing early intervention to support cognitive and emotional development in preterm infants.

In the absence of major brain lesions, these neurodevelopmental difficulties are both due to the disruption of normal brain development and to prematurity itself, as well as to an adverse postnatal environment. Preterm birth interrupts abruptly the brain maturation and can result in delayed or abnormal brain development during critical periods involving glial cell proliferation, synaptogenesis, pruning, and initiation of myelination (Volpe, 2001). Therefore preterm infants are at high risk for injury to the gray and white matter (Inder et al., 1999), delay in cortical maturation (Dubois et al., 2008), brain tissue volume alterations (Peterson et al., 2003; Nosarti et al., 2004; Inder et al., 2005; Mewes et al., 2006; Keunen et al., 2012; Cismaru et al., 2016), as well as impaired connectivity with long-term effects on socio-emotional and cognitive outcomes (Peterson et al., 2000; Treyvaud et al., 2013; Matthews et al., 2018). Moreover, preterm infants are exposed for weeks in the neonatal intensive care unit (NICU), to important factors of stress such as an atypical sensory environment (including high levels of noise and light), maternal separation and exposure to routine pain procedures. All these factors have short-term effects with behavioral and physiological stress responses (Peng et al., 2009), as well as long-term effects on their emotional and cognitive development (Montagna and Nosarti, 2016). For example, numerous studies have focused on the negative effect of noise and found that intense sounds act as stressful events on physiological self-regulatory abilities (Wachman and Lahav, 2011). The stress generated by these inadequate sensory stimulations leads to significant changes in the hypothalamic-pituitary-adrenal axis, as well as changes in brain development which could in turn impact the subsequent neurodevelopmental outcomes of preterm infants (Mooney-Leber and Brummelte, 2017).

In this context, there is currently a major need of developing intervention which aims to support the sensory and emotional development of preterm newborns by offering them a physical and human environment adapted to their needs. Developmental care programs are designed to limit overstimulations, pain and stress for preterm infants in the NICU, and to promote their well-being through various interventions, such as reduction of light and sound, skin-to-skin contact or massage therapy. These programs have already shown positive effects on neurodevelopmental outcomes in children born preterm (Spittle and Treyvaud, 2016). Environmental enrichment by music might be a non-invasive intervention to reduce preterm infants' stress during their hospitalization in the NICU.

Listening to music is a complex cerebral process, as it involves auditory, cognitive, motor, and emotional functions soliciting widespread activation of various neuronal networks (Koelsch, 2014; Sihvonen et al., 2017). Studies showed that listening to music had positive effects on stress and anxiety reduction in healthy adults (Linnemann et al., 2015a; Panteleeva et al., 2018) and newborns (Rossi et al., 2018), as well as for pain-reduction in patients with chronic pain disease (Linnemann et al., 2015b) or in postoperative patients who had various types of major surgery (Hole et al., 2015). These studies suggest that music intervention may enhance self-regulatory abilities. Music seems to be a relevant intervention in the management of stress, anxiety and pain in vulnerable population, such as preterm newborns, which could in turn have positive effects on their long-term neurodevelopment (see for a review, Anderson and Patel, 2018).

Recent studies have considered the effects of music listening in preterm infants and many have shown that proposing harmonious and regular sounds had short-term beneficial effects (during NICU stay and until hospital discharge), such as stabilizing heart and respiratory rate, reducing apnea or bradycardia episodes, improving resting energy expenditure and feeding, better weight gain and more mature sleep patterns

(Anderson and Patel, 2018). Music listening has been shown to activate brain regions related to emotional processing in adults (Koelsch, 2014) and even in full-term newborns (Perani et al., 2010). Furthermore, a recent study has shown that repeated listening to familiar music (heard daily during the NICU stay) enhanced the functional connectivity of preterm infants between the auditory cortices and the subcortical brain regions at term-equivalent age. This result might not only reflect that preterm infants recognized the known music but also that they perceived it as more arousing and pleasant (Lordier et al., 2018). Other studies have reported positive effects on behavioral development after exposure to a breathing bear (Thoman et al., 1991; Ingersoll and Thoman, 1994), to the sound of a heartbeat (Barnard and Bee, 1983), or to voices (Nöcker-Ribaupierre et al., 2015; Filippa et al., 2017; Best et al., 2018; Saliba et al., 2018) during the NICU stay. However, the long-term effects of music listening in the NICUs on preterm infant's cognitive and emotional development have never been explored so far.

Self-regulatory abilities, which are impaired in the preterm survivors (Langerock et al., 2013; Witt et al., 2014; Lejeune et al., 2015), have be shown to be enhanced by music listening in different clinical population, such as patients with chronic pain disease or those who had various types of major surgery (Hole et al., 2015; Linnemann et al., 2015b), as well as in full-term newborns (Rossi et al., 2018). Assessment of the long-term effect of music interventions in preterm children should focus on these specific outcomes.

The aim of this study was to evaluate long-term effects of music listening in the NICU on cognitive and emotional development in preterm children by comparing them to a preterm control group with no previous music exposure and to a full-term group at 12 and 24 months. The cognitive and emotional abilities of the preterm music group were expected to be higher than those of the preterm control group, as well as to be closer to those of the full-term group.

MATERIALS AND METHODS

Participants

The initial cohort included 39 preterm infants (gestational age at birth < 32 weeks) and 24 full-term infants, born between March 2013 and October 2015, who were participants in a longitudinal study assessing the effects of early music exposure during the NICU stay on brain processing (Lordier et al., 2018) and neurobehavioral development. Infants were recruited at the neonatal units of the University Hospital of Geneva. Written informed parental consent was obtained for each newborn prior to participation. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University Hospital of Geneva.

The present study concerned the cognitive and emotional evaluation of these children at 12 and 24 months of age, which took place in the follow-up unit of the University Hospital of Geneva. Nineteen participants (7 full-term and 12 preterm) from the initial cohort did not participate in the follow-up assessment (**Figure 1**). Exclusion criteria for all newborns were major brain lesions detected on early MRI, such as intraventricular hemorrhage grade III with or without apparent periventricular hemorrhagic infarction, or cystic periventricular leukomalacia. The final sample consisted of 44 children (17 full-term and 27 preterm). There was no significant difference in demographic and perinatal variables between full-term children who participated in the follow-up and those who dropped out. For the preterm group, there was only one significant difference for the family's socioeconomic status (SES), $t(35) = 2.760$, $p = 0.009$. The family's SES is a 12-point scale based on paternal occupation and maternal education (range from 2 – the highest SES – to 12 – the lowest SES). The SES of the families of preterm children who dropped out (mean = 3.20, $SD = 0.9$) was higher than that of the families of preterm children who participated in the follow-up assessment (mean = 6.22, $SD = 3.4$).

Preterm infants were randomized to either music intervention or control condition (without music). **Figure 1** illustrates the flow chart of the participants in the study. The preterm-music group consisted of 13 children at 12 months (mean corrected age = 14.7 months, SD = 2), and 10 children at 24 months (mean corrected age = 24.9 months, $SD = 1.03$). The preterm-control group consisted of 10 children at 12 months (mean corrected age = 14 months, $SD = 1.2$), and 7 children at 24 months (mean corrected age = 25.6 months, $SD = 1.2$). The full-term group was composed of 12 children at 12 months (mean age = 14.8 months, $SD = 1.9$), and 15 children at 24 months (mean age = 25.8 months, $SD = 1.9$). The mean ages of the three groups did not differ significantly at both assessment ages (all $ps > 0.05$). The SES of the families of preterm children (mean = 6.22, $SD = 3.4$) was marginally lower than that of the families of full-term children (mean = 4.47, $SD = 2.8$), $t(42) = 1.857$, $p = 0.07$.

Table 1 presented demographic and perinatal variables of the three groups. The preterm-music and the preterm-control groups did not differ significantly on these demographic and perinatal variables.

Procedure

Music Intervention

Preterm infants were randomized to either music intervention (preterm-music) or control condition (preterm-control group). Parents, caregivers and music intervention providers were blind to group assignment. The preterm-music group listened to a music especially created by Andreas Vollenweider[1] during 8 min with headphones, from gestational age of 33 weeks until hospital discharge or term-equivalent age. The music was composed of background, bells, harp, and punji. Three tracks were created in collaboration with a nurse specialized in developmental care. Music was presented to the baby according to the state of wakefulness, following his biological rhythm: one was composed with the aim of helping the baby to wake up, one to maintain the child in a state of calm awakening, and the last one to help the baby to fall asleep. The nurse determined the readiness for music exposure and chose the track based on a neonatal behavioral assessment scale (Martinet et al., 2013). The intervention was performed

[1] http://vollenweider.com/en

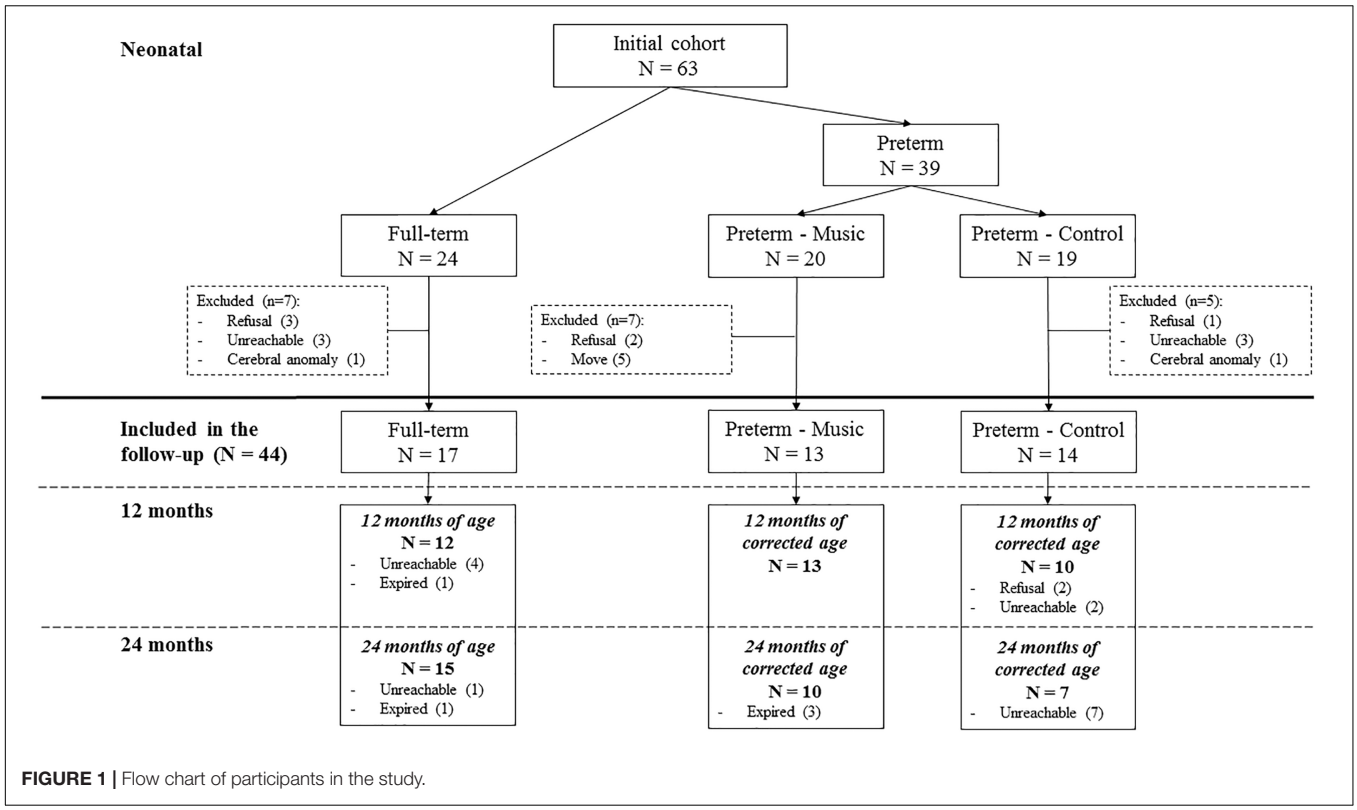

FIGURE 1 | Flow chart of participants in the study.

only when the baby was lying in the bed. The music extract presented a high degree of homogeneity and repetitions, and it was structured by a continuous background, with short and repetitive melodies in a reduced pitch range. The sound level ranged from 30 dBA (background) to 65 dBA (peak with the bells).

Preterm-music infants listened to music about 5 times per week and preterm-control infants had open headphones put on without music at the same frequency. The mean number of music listening was 24.58 times (*SD* = 9.49) for the preterm-music group and the mean number of having open headphones was 23 times (*SD* = 6.28) for the preterm-control group. More details about the music intervention can be found in Lordier et al. (2018).

Full-term children were recruited at the maternity of the same hospital where they underwent magnetic resonance imaging including an fMRI music paradigm in their first days of life (Lordier et al., 2018). They were thus exposed to music only once during this fMRI. They were then contacted for follow-up assessments at 1 and 2 years.

Cognitive and Emotional Evaluation

The children were tested individually in a quiet room with at least one reference person present during a 1-h session. They were seated on the reference person's lap, or in front of a small table on a small chair. All the evaluations were videotaped with written informed parental consent for subsequent analysis and were done by trained psychologists or developmental pediatricians who were blind to the music group assignment. At 12 months, children were evaluated on the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III; Bayley, 2006), then with 4 episodes of the Laboratory Temperament Assessment Battery (Lab-TAB; Goldsmith and Rothbart, 1999). At 24 months, the children were evaluated with the same tests, as well as with 3 additional episodes of the Effortful Control Battery (Kochanska et al., 2000). The 4 episodes from the Lab-TAB were Puppet game, Attractive toy placed behind barrier, Unpredictable mechanical toy, and Blocks, assessing expression of joy, anger, fear, and sustained attention, respectively. The 3 episodes from the Effortful Control Battery were Snack delay, Wrapped gift, and Tower, measuring the child's ability to delay (wait for a pleasant event) twice and to suppress or initiate activity to signal (take turns), respectively. Two coders scored the episodes independently for 16% of the sample after thorough training on the scoring methods. Inter-rater reliability was calculated using Pearson correlations on the means for each variable by episode. Correlations ranged from 0.59 to 1 with a mean *r* of 0.83.

Measures

Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III)

The BSID-III is a standardized battery of tests that assesses development of different domains in 1- to 42-month-old children and generates scores for 3 composite indices (cognitive, language, and motor). Raw scores were converted into standard scores based on adjusted age.

Laboratory Temperament Assessment Battery (Lab-TAB)

Lab-TAB coding involves facial, vocal, and bodily measures. For each episode, the measures were coded on a scale from 0 to 3. A higher score indicates higher emotional reactivity.

The puppet game was used to assess joy and involved the presentation of a scripted puppet show lasting about 1 min. This episode consisted of presenting 2 puppets who interacted with the child and tickled him three times. Scoring was performed in four equivalent time intervals (introduction, first tickle, second tickle, and third tickle). For each time interval, intensity of smiling, positive vocalizations and positive motor activity were coded. These scores were averaged to compute a score of Joy.

The attractive toy placed behind barrier was used to assess anger. It consisted of presenting an attractive toy to the child. Once he was playing with interest, the toy was gently removed from his hand and placed behind a transparent Plexiglas barrier for 30 s (2 trials). Scoring for each trial was performed in 6 time intervals of 5 s. For each time interval, intensity of facial anger, distress vocalizations and struggle were coded. These scores were averaged to compute a score of Anger for each trial.

The unpredictable mechanical toy was chosen to elicit fear and involved the presentation of a mechanical robot placed on a table in front of the child. The robot went toward the child, stopped in front of him and barked, and then moved back. The episode lasted 15 s and included 2 trials. Scoring for each trial was performed in 3 time intervals of 5 s. For each time interval, intensity of facial fear, distress vocalizations, bodily fear, escape, and startle response were coded. All measures were then averaged across time intervals to compute a score of Fear for each trial.

The Blocks episode measures sustained attention. The child played freely with decorated cubes for 3 min. Each minute was divided into 6 time intervals of 10 s. Each time interval was coded for intensity of facial interest, duration of observation and duration of manipulation. All scores were averaged to compute a composite score of Sustained attention.

TABLE 1 | Population characteristics.

	Full-term	Preterm-control	Preterm-music	Preterm-control vs. Preterm-music
	n = 17	*n* = 14	*n* = 13	
	n(%) or mean (*SD*)	*n*(%) or mean (*SD*)	*n*(%) or mean (*SD*)	*p*-value[a]
Sex, number of girls	9 (52.9)	7 (50)	8 (61.5)	0.547
Socioeconomic score[b]	4.47 (2.8)	6.07 (3.3)	6.38 (3.6)	0.867
Gestational age at birth (weeks)	39.57 (1)	29 (2.2)	29.14 (2.3)	0.905
Birth weight (g)	3271 (410)	1207 (274)	1217 (377)	1.000
Small for gestational age[c]	1 (5.9)	3 (21.4)	1 (7.7)	0.315
Birth height (cm)	49.3 (1.4)	37.2 (4)	38 (3.4)	0.519
Birth head circumference (cm)	34.2 (1.3)	26.6 (2.2)	27.1 (3.1)	0.519
Broncho-pulmonary dysplasia	0	5 (35.7)	2 (15.4)	0.228
Intraventricular hemorrhage (grade I–II)	0	3 (21.4)	4 (30.8)	0.580
Early and late onset sepsis	0	3 (21.4)	0	0.077
Patent ductus arteriosus	0	1 (7.1)	1 (7.7)	0.957

[a] *Pearson's chi-square and Mann–Whitney U-tests were used for the comparison of the variables between the preterm-music and preterm-control groups.* [b] *The socioeconomic status (SES) was calculated using the Largo et al. (1989) 12-point scale based on paternal occupation and maternal education (range from 2 – the highest SES – to 12 – the lowest SES).* [c] *Small for gestational age: <10th percentile for birth weight as a function of gestational age and gender.*

Effortful Control Battery

The Snack Delay measures the child's ability to delay gratification. Children were asked to place their hands on a mat on the table and not to touch or eat a treat placed in front of them under a transparent cup until the experimenter rang a bell (4 trials, with delays of 10, 20, 30, and 15 s, respectively). In the middle of each trial, the experimenter picked up the bell but did not ring it. For each trial, an inhibition score was computed on a scale from 1 to 9 (1 = child eats snack before experimenter lifts bell; 4 = child touches snack after experimenter lifts bell; 7 = child waits until bell rung). One point was added for keeping hands on the mat only before or after the experimenter lifted the bell, and 2 points were added for keeping hands on the mat during the entire trial.

The Wrapped Gift also measures the child's ability to delay gratification. During the first part of the episode, children were told that they would receive a gift but that they could not peek while the gift was being wrapped. The experimenter asked the child to sit down with his or her back to him as he noisily wrapped (60 s). During the second part, the wrapped gift was placed on the table, and the child was told to stay on his or her chair and not to touch the gift until the experimenter returned with a bow (180 s). Scoring during the first part corresponded to the Turn score on a scale from 1 to 5 (1 = child turns around and continues to peek; 3 = child peeks over shoulder; 5 = child does not peek). Scoring during the second part was divided into 2 scores on a scale from 1 to 4: the touch score (1 = child opens gift; 4 = child never touches the gift) and the Seat score (child is on the seat for a total time of 1 = less than 30 s; 2 = less than 1 min; 3 = less than 2 min; 4 = more than 2 min).

The Tower assesses the ability to take turns by suppressing an impulsive motor response. Children were invited to take turns with the experimenter to build a tower with wooden blocks. The experimenter demonstrated turn-taking to ensure that the child understood what it meant. The episode included 2 trials and an Inhibition scores was computed for each trial. The total number of the placed blocks (multiplied by 10) was divided by the number of blocks put by the child. If a child took turns with the experimenter every time, she or he placed as many blocks as the

experimenter (20 was the highest score). A penalty of −5 points was given for intentionally knocking down the tower.

Statistical Analysis

All statistical analyses were conducted using SPSS 25.0 (IBM SPSS Statistics, IBM Corporation). Kolmogorov–Smirnov analyses were performed to verify the normality of the data. The not normally distributed scores were then transformed into rank-ordered scores (Conover and Iman, 1982). Moreover, all of the analyses were performed to control for the effects of between-group differences in SES, as well as for the age at assessment (chronological age for full-term children; corrected age for preterm children).

Analyses of covariance (ANCOVAs) were run on raw scores for the normally distributed data and on the ranked dependent variables for the not normally distributed ones. These analyses were performed for each dependent variable with the group (preterm music vs. preterm control vs. full-term) as the between-subjects factor. For the tasks comprising several trials, repeated-measures ANCOVAs were conducted with the trial (trial 1 vs. trial n) as the within-subjects factor and the group (preterm music vs. preterm control vs. full-term) as the between-subjects factor. To further investigate the significant group effects, contrasts were conducted. Effect sizes for the overall ANCOVAs were reported (calculated by the SPSS software), as well as those for the contrasts [calculated according to Field (2009), p.390], using the values of t and df). The significant threshold was 0.05 and the marginal threshold was 0.07.

RESULTS

At 12 Months

The results of the evaluation of children at the age of 12 months are presented in **Table 2**.

Bayley Scale

No significant group effect was observed.

Lab-TAB

For the unpredictable toy episode, results revealed a significant group × trial interaction, $F(2,21) = 10.12$, $p < 0.001$, $\eta_p^2 = 0.49$. A significant group effect was only observed for the second trial (Anger-Trial 2), $F(2,21) = 5.612$, $p = 0.011$, $\eta_p^2 = 0.35$. Contrasts indicated that the full-term group had a higher score of fear than the preterm-control group [$F(1,21) = 10.93$, $p = 0.003$, $\eta_p^2 = 0.59$] and the preterm-music group [$F(1,21) = 4.63$, $p = 0.043$, $\eta_p^2 = 0.42$] during the second trial, showing that the difference of fear reactivity was less important between the preterm-music and the full-term groups ($\eta_p^2 = 0.42$) than between the preterm-control and the full-term groups ($\eta_p^2 = 0.59$). Contrasts also showed that a significant increase in fear reactivity was observed between Trial 1 and Trial 2 in the full-term group [$F(1,21) = 12.03$, $p = 0.002$, $\eta_p^2 = 0.60$], whereas the opposite was observed in the preterm-control group [$F(1,21) = 7.05$, $p = 0.015$, $\eta_p^2 = 0.50$]. There was no significant change in fear reactivity between the two trials in the preterm-music group [$F(1,21) = 0.34$, $p = 0.565$, $\eta_p^2 = 0.13$]. No other significant effect was observed.

At 24 Months

The results of the evaluation of children at the age of 24 months are presented in **Table 3**.

Bayley Scale

No significant group effect was observed.

Lab-TAB

For the toy behind barrier episode, results revealed a significant group effect for the second trial (Anger-Trial 2), $F(2,23) = 3.659$, $p = 0.042$, $\eta_p^2 = 0.24$. Contrasts indicated that the full-term group had a higher score of anger than the preterm-control group [$F(1,23) = 5.988$, $p = 0.022$, $\eta_p^2 = 0.45$] and the preterm-music group [$F(1,23) = 4.26$, $p = 0.05$, $\eta_p^2 = 0.39$] during the second trial, showing that the difference of anger reactivity was less pronounced between the preterm-music and the full-term groups ($\eta_p^2 = 0.39$), than between the preterm-control and the full-term groups ($\eta_p^2 = 0.45$). No other significant effect was observed.

Effortful Control Battery

No significant effect between groups was observed.

DISCUSSION

The present study aimed to assess the effects of music listening in the NICU at 12 and 24 months of age on cognitive and emotional abilities in preterm children by comparing them to a preterm control group with no previous music exposure and to a full-term group. Results showed that the scores of the two groups of preterm children, music and control, differed from those of the full-term children for fear reactivity at 12 months of age and for anger reactivity at 24 months of age. Interestingly, these significant differences were less important between the preterm-music and the full-term groups than between the preterm-control and the full-term groups. These results will be discussed in regards to music listening in the NICU.

Firstly, during the fear-eliciting episode of the Lab-TAB, the full-term group aged 12 months expressed a higher level of fear reactivity than the preterm-control and the preterm-music groups during the second trial. This result is in accordance with previous findings showing that 12-month-old preterm infants perceived the unpredictable mechanical dog episode as less frightening than did the full-terms (Langerock et al., 2013). Furthermore, we did not find any difference at 24 months of age. In line with these findings, Lejeune et al. (2015) showed no difference between 24-month-old preterm and full-term children using the same fear-eliciting episode. However, at 42 months, preterm children displayed higher fear reactivity during another fear-eliciting episode of the Lab-TAB (the mask) than their full-term peers (Witt et al., 2014). This different developmental trajectory of fear reactivity in the two populations is in favor of a developmental delay hypothesis in the preterm population.

Interestingly, the effect sizes also revealed that the difference of fear reactivity was less important between the preterm-music and the full-term groups (η_p^2 = 0.42) than between the preterm-control and the full-term groups (η_p^2 = 0.59). Moreover, a significant increase in fear reactivity was observed between Trial 1 and Trial 2 in the full-term group, while the opposite

TABLE 2 | Results of the Bayley and the Lab-TAB at 12 months according to the group.

	Preterm-control *n* = 10	Preterm-music *n* = 13	Full-term *n* = 12		
	mean (*SD*)	mean (*SD*)	mean (*SD*)	*F*	*p*
Bayley scale					
Cognitive	106 (11)	100.4 (9.7)	110 (9.5)	1.817	0.180
Motor	100.2 (13.2)	99.3 (5.7)	105.7 (9.8)	0.708	0.501
Langage	97.2 (9.5)	95.5 (8.2)	99.7 (5.9)	0.385	0.684
Puppet game					
Joy	1.06 (0.5)	0.99 (0.5)	0.88 (0.5)	0.229	0.797
Toy behind barrier					
Anger-Trial 1	0.78 (0.7)	0.68 (0.4)	0.72 (0.5)	0.260	0.773
Anger-Trial 2	0.57 (0.4)	0.56 (0.7)	0.84 (0.5)	0.745	0.485
Unpredictable toy					
Fear-Trial 1	0.41 (0.4)	0.27 (0.3)	0.42 (0.2)	0.099	0.905
Fear-Trial 2	0.18 (0.2)*	0.35 (0.4)§	0.63 (0.3)§ *	5.612	**0.011**
Blocks					
Sustained attention	2.09 (0.6)	2.03 (0.6)	2.17 (0.8)	0.979	0.387

*Boldface entries indicate significant difference ($p < 0.05$). §,*Significant contrasts between two groups.*

TABLE 3 | Results of the Bayley, the Lab-TAB and the Effortful Control Battery at 24 months according to the group.

	Preterm-control *n* = 7	Preterm-music *n* = 10	Full-term *n* = 15		
	mean (*SD*)	mean (*SD*)	mean (*SD*)	*F*	*p*
Bayley scale					
Cognitive	98.6 (6.9)	100 (10.8)	101.7 (7.9)	0.015	0.985
Motor	102.1 (8.1)	101.5 (9.7)	106.3 (13.5)	0.555	0.581
Langage	92.1 (8.5)	92.1 (8.4)	95.9 (12)	0.283	0.756
Puppet game					
Joy	0.83 (0.6)	0.87 (0.6)	0.98 (0.6)	0.240	0.789
Toy behind barrier					
Anger-Trial 1	0.49 (0.3)	0.49 (0.3)	0.68 (0.4)	1.240	0.308
Anger-Trial 2	0.44 (0.4)*	0.51 (0.3)§	0.82 (0.4)§ *	3.659	**0.042**
Unpredictable toy					
Fear-Trial 1	0.74 (0.4)	0.77 (0.6)	0.76 (0.6)	0.093	0.912
Fear-Trial 2	0.76 (0.5)	0.88 (0.8)	0.98 (0.7)	0.861	0.438
Blocks					
Sustained attention	2.31 (0.4)	2.49 (0.3)	2.59 (0.3)	1.457	0.254
Snack delay					
Inhibition-Trial 1	9 (0)	8.2 (1.5)	7.27 (2.3)	1.881	0.178
Inhibition-Trial 2	7 (4)	7.2 (2.8)	7.45 (2.8)	0.075	0.928
Inhibition-Trial 3	6.75 (3.9)	7.1 (2.5)	7.2 (2.7)	0.110	0.896
Inhibition-Trial 4	8 (2)	6.6 (2.7)	7.82 (1.7)	0.774	0.475
Wrapped gift					
Peak and turn	2.83 (1.8)	1.44 (0.7)	1.86 (1.4)	2.071	0.148
Touch	3.33 (1)	3.33 (1.1)	3.29 (1)	0.011[a]	0.989
Seat	2.33 (1.5)	2 (1.3)	2.14 (1.1)	0.099	0.906
Tower					
Inhibition-Trial 1	16.6 (2.3)	17.2 (1.6)	14.8 (2)	2.138	0.157
Inhibition-Trial 2	18.1 (2.1)	17.1 (1.7)	15.9 (2.8)	1.719	0.218

*Boldface entries indicate significant difference ($p < 0.05$). [a]Normalized by rank-ordered transformation for ANCOVAs. §,*Significant contrasts between two groups.*

was observed in the preterm-control group. The preterm-music group did not show any significant change in fear reactivity between the two trials. Taken together, these results suggest that music exposure in NICU would have a positive impact on fear processing. Recently, a study has revealed a processing bias toward fear: when preterm adults were presented with different facial emotion and they had to identify the emotion, they were more likely to report fear than another negative emotion (Gao et al., 2017). The authors suggested that this bias for fear could reflect a dysregulation of the neuronal distributed fear system. Our results showing that the processing of fear in the preterm-music group is closer to that of the full-term group, is promising as music listening in the NICU could have long-term positive effects on fear processing and regulation.

Cismaru et al. (2016) compared amygdala volumes in full-term infants and preterm infants at term-equivalent age, and related preterm infants' amygdala volumes with their performance on the unpredictable mechanical dog episode at 12 months. They found that amygdala volumes were smaller in preterm infants than in full-term infants. They also observed that amygdala volumes were larger in infants showing an escape response of fear compared to the infants showing no escape response. In other words, 12-month-old preterm infants display a reduced fear reactivity and it seems to be related to smaller amygdala volumes. Our results suggest that music listening in the NICU could have some positive effects on fear processing and regulation and it could also have a positive impact on amygdala volumes or related connected brain regions. It would be interesting to address this question in further studies.

Secondly, during the anger-eliciting episode of the Lab-TAB, the 24-month-old full-term group expressed a higher level of anger reactivity than the preterm-control and the preterm-music groups during the second trial. A previous study using the same task found contradictory results with no significant difference at 24 months observed between full-term and preterm children (Lejeune et al., 2015). Nevertheless, it should be noted that the present study used corrected age, while the previous one used the chronological age. This difference could explain the discrepancy between the results. More importantly, our results indicated that the difference of anger reactivity was less pronounced between the preterm-music and the full-term groups ($\eta_p^2 = 0.39$), than between the preterm-control and the full-term groups ($\eta_p^2 = 0.45$). It seems that the music intervention would also have a positive impact on the processing of anger with an anger reactivity in the preterm-music group closer to that of the full-term group.

It is also interesting to note that the significant group differences for fear and anger regulation were observed only during the second trial of the episodes. It is possible that preterm children had greater difficulties in maintaining an optimal level of emotional processing and regulation when the emotion-eliciting episodes were repeated. Previous findings indicated differences in emotion regulation strategies between preterm and full-term children (Clark et al., 2008; Evrard et al., 2011). Preterm children could have altered emotion regulation strategies that did not allow for optimal emotional regulation over time. It would explain why significant differences appeared between preterm and full-term children only in the second trial, and consequently why the potential positive impact of music intervention could only be observed in the second trial.

In addition, the three groups did not significantly differ for the cognitive, language, and motor scales of the BSID-III. This result contrasts with previous researches showing that preterm infants achieved lower mean scores on all of the Bayley-III scales than full-term ones at 12 and 24 months of age (Yu et al., 2013; Gasparini et al., 2017). Finally, no significant group difference was found for joy, sustained attention and inhibition abilities. Previous studies found different results with higher levels of joy reactivity in 12-month-old preterm children, different attention scores in 12- and 24-month-old preterm children compared to full-term children, and inhibition difficulties at 24 months (Langerock et al., 2013; Lejeune et al., 2015). The small sample size of the present study could explain these differences of result. Future studies with a larger sample are necessary to verify these preliminary findings.

Preterm children and adolescents are at greater risk for emotional problems (Johnson and Marlow, 2011). Indeed, numerous studies reported a higher prevalence of internalizing problems in this population with an increased risk for anxiety symptoms, depressive symptoms, and withdrawn behavior (Guedeney et al., 2012; Somhovd et al., 2012; Montagna and Nosarti, 2016). Dimitrova et al. (2018) have recently shown that early emotional problems in 18-month-old preterm children predicted later internalizing problems at 11 years of age, but this link was moderated by the severity of perinatal stress. Preterm children who experienced high perinatal stress were at increased risk for emotional difficulties during preadolescence. Emotional problems also seem to persist into adulthood in the preterm population (Montagna and Nosarti, 2016). Our results regarding fear and anger processing and regulation suggest that music listening in the NICU may moderate the effects of preterm birth on later emotion mechanisms, especially in the more vulnerable preterm infants.

In addition, other factors may affect cognitive and emotional development, such as mother-child interaction, maternal anxiety and maternal sensitivity (Forcada-Guex et al., 2006; Zelkowitz et al., 2011; Bouvette-Turcot et al., 2017). For example, among the possible mother-infant dyadic patterns of interaction, the controlling pattern (with a controlling mother and a compliant child) was more often observed among preterm than full-term dyads at 6 months of age and was related to poorer developmental outcome at 18 months of age (Forcada-Guex et al., 2006). Interestingly, a recent study suggests that high maternal sensitivity during mother-infant interaction when the infant was 18 months old is a long-term resilience factor that prevents the development of internalizing disorders in 11-year-old preterm children (Faure et al., 2017). These studies highlight the importance of considering mother-child interaction, parental anxiety and maternal sensitivity as factors to control in future studies.

Introducing music in the NICU had positive effects on brain development in preterm infants. Indeed, Lordier et al. (2018) showed that listening to a familiar music every day during the NICU stay enhanced preterm infants' connectivity between the

right primary auditory cortex and the left caudate nucleus and between the primary auditory cortices and the left putamen and the superior temporal gyrus at term-equivalent age. This result might reflect that they recognized the music but also perceived it as more arousing and pleasant. Koelsch (2014) indicated that music elicited changes in the cerebral regions underlying emotion (limbic and paralimbic areas) in adults, similarly to full-term newborns (Perani et al., 2010). Moreover, music listening had also positive effects on stress and anxiety reduction, suggesting that it improved emotion regulation abilities (Van Goethem and Sloboda, 2011; Linnemann et al., 2015a; Panteleeva et al., 2018). Our preliminary findings are consistent with the literature supporting that music listening has positive effects in emotion processing and regulation. For the first time, the present study suggests some positive long-term effects of music listening in the NICU on neurodevelopmental outcomes in preterm children, and more specifically on emotion mechanisms.

There is a lack of precise guidelines for the choice of music for newborns: live or recorded, instrumental music or parents singing? Live music has been shown to have a larger effect on heart rate and sleep than recorded music (Garunkstiene et al., 2014). Furthermore, one main advantage of live music is that the musician can adapt his music to the baby's reactions. However, the use of live music needs a musician to be present for each baby and at the right time, leading to some difficulties to conduct live intervention during a long duration in NICUs and standardization of the music intervention becomes impossible. A second major concern in these developmental care interventions is the involvement of parents. Indeed, developmental care programs have shed light on the importance for the parents to be partners in their infant's care (Craig et al., 2015; Bieleninik et al., 2016). Recently, two reviews described physiologic and behavioral stabilization effect of maternal voice intervention in NICU care (Filippa et al., 2017; Provenzi et al., 2018). It is however important to mention that the present music intervention does not aim to replace the maternal presence/voice, but rather to complete its beneficial effects. Indeed, it is not possible for all mothers to be present every day with their baby in the NICU (for example, other children to take care). Future studies should compare the effect of mother speaking/singing versus music intervention on neurodevelopmental outcomes in preterm infants.

Limitations to the generalizability of our findings should be addressed. First, it included a relatively small sample size. Second, the attrition rate was quite important. Moreover, not all children could be assessed at both ages: some could only be seen at 12 months and others only at 24 months. This reveals the great difficulty of conducting longitudinal follow-up studies. Finally, Bonferroni corrections could also have been conducted given the multiple comparisons. However, since this exploratory study presented preliminary results with a relatively modest sample size, we did not perform such a threshold correction. From our data, we performed a calculation of the sample size (with power goal = 0.8) needed for a randomized controlled trial to answer the original research question, i.e., does music exposure in the NICU have an effect on emotional regulation (Unpredictable toy at 12 months)? These analyses indicate that it will be necessary to include at least 52 children (intervention and control groups). In their recent review of literature, Anderson and Patel (2018) underlined "the pressing need to examine the long-term neurodevelopmental outcomes of children who undergo music interventions in the NICU." The present study provides preliminary, but promising, scientific findings on the beneficial long-term effects of the music intervention in preterm children. However, future studies are needed with larger number of participants, in order to confirm and complement our results.

CONCLUSION

In conclusion, for the first time, the current study suggests some positive long-term effects of music listening in the NICU on emotion processing and regulation at 12 and 24 months of age in preterm children. Our findings bring new insights for supporting music intervention in the NICU. It would be interesting to investigate the later emotion processing of these infants in order to know if this positive effect would persist during childhood.

DATA AVAILABILITY

All data of this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

FL and LL conceptualized and designed the study, collected the data, performed and interpreted data analyses, and drafted the initial manuscript. MP and LS collected the data. DG conceptualized and designed the study, critically reviewed, and revised the manuscript. PH obtained funding for the study, conceptualized and designed the study, critically reviewed, and revised the manuscript. MF interpreted data analyses and drafted the initial manuscript. CBT conceptualized and designed the study, interpreted data analyses, and drafted the initial manuscript. All authors approved the final manuscript as submitted.

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SYSTEMATIC REVIEW
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Prenatal Risk Factors for Adverse Developmental Outcome in Preterm Infants—Systematic Review

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Background: Preterm infants are still at an increased risk for suboptimal neurodevelopmental outcomes when compared with term born infants. The development of a child born preterm can be jeopardized by suboptimal conditions during pregnancy, in addition to the suboptimal growth environment postnatally compared to the normal *in utero* environment. This review summarizes the literature on the role of chorioamnionitis, placental insufficiency, and maternal smoking on the developmental outcomes of preterm infants.

Methods: A systematic database search was performed to identify all original articles published on or before September 12, 2018 that evaluated the impact of clinical or histological chorioamnionitis, abnormal prenatal fetal and placental blood flow, and prenatal smoking exposure on the neuropsychological and cognitive outcomes of preterm infants. We identified a total of 54 studies. Thirty five original articles evaluated the effects of clinical or histological chorioamnionitis; 15 studies evaluated the effects of abnormal blood flow patterns; and four studies evaluated the effects of maternal smoking during pregnancy.

Results: The studies on prenatal risk factors showed conflicting results about the impact on the neurodevelopment of preterm infants. The majority of the studies did not show that chorioamnionitis poses a direct risk to the development of preterm infants. The role of abnormal prenatal placental and fetal blood flow on the development of preterm infants remained inconclusive because the sample sizes were often small and methodological problems complicated the interpretation of the data. Maternal smoking during pregnancy was assessed only in one cohort which showed that maternal smoking is a risk for suboptimal cognitive and neuropsychological development in preterm infants.

Conclusions: This review summarizes the data on several prenatal risk factors which play a role in the developmental outcomes of preterm infants. To optimize the developmental outcomes, we need to first optimize the fetal wellbeing before birth. More research that extends from the fetal life to long-term developmental outcomes is needed.

Keywords: chorioamnionitis, smoking, doppler, preterm, development

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INTRODUCTION

Although the perinatal and neonatal care of preterm infants is constantly improving, preterm infants are still at increased risk for suboptimal cognitive and neuropsychological outcomes when compared with term infants. Preterm infants will inevitably have a different developmental environment compared to their physiological *in utero* environment, which poses a risk for the developing brain. This risk increases further with decreasing gestational age (Munck et al., 2010; Lind et al., 2011; Cheong et al., 2017; Hirvonen et al., 2017; Luu et al., 2017; Twilhaar et al., 2018). In addition to medical risk factors associated with preterm birth and intensive care needed in these situations, the premature babies are exposed to other risk factors associated with poor neurodevelopmental outcome, such as parental separation, stress, anxiety, and depression. Also the ability to succesfully breast feed the baby is often compromised in a case on very preterm delivery, which is an additional risk factor for mother-infant interaction and neurodevelopment (Flacking et al., 2012). Further more, there are several prenatal factors related to prematurity which have been suggested to increase the risk of developmental deficits in preterm infants. This review summarizes the research of prevalent prematurity associated prenatal risk factors with accumulating new research on later cognitive and neuropsychological outcomes of preterm infants.

Choriamnionitis is an important cause of preterm delivery. The incidence of chorioamnionitis increases with decreasing gestational age, with nearly all spontaneous preterm deliveries occurring around 24 weeks of gestation being associated with chorioamnionitis (Andrews et al., 2000; Goldenberg et al., 2000, 2008; Goldenberg, 2002). Although some studies have shown that histological and/or clinical chorioamnionitis are associated with suboptimal neurodevelopment in preterm infants, the findings are inconsistent as shown in previous reviews (Ylijoki et al., 2012; van Vliet et al., 2013; Maisonneuve et al., 2017). Whether chorioamnionitis leads to impaired outcomes compared to non-exposed preterm infants born at similar gestational age remains unclear. As inflammation may enhance maturation, chorioamnionitis may also have beneficial effects for preterm infants.

An abnormal placental and fetal blood flow pattern has been shown to occur in about 20% of all very preterm births (Leppänen et al., 2009). It is well known that increased impedance in the umbilical artery flow is associated with an increased perinatal mortality and morbidity, especially in growth restricted fetuses (Karsdorp et al., 1994). To cope with placental insufficiency, the fetus increases blood flow to the brain. This so called "brain sparing" is reflected as an increased ratio between umbilical artery (UA) and middle cerebral artery (MCA) pulsatile indices. Brain sparing has been associated with decreased total brain volume, cortical gray matter volume, and cerebral volumes (Tolsa et al., 2004; Maunu et al., 2007), as well as increased incidence of brain pathology (Leppänen et al., 2009). It has been hypothesized that impaired fetal brain growth may lead to an impaired neurocognitive outcome. However, the data are inconclusive, and there are no previous reviews highlighting the matter.

Maternal smoking during pregnancy is the most common preventable factor causing adverse effects on fetal development. It is associated with an increased risk of preterm birth and low birth weight (Andres and Day, 2000). The risk of sudden infant death syndrome has also been associated with maternal smoking during pregnancy (Mitchell and Milerad, 2006). In addition, maternal smoking has been associated with adverse effects on fetal brain development in term and preterm infants (Ekblad et al., 2010, 2015), and with long term adverse effects in exposed infants, such as psychiatric morbidity (Ekblad et al., 2010) and neurodevelopmental problems (Clifford et al., 2012; Polanska et al., 2015). Study populations have mainly been full-term children. In preterm children, the association of maternal smoking during pregnancy with cognitive and neuropsychological outcomes is less studied, and there are no previous reviews concerning the matter.

In this review, we aim to evaluate the significance of selected prenatal risk factors related to prematurity, such as smoking during pregnancy, abnormal prenatal blood flow patterns, and chorioamnionitis, on the neurodevelopment of preterm children. This information is important for the professionals involved in the follow-up of pregnant women, as well as the professionals, such as psychologists, speech therapists, pediatricians, child neurologists and teachers who are involved in the follow up, therapy and education of preterm children.

METHODS

We performed a systematic electronic database search in the PubMed database (including MeSH search) to identify all original articles published on or before September 12, 2018 that evaluated the impact of clinical or histological chorioamnionitis, prenatal smoking exposure, and abnormal prenatal fetal and placental blood flow on neuropsychological and cognitive outcomes in preterm infants. With chorioamnionitis, the search terms used for the search were *Chorioamnionitis* combined with *Development*. Articles published before October 5, 2011 were part of a previously published review article (Ylijoki et al., 2012). We chose to use the same search terms to be able to combine the search results and update the previous findings with recent publications. With prenatal blood flow (Doppler velocimetry), the search terms used were *doppler* combined with *cognitive outcome* and *placental doppler* combined with *outcome*. With maternal smoking the search terms used for the search were *Smoking*, *Pregnancy* and *Neurodevelopment* or *Cognitive Development*. In addition, we performed a manual search of the reference lists of all included articles from the database search.

Study Selection

In the first phase, the publications were selected based on titles and abstracts to exclude irrelevant publications. Only publications written in English were included. Based on the full text articles, publication were excluded if they did not provide

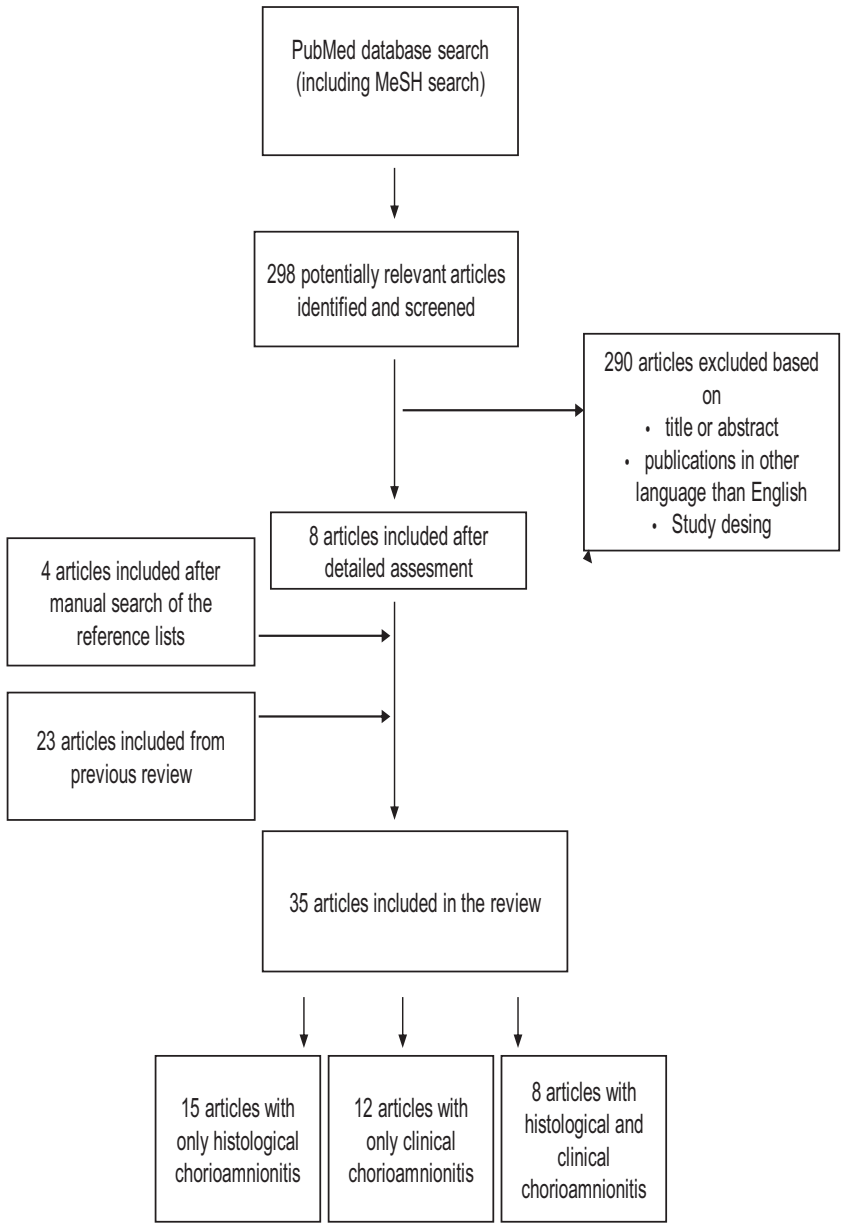

FIGURE 1 | Study selection process for articles about the association between chorioamnionitis and cognitive and neuropsychological outcome in preterm infants. The search was performed for articles published between October 5, 2011and September 12, 2018. Articles published before October 5, 2011 was identified earlier as a part of our previous review.

answer to the question of interest. **Figures 1–3** show the flow chart of the literature search.

We only included articles with preterm infants (born before 37 weeks of gestation). The developmental outcomes in the included articles were neuropsychological and cognitive development. The methods of evaluation varied greatly among the different studies.

Chorioamnionitis (i.e., inflammation of the fetal membranes) can be classified as either histological or clinical chorioamnionitis. Clinical chorioamniotis is usually diagnosed based on a combination of clinical signs (ruptured membranes, maternal or fetal tachycardia, maternal leukocytosis, foul-smelling amnionitic fluid), but the criteria of the diagnosis in the articles was variable. Histological chorioamnionitis is based on the appearance of neutrophils in the placental tissue. Histological chorioamnionitis can be further classified as maternal or fetal chorioamnionitis (also called funisitis).

Studies using doppler ultrasound to assess fetoplacental blood flow from the umbilical artery, fetal median cerebral artery, and/or the aortic isthmus were included in this review.

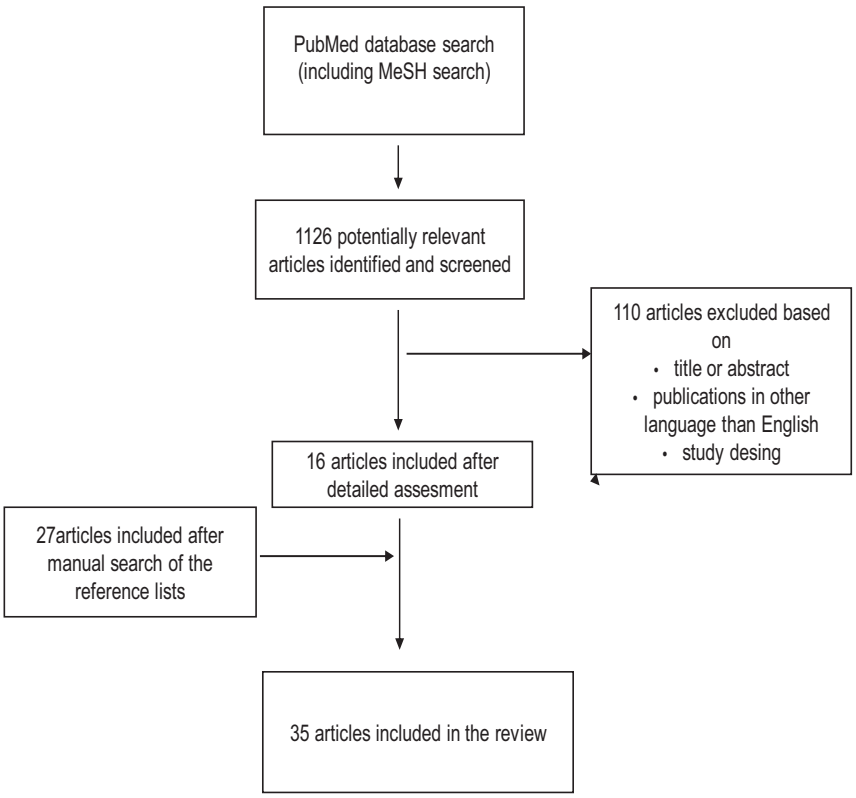

FIGURE 2 | Study selection process for articles about the association between abnormal placental and fetal blood flow and cognitive and neuropsychological outcome in preterm infants.

All of the articles about the effects of smoking were about maternal smoking during pregnancy.

RESULTS

We identified 54 studies about prenatal risk factors related to prematurity and their impact on cognitive and neuropsychological outcome. Thirty-five original articles evaluated the effects of clinical or histological chorioamnionitis; 15 studies evaluated the effects of abnormal prenatal Doppler velocimetry; and four studies evaluated the effects of maternal smoking during pregnancy.

Chorioamnionitis

The majority of the studies found no independent effect of chorioamnionitis on abnormal neurodevelopment (Morales, 1987; Dexter et al., 1999, 2000; Ambalavanan et al., 2000; Kosuge et al., 2000; Vermeulen et al., 2001; Dammann et al., 2003; Kent et al., 2005; Polam et al., 2005; Mu et al., 2007; Redline et al., 2007; Andrews et al., 2008; Helderman et al., 2012; Nasef et al., 2013; Soraisham et al., 2013; Manuck et al., 2014; Pappas et al., 2014; Källén et al., 2015; Miyazaki et al., 2016; Vander Haar and Gyamfi-Bannerman, 2016; Bierstone et al., 2018). There was a similar proportion of studies without association in the groups of histological and clinical chorioamnionitis. However, all studies with associations showed that chorioamninitis was a risk factor, not a protective factor, for later development. The results of the studies about chorioamnionitis are summarized in **Table 1**.

Only seven of the 20 studies about histological chorioamnionitis demonstrated that histological chorioamnionitis was a risk factor for suboptimal development. Two studies found an association between histological chorioamnionitis and speech delay (Suppiej et al., 2009) and lower mental developmental index (Hendson et al., 2011) at 18 months of corrected age in very low birth weight and very low gestational age infants, but multivariate analyses were not performed in one of them (Suppiej et al., 2009). Rovira et al. (2011) found an association with severe disability in very low birth weight infants at 2 years of age in logistic regression analyses but gestational age was not included in the analyses. Two studies (Salas et al., 2013; Lee et al., 2014) found that histological chorioamnionitis and funisitis were associated with weaker language performance at 18–24 months of corrected age, but one of them (Salas et al., 2013) did not include gestational age in multivariate analyses. Mittendorf et al. (2003) found that funisitis predicted impaired neurodevelopment at 18 months of corrected age. Ylijoki et al. (2016) found that histological chorioamnionitis, but not funisitis associated with slightly weaker memory and learning functions as well as weaker cognitive performance at 5 years of age. Nine articles of histological chorioamnionitis (all of

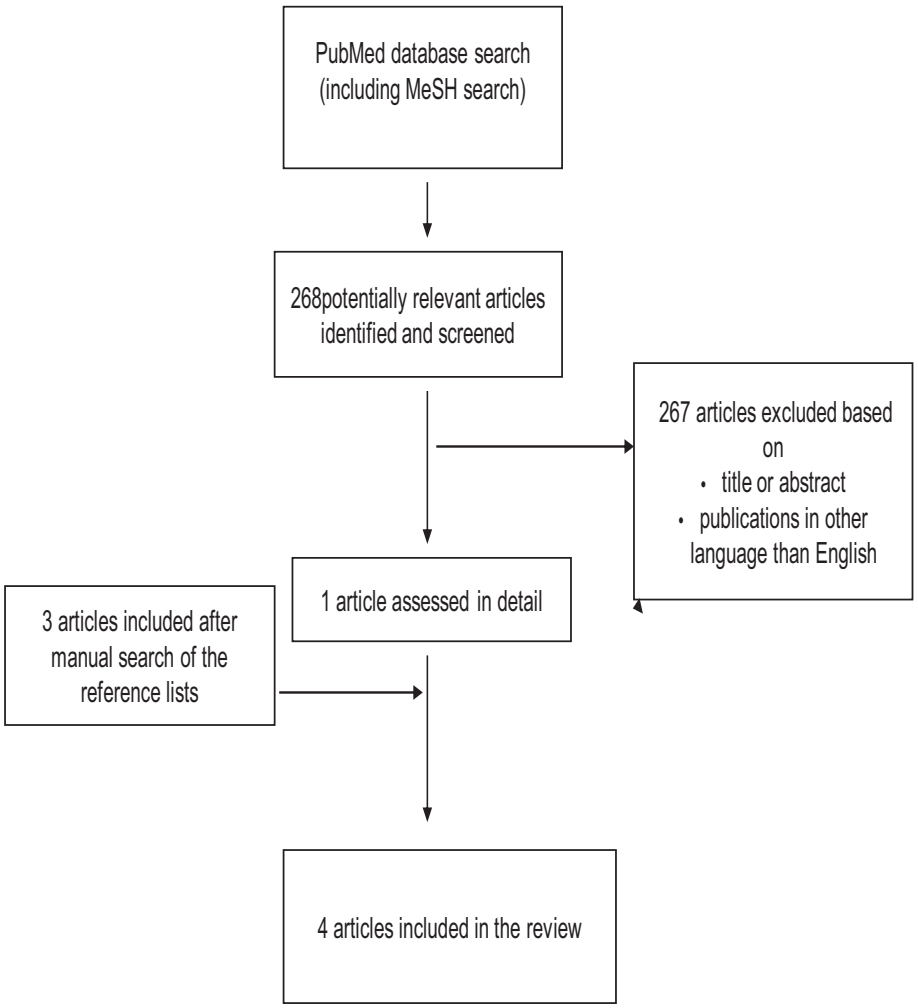

FIGURE 3 | Study selection process for articles about the association between smoking during pregnancy and cognitive and neuropsychological outcome in preterm infants.

them discussed above) separately evaluated the effects of funisitis on the neurodevelopment of preterm born children. Four of these found that funisitis independently increased the risk for the developmental problems (Mittendorf et al., 2003; Redline et al., 2007; Rovira et al., 2011; Salas et al., 2013), while five studies did not find any associations (Redline et al., 2000; Helderman et al., 2012; Soraisham et al., 2013; Lee et al., 2014; Ylijoki et al., 2016).

Only five of 15 studies found an association between clinical chorioamnionitis and neurodevelopmental impairments in preterm born children. Hardt et al. (1985) found lower cognitive scores in children born after preterm rupture of membranes with chorioamnionitis compared to those without chorioamnionitis at 12 months corrected age. Wilson-Costello et al. (1998), Rovira et al. (2011), and Nasef et al. (2013) have reported an association between clinical chorioamnionitis and cognitive, verbal, and motor performance, as well as with neurological disability at 18–24 months of corrected age. Versland et al. (2006) had similar findings about increased risk for cognitive impairment up to 11 years of age.

In addition, there were four studies in which clinical and histological chorioamnionitis was not separated (Gray et al., 1997; Fung et al., 2003; Schlapbach et al., 2010; Pappas et al., 2014). One of these studies found an association between chorioamnionitis and lower cognitive scores at 18–22 months of corrected age (Pappas et al., 2014), while the others did not.

Placental and Fetal Blood Flow

Eleven studies reported outcomes in association with umbilical artery blood flow. Seven of these did not find an association between abnormal flow in the umbilical artery and neurocognitive outcome (Valcamonico et al., 2004, 2007; Kirsten et al., 2007; Leppänen et al., 2009; Shand et al., 2009; Torrance et al., 2010; Eger et al., 2013). Male fetuses with intrauterine growth restriction and absent or reversed end diastolic flow in the umbilical artery have performed worse on cognitive

TABLE 1 | The outcomes of the 35 included articles divided according to the definition of chorioamnionitis (clinical, histological or clinical, and/or histological) and the association with developmental outcomes (abnormal cognitive outcome ≤/>2 years of age or other).

			Abnormal cognitive outcome ≤2 years of age	Abnormal cognitive outcome >2 years of age	Other
			OR (95% CI)	OR (95% CI)	OR (95% CI)
Histological chorioamnionitis $n = 20$	VLBW/VLGA infants $n = 18$	(Dexter et al., 2000) (164/287)	ns		
		(Kosuge et al., 2000) (44/81)	ns		
		(Kent et al., 2005) (72/220)	ns		
		(Polam et al., 2005) (102/177)	ns		
		(Mu et al., 2007) (54/95)	ns		
		(Redline et al., 2007) (69/129)		ns	
		(Andrews et al., 2008) (?/261)		ns	
		(Suppiej et al., 2009) (41/104)			$p < 0.05$ 33 vs. 9% for speech delay
		(Hendson et al., 2011) (303/628)			coefficient = 3.93 (7.52–0.33) for MDI at 18 moths corrected age
		(Rovira et al., 2011) (87/177)			$p = 0.03$ 18 vs. 5% for association of funisitis with severe disability
		(Helderman et al., 2012) (?/921)	ns		
		(Soraisham et al., 2013) (197/384)		ns	
		(Salas et al., 2013) (148/347)			RR 2.57 (1.02–6.46) for BSID III language score <70, RR 2.52 (1.11–5.72) for NDI
		(Nasef et al., 2013) (95/274)	ns		
		(Pappas et al., 2014) (910/2390)	ns		
		(Lee et al., 2014) (60/138)			β = −8.6 (−14.7 to −2.5) for language composite score
		(Ylijoki et al., 2016) (45/117)	ns	b = −7.22 (−14.31 to 0.13)	b = −1.29 (−2.40 to 0.18) for memory and learning functions in NEPSY
		(Bierstone et al., 2018) (145/350)	ns		
	Other premature infants $n = 2$	(Mittendorf et al., 2003) (21/121)	1.3 (1.1–1.9)		
		(Miyazaki et al., 2016) (1,235/4,078)		ns	
Clinical chorioamnionitis $n = 15$	VLBW/ VLGA infants $n = 8$	(Wilson-Costello et al., 1998) (30/144)	3.79 (1.3-10.8)		
		(Dexter et al., 1999) (71/330)	ns		
		(Ambalavanan et al., 2000) (57/218)	ns		
		(Andrews et al., 2008) (?/261)	ns		
		(Rovira et al., 2011) (56/177)			$p = 0.045$ 49 vs. 31% for association of chorioamnionitis with any disability
		(Nasef et al., 2013) (33/274)	0.2 (0.06–0.9)		0.3 (0.09–1.0) for language score below average, 0.2 (0.04–0.8) for motor score below average (Bayley-III)
		(Källén et al., 2015) (155/1,011)		ns	ns
		(Ylijoki et al., 2016) (16/117)	ns	ns	ns
	Other premature infants $n = 7$	(Hardt et al., 1985) (42/127)	$p = 0.017$ 12 vs. 10%		
		(Morales, 1987) (92/698)	ns		
		(Vermeulen et al., 2001) (70/185)	ns		
		(Dammann et al., 2003) (36/294)		ns	
		(Versland et al., 2006) (13/130)		$p = 0.04$ difference of mean 11.0%	

(Continued)

TABLE 1 | Continued

			Abnormal cognitive outcome ≤2 years of age	Abnormal cognitive outcome >2 years of age	Other
			OR (95% CI)	OR (95% CI)	OR (95% CI)
		(Manuck et al., 2014) (384/1,771)	ns		
		(Vander Haar and Gyamfi-Bannerman, 2016) (194/157)	ns		ns
Clinical and/or histological chorioamnionitis $n = 4$	VLBW/ VLGA infants $n = 4$	(Gray et al., 1997) (16/189)	ns		ns
		(Fung et al., 2003) (105/388)	ns		
		(Schlapbach et al., 2010) (33/99)	ns		ns
		(Pappas et al., 2014) (910/2390)	2.4 (1.3–4.3)		
	Other premature infants $n = 0$				

Articles which analyzed histological and clinical chorioamnionitis separately are presented twice in the table. Articles including only very low birth weight (VLBW) infants (birth weight <1,501 g) and/or very low gestational age (VLGA) infants (born <32 weeks of gestation) are shown separately. The number of study subjects with chorioamnionitis in relation to the total number of study subjects are shown in parenthesis, "?" is used when the number of study subjects with abnormal blood flow patterns is not reported in the article.

tests than those with appropriate growth for gestational age (Morsing et al., 2011). Similarly, preterm neonates with absent or reversed end diastolic flow have been shown to have more cognitive, mental, and motor disabilities than appropriately grown controls (Vossbeck et al., 2001). Unfortunately, in both of these studies, blood flow patterns were not assessed in the groups of appropriately grown fetuses, which is a serious methodological limitation. Pathological flow in the umbilical artery has also been associated with moderate or severe neurological impairment in children with intrauterine growth restriction, but not in those with normal prenatal growth (Spinillo et al., 2005). Growth restricted preterm children with suboptimal neurological outcomes at 1 year of age had higher pulsatility index in the umbilical artery flow than those with normal neurodevelopmental outcome in univariate analyses (Kaukola et al., 2005).

An increased placenta-cerebral ratio (UA/MCA-ratio) reflecting brain sparing in a fetus has been associated with adverse cognitive performance in very low birthweight children (Leppänen et al., 2009). An increased UA/MCA ratio was associated with impaired cognitive outcomes at 5 years of age, but not with neurodevelopmental outcomes at 3 years of age in the same patient population (Scherjon et al., 1998, 2000).

Some studies have suggested that retrograde (Fouron et al., 2001, 2005) or abnormal blood flow in the aortic isthmus (Leppänen et al., 2009) is associated with non-optimal neurodevelopment. However, retrograde flow did not associate with abnormal outcomes in one study with six patients with retrograde flow (Kaukola et al., 2005). The results of the articles about placental and fetal blood flow are summarised in **Table 2**.

Maternal Smoking During Pregnancy

The four articles assessing the association between prenatal smoking exposure and cognitive and neurodevelopmental outcomes in preterm infants were based on the same cohort studied in Austria at 12 months of corrected age (Kiechl-Kohlendorfer et al., 2009), 24 months of corrected age (Kiechl-Kohlendorfer et al., 2010), and 5 years of corrected age (Kiechl-Kohlendorfer et al., 2013; Gnigler et al., 2015). Smoking information was based on maternal self-report after birth. The mothers who refused to report their smoking status were classified as smokers. These articles showed associations between prenatal smoking exposure and adverse developmental outcomes at 12 and 24 months of corrected age (Kiechl-Kohlendorfer et al., 2009, 2010), as well as an association with numerical skills and processing speed at 5 years of corrected age (Kiechl-Kohlendorfer et al., 2013; Gnigler et al., 2015). The results of these articles are summarized in **Table 3**.

DISCUSSION

There is conflicting data about the impact of prenatal risk factors on long-term development of preterm infants. It is challenging to do longitudinal studies extending from fetal risk factors or well-being to eventual long-term developmental outcomes, as so many of the studies have only small number of patients. Therefore, a summarizing, critical review of all the data is valuable.

A review of the effects of chorioamnionitis on the development of preterm infants helps clinicians get an overview of the heterogeneous studies with inconsistent results regarding this topic. Inconsistencies are partly due to small sample sizes, differences in patient populations, evaluation methods, and age points. This review classifies the studies according to the distinction between histological and clinical chorioamnionitis. Altogether, it seems that the majority of the publications do not support the belief that chorioamnionitis poses an independent risk for adverse development in preterm born children. This review does not give further support either to the hypothesis that clinical chorioamnionitis and funisitis are more deleterious to the developing central nervous system than histological chorioamnionitis.

One factor possibly explaining this complexity might be the maturation enhancing effects of chorioamnionitis on immature infants. Animal models have shown that chorioamnionitis significantly enhances lung maturation (Kramer et al., 2009), which might also be seen clinically in the lungs of preterm

TABLE 2 | The outcomes of the 15 included articles divided according to the placental or fetal blood flow measurements used (umbilical artery blood flow, increased placenta-cerebral ratio, retrograde flow in the aortic isthmus) and developmental outcomes (abnormal cognitive outcome ≤/>2 years of age or other).

			Abnormal cognitive outcome ≤2 years of age	Abnormal cognitive outcome >2 years of age	Other
			OR (95% CI)	OR (95% CI)	OR (95% CI)
Umbilical artery blood flow $n = 11$	VLBW/ VLGA infants $n = 7$	(Eger et al., 2013) (38/71)		ns	
		(Morsing et al., 2011) (34/68)		$p = 0.007$ for FSIQ and $p = 0.005$ for VIQ in boys	
		(Valcamonico et al., 2007) (34/58)			ns
		(Kaukola et al., 2005) (?/17)			$p = 0.005$ for neurodevelopmental impairment at 1 year of corrected age
		(Shand et al., 2009) (39/119)	ns		
		(Vossbeck et al., 2001) (40/80)			$p = 0.005$ for cognitive development at 1–8 years of age
		(Leppänen et al., 2009) (17/83)	ns		
	Other premature infants $n = 4$	(Kirsten et al., 2007) (50/190)	ns	ns	$p = 0.03$ for performance subscale at 2 years of age
		(Valcamonico et al., 2004) (14/25)		ns	
		(Torrance et al., 2010) (?/71)	ns		
		(Spinillo et al., 2005) (75/266)			$p = 0.05$ for neurodevelopmental impairment at 12–24 months of age in growth restricted fetuses
Increased placenta-cerebral ratio $n = 3$	VLBW/ VLGA infants $n = 1$	(Leppänen et al., 2009) (16/83)	$p = 0.01$		
	Other premature infants $n = 2$	(Scherjon et al., 1998) (34/96)			ns
		(Scherjon et al., 2000) (28/73)		$p < 0.02$, 54 vs. 20%	
Aortic isthmus blood flow $n = 3$	VLBW/ VLGA infants $n = 1$	(Kaukola et al., 2005) (6/17)			ns
		(Leppänen et al., 2009) (?/83)	$p = 0.03$		
	Other premature infants $n = 2$	(Fouron et al., 2001) (5/44)		Relative risk 2.05 (1.49–2.83)	
		(Fouron et al., 2005) (?/48)			$p = 0.007$ for non-optimal neurodevelopmental outcome at 2–5 years of age

Articles including only very low birth weight (VLBW) infants (birth weight <1,501 g) and/or very low gestational age (VLGA) infants (born <32 weeks of gestation) are shown separately. The number of study subjects with abnormal blood flow patterns in relation to the total number of study subjects are shown in parenthesis, "?" is used when the number of study subjects with abnormal blood flow patterns is not reported in the article.

infants, although this comes with inflammatory consequences (Jobe, 2012). Therefore, the clinical effects of chorioamnionitis on the developmental outcome of preterm infants is complex. There are a few studies that show that histological chorioamniniotis has a protective effect on mortality rates (Hendson et al., 2011) and neurodevelopment of preterm infants when compared with placental underperfusion (van Vliet et al., 2012). Therefore, it seems that chorioamnionitis might have beneficial effects, in addition to the deleterious effects, on the developing preterm infant. Another factor modifying the effects of chorioamnionitis might be prenatal glucocorticoids. Most preterm infants are exposed to the anti-inflammatory effects of prenatal glucocorticoids. It is likely that this immunomodulation attenuates the effects of chorioamnionitis. Indeed, a meta-analysis has shown that prenatal steroid administration is associated with a reduced risk for brain lesions in clinical and histological chorioamnionitis (Been et al., 2011). One study found that histological chorioamnionitis was associated with CP only in those infants who had not been given two doses of prenatal corticosteroids (Kent et al., 2005). However, our review also includes recent publications with patients who had a high prenatal glucocorticoid administration rate where chorioamnionitis still seemed to be a significant risk factor for suboptimal development.

We can conclude that the available evidence does not suggest chorioamnionitis is a major independent risk factor for suboptimal cognitive and neuropsychological development in preterm born children. As there are no "healthy" preterm controls without other risk factors, we can only conclude that chorioamnionitis may not be a greater risk for the brain of a

TABLE 3 | The outcomes of the four included articles about maternal smoking during pregnancy divided according the developmental outcomes (abnormal cognitive outcome ≤/>2 years of age or other).

	Abnormal neurodevelopmental outcome ≤2 years of age	Abnormal cognitive outcome >2 years of age
	OR (95% CI)	OR (95% CI)
(Kiechl-Kohlendorfer et al., 2009) (41/205)	$P = 0.016$	
(Kiechl-Kohlendorfer et al., 2010) (30/142)	3.36 (1.38–8.17)	
(Kiechl-Kohlendorfer et al., 2013) (34/135)		Delayed numerical skills: OR 4.26 (1.56–11.65)
(Gnigler et al., 2015) (44/161)		Reduced processing speed; OR 3.05 (1.43–6.52)

All articles include only very low gestational age (VLGA) infants (born <32 weeks of gestation). The number of study subjects with prenatal smoking exposure in relation to the total number of study subjects are shown in parenthesis.

preterm infant than other underlying pathologies already present before preterm delivery.

Most of the data on the association of prenatal fetoplacental blood flow and later neurocognitive development is focused on umbilical artery blood flow. The pulsatility index of umbilical artery flow reflects the number of tertiary villous arterioles in the placenta (Acharya et al., 2004). Thus, pulsatility index of the umbilical artery flow is known to increase in placental insufficiency. Most of the studies did not find an association between increased pulsatility in the umbilical artery flow and later neurocognitive outcomes in preterm born infants. However, there are some data on abnormal umbilical blood flow and impaired neurodevelopment in fetuses suffering from placental insufficiency (Kaukola et al., 2005). However, the sample sizes were often small and methodological problems complicated the interpretation of the data.

The so called "brain sparing" effect has been considered protective to fetuses with growth restriction. Thus, it is interesting that an increased placenta-cerebral ratio has been associated with impaired cognitive outcomes in some (Scherjon et al., 2000; Leppänen et al., 2009), although not all studies (Scherjon et al., 1998). The data is too sparse to draw conclusions on the effect of aortic isthmic blood flow on infant neurocognitive development.

Prenatal smoking exposure has been associated with many adverse effects on fetal health as well as an increased risk for diseases in later life. The data show a negative association between prenatal smoking exposure and later cognition in preterm infants up to 5 years of age. However, it is challenging to prove a causal link between prenatal smoking exposure and cognitive outcomes in later life because genetic and familial factors are known confounders (Gilman et al., 2008; Knopik, 2009). Maternal education, which is one important familial factor, was adjusted for in the analyses of these studies with preterm infants (Kiechl-Kohlendorfer et al., 2009, 2010, 2013; Gnigler et al., 2015). The familial factors include also disadvantageous parenting and family functioning. Therefore, more precise measurements of genetic and familial influences should be taken into account (e.g., by sibling design). It has been shown in a sibling design study that the unexposed sibling was also at an increased risk of poor school performance at the age of 15 years Lambe et al. (2006). On the other hand, there is a large population study showing that the effects of prenatal smoking exposure remained also in a sibling design study (Ekblad et al., 2017). Individual genetic polymorphisms might also modify the effect of prenatal smoking exposure on cognitive functioning (Morales et al., 2009). Women who smoke during pregnancy may also be more likely to engage in other unhealthy behaviors, like alcohol drinking during pregnancy when smoking serves as an indicator for unhealthy lifestyle. A Finnish study showed that heavy smoking before pregnancy was associated with lower cognitive scores in children aged 56 months even if the mother did not smoke during pregnancy (Heinonen et al., 2011). The authors of the aforementioned study speculate that the association might be explained by maternal smoking-related health habits and status. In future studies, it would be interesting to also control also for cognitive abilities and executive functions of the parents.

The lack of reliable validation of smoking exposure is one significant limitation in most of the studies on the effects of smoking exposure. Smoking exposure can be verified by measuring cotinine, a metabolite of nicotine, in the saliva or hair of a mother during pregnancy (Shipton et al., 2009). Cotinine measurements would also reveal a significant environmental smoking exposure. In addition, there might be different effects of smoking exposure at different stages of pregnancy, and so evaluating changes in cotinine levels throughout pregnancy might be relevant.

One limitation of this review is the heterogeneous and rather small patient populations in the original articles. The inclusion criteria vary between the studies, and thus the comparison of the studies is difficult. The same applies to the outcome variables and time points. Also, some of the studies were done in the 1980s and 1990s, and it is well known that the treatments and outcomes of preterm infants have developed during the subsequent years.

This review summarizes the data on several prematurity related prenatal risk factors which play a role in the developmental outcomes of preterm infants. To optimize the developmental outcomes of this patient population we need to first optimize the fetal well-being before birth. More longitudinal research with large patient populations that extends from the fetal life to long-term developmental outcomes is needed. To draw definite conclusions about clinical practices such as the right timing of the delivery requires randomized controlled trials. It is also crucial to implement all practices which protect brain development and improve later neurodevelopmental outcomes of immature preterm infants.

AUTHOR CONTRIBUTIONS

MY, EE, ME, and LL took part in the design of the study, the interpretation of data, and drafted the initial manuscript. All authors have approved the final manuscript

as submitted and agree to be accountable for all aspects of the work.

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REVIEW
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Pain, Parental Involvement, and Oxytocin in the Neonatal Intensive Care Unit

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Preterm infants (PTI) typically experience many painful and stressful procedures or events during their first weeks of life in a neonatal intensive care unit, and these can profoundly impact subsequent brain development and function. Several protective interventions during this sensitive period stimulate the oxytocin system, reduce pain and stress, and improve brain development. This review provides an overview of the environmental risk factors experienced by PTI during hospitalization, with a focus on the effects of pain, and early maternal separation. We also describe the long-term adverse effects of the simultaneous experiences of pain and maternal separation, and the potential beneficial effects of maternal vocalizations, parental contact, and several related processes, which appear to be mediated by the oxytocin system.

Keywords: prematurity, pain, parents, early separation, early contact

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INTRODUCTION

Recent improvements in neonatal intensive care units (NICUs) have contributed to the increased survival rates of extremely PTI (<28 weeks gestation) and infants with extremely low birth weight (<1000 g) (Saigal and Doyle, 2008). However, preterm infants (PTIs; <37 weeks gestation) remain at high risk for development of disabilities, and recent research indicates they experience a broad and complex spectrum of adverse neurodevelopmental outcomes (Adams-Chapman et al., 2018). More specifically, PTIs have an increased risk of brain injury and disruption of brain maturation, which can manifest as an increased risk of cerebral palsy, cognitive deficits, and psychiatric disorders, such as attention deficit hyperactivity disorder or autism spectrum disorder. Moreover, PTIs may experience modifications of the hypothalamic-pituitary-adrenal (HPA) axis, although stimulation of the OXT system can protect against these modifications (Grunau et al., 2007). Several studies suggested that the impaired brain maturation of PTIs is at least partly a consequence of their atypical early-life environment, and their exposure to various stressors, such as physical pain and maternal separation (Anand and Scalzo, 2000; Grunau et al., 2006; Flacking et al., 2012).

Abbreviations: OXT, oxytocin; PTI, preterm infants; VPI, very preterm infants.

This review examines the environmental risks factors to which PTIs are exposed during their early lives in NICUs, with a focus on exposure to pain and early maternal separation. We also review the impact of the long-term and simultaneous exposure to these risk factors on the OXT system. In everyday clinical practice, PTIs may undergo painful procedures while separated from their parents. It is possible that the simultaneous experience of these two negative early experiences – pain and parental separation – has a synergistic and negative impact on infant development. Similarly, it is possible that interventions which prevent these early negative experiences could have cumulative positive effects.

Thus, we also examine the effect of parental presence on protection against the short-term and long-term effects of pain in PTIs.

This review is needed as to date, no studies scrutinized the cumulative impact of separation and pain on the specific hormone of the OXT. Moreover, no reviews, at our knowledge, formulated a clear and evidence-based theoretical framework explaining the role of OXT in early family-based interventions.

The main reason for reviewing the role of OXT, instead of other hormones, is that OXT plays a pivotal role both in pain perception and early separation, which are both negative and stressful events that PTI in the NICU experience during the first weeks of hospitalization. OXT is involved in the attachment process and has analgesic properties, demonstrated in preclinical models, while other hormones (such cortisol) are specifically linked to stress or to the long-term effects of pain, but not specifically to attachment and early separation.

Our specific objectives were to examine the hypothesis that increased parental care using early vocal contact (EVC; Filippa et al., 2017a), and skin-to-skin-contact (SSC), provides benefits to PTIs undergoing painful procedures in the NICU, and that this effect is mediated by the OXT system. We also review the potential applications and opportunities for research in the field of risk aversion for neonatal pain and reduced parental care in PTIs, and propose protective actions that may help to improve the developmental outcomes of PTIs.

PROCEDURAL PAIN AND EARLY SEPARATION IN THE NICU

Epidemiology of Procedural Pain and Early Separation in the NICU

Preterm infants (PTIs; <37 weeks gestation), especially very PTI (VPIs; 28–32 weeks gestation), and extremely preterm infant (<28 weeks gestation), are hospitalized in NICUs where they receive early postnatal exposure to an environment that differs markedly from the *in utero* environment (Kuhn et al., 2011). In particular, the NICU exposes them to excessive deleterious sensory stimuli, and deprives them of biologically meaningful sensory stimuli. The auditory environment of the NICU is characterized by frequent loud and high-pitched sounds, and this noise triggers stress responses, reduces physiological well-being, and disrupts sleep (Kuhn et al., 2012, 2013). The NICU also limits access to the vocal signature of the mother's voice.

Furthermore, VPIs are frequently exposed to stressful and painful stimuli during care in the NICU. This burden results in a "distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive, and social components" (Williams and Craig, 2016), and still occurs in nearly all NICUs, although some recent epidemiological studies reported improvements in reducing procedural pain and improving analgesia (Simons et al., 2003; Carbajal et al., 2008, 2015; Johnston et al., 2011; Roofthooft et al., 2014; Allegaert and van den Anker, 2016). In France, the Epidemiology of Procedural Pain in Neonates study (EPIPPAIN 1), conducted in 2005, showed that each neonate experienced a mean of 10 painful procedures per day of hospitalization (Carbajal et al., 2008). A recent systematic review of observational studies that assessed painful procedures in neonates found that each newborn can undergo 7.5–17.3 procedures per day (Cruz et al., 2016). The most common procedures involve skin breakage and nasal or tracheal suctioning. Moreover, there are wide variations in the administration of analgesics to PTIs among countries, and among units in the same country (Carbajal et al., 2008). For example, a European prospective study (EUROPAIN) showed that the use of sedation or analgesia for PTIs varied from 0 to 100% among different centers (Carbajal et al., 2015). In addition, discomfort and stress related to human interventions during "standard" routine care, even supposedly non-painful procedures (diaper changes, temperature measurements, and mouth care), add to this burden (Sizun et al., 2002; Catelin, et al., 2005). Repeated exposure to odors that irritate the trigeminal system, such as those from health care products, can also trigger behavioral, and cortical pain responses (Frie et al., 2018). Routine nursing interventions can even potentiate the pain associated with many procedures. For example, blood collection taken immediately after routine clustered care provoked a greater pain response than blood collection following a period of rest (Holsti et al., 2006).

Very preterm infants also experience early and prolonged separation from their parents, and this separation is a well-known critical stressor for the infant (Flacking et al., 2012). Parental presence in a NICU is greater when there is implementation of infant- and family-centered developmental care, and when the NICU design supports increased parental presence by providing single family rooms (Lester et al., 2016). Studies that compared parental access and involvement of parents in the care of infants in Europe indicated increased parental presence in the Nordic countries (Greisen et al., 2009; Pallas-Alonso et al., 2012). Significant discrepancies remain among the NICUs in Europe, and there is evidence that providing parents the opportunity to stay overnight in a NICU prolongs the time they can spend with their newborns (Raiskila et al., 2017). Separation is also stressful to parents, and separated PTIs lack feedback from their parents, to whom they are normally sensorially tuned. Sensory interactions and biologically meaningful stimuli from the mother and father support early bonding and attachment (Flacking et al., 2012; Korja et al., 2012).

Repeated procedural pain and early parental separation by a PTI can also have negative consequences later in life.

Long Term Consequences of Procedural Pain and Early Separation in the NICU

There is evidence that the NICU environment itself may interfere with the neurodevelopment and growth of VPIs. For instance, excessive noise contributes to the neurocognitive burden of PTIs (Graven, 2000; Wachman and Lahav, 2011), or excessive sensory deprivation and isolation in the incubator (Lagercrantz, 2016) may contribute to the neurocognitive burden of PTIs, and may lead to attention deficit hyperactivity disorder (Gray and Philbin, 2004) and/or alterations in early communication skills. In addition, the pain and stress that VPIs experience during care can impact brain growth and function (Smith et al., 2011). An increased exposure to invasive procedures during care of PTIs is associated with a lower IQ at school age (Vinall et al., 2014). Thus, untreated neonatal pain can impair multiple aspects of brain development, including cognition, emotional responses, and motor function. Receipt of analgesics is also associated with cognitive decline in these infants (Anand et al., 2004; Grunau et al., 2009), and excessive analgesia, such as during situations that are not painful, might increase these potential detrimental effects of analgesia. Thus, care strategies should attempt to reduce experiences of acute pain and unnecessary use of pharmacological analgesics. Another goal of pain management in neonates is to maximize the newborn's capacity to cope with and recover from painful experiences (Carbajal et al., 2004).

Early separation of PTIs from their parents can also adversely impact their neurodevelopment, and can have adverse consequences later in life for the PTIs and their parents (Flacking et al., 2012). Parental separation limits the opportunities for early engagement in intimate contact with the parents (Bialoskurski et al., 1999). Early mother-infant separation also has a long-term impact on the infant's autonomic, neuroendocrine, and immune systems (Sanchez et al., 2001). Over the long-term, this separation can alter the neurocognitive outcomes of PTIs, and their emotional experiences can negatively impact their emotional processing, and the health of the parents (Morelius et al., 2007; Korja et al., 2012; Montirosso et al., 2012a, 2014; Kommers et al., 2016). For instance, a recent study found that reduced bonding of mothers with VPI was associated with less intimacy with the infant, and with infant difficulties in regulation of socio-emotional stress at 3-months of age (Provenzi et al., 2017).

Effects of Procedural Pain and Mother-Infant Separation on the OXT System

Procedural Pain and the OXT System

Pain associated with routine care or a medical procedure may alter the infant's OXT system and have long-term effects (see section "Role of OXT in Modulation of Pain"). Although many studies have established the analgesic effects of OXT, little is known about the potential effect of chronic or procedural pain on regulation of the OXT system. One study showed that children with recurrent abdominal pain of psychosomatic origin had low concentrations of plasma OXT and high concentrations of cortisol (Alfvén et al., 1994). One approach to evaluate the association of the OXT system with pain is to analyze the impact of painful procedures on cortisol levels and the HPA axis, because these systems are known to interact with the OXT system. For instance, OXT can inhibit the function of the HPA axis at several levels during the production of cortisol (Neumann, 2002; Moberg and Prime, 2013): corticotrophin releasing factor release from the hypothalamus, release of adrenocorticotropic hormone from the anterior pituitary, and cortisol release into the circulation from the adrenal cortex.

Thus, additional studies of the HPA axis may provide a better understanding of the relationship of long-term exposure to pain with the OXT system. In animals, perinatal unpredictable stressors that stimulate the HPA axis also reduce OXT levels in the hypothalamus (Lee et al., 2007). Repeated exposure to pain is associated with increased activation of the HPA axis, and the OXT system can modulate this effect (Petersson et al., 1999). When OXT action is dampened in rodents by treatment with a selective OXT receptor antagonist, this reduces pair bonding and stimulates the HPA axis (Detillion et al., 2004). Thus, in animals, early and repeated exposure to pain has detrimental effects on behavior, presumably due to upregulation of the HPA axis. However, studies in humans indicated that a painful procedure in neonates could increase or have no impact on cortisol levels (Magnano et al., 1992; Herrington et al., 2004; Cignacco et al., 2009). A series of studies by Grunau et al. (2004, 2005) showed that exposure of PTIs to more neonatal pain had different effects on HPA responsiveness throughout development. In particular, cortisol responses were dampened while infants were still in the hospital with ongoing environmental stress, but then increased later during infancy.

Although there is strong evidence for the role of the OXT system in pain modulation, to our knowledge, no studies have yet evaluated the effect of early and repeated pain experience on the regulation of the OXT system in PTIs.

Early Mother-Infant Separation and the OXT System

Changes in the responsivity to social behaviors are critical determinants for development of bonding and attachment processes during the sensitive period soon after birth, and early separation of the infant from the mother has a negative impact on these processes (Hofer et al., 1993). However, the relationship of these changes with the OXT system is still uncertain. We examine this issue below by a review of animal and human studies that used different approaches to address this issue.

Numerous researchers have examined the impact of the OXT system on the early development of animals. For example, manipulating different crucial elements during early development indicated that the OXT system responded during periods of separation and reunion of mothers and offspring (Veenema, 2012). In rodents, long periods of early maternal separation led to reduced maternal care, and also affected regulation of the OXT system. At the physiological level, offspring that receive less maternal licking and grooming exhibit decreased estrogen-mediated up-regulation of OXT receptor binding and c-fos immunoreactivity in hypothalamic regions that are implicated in maternal care, such as the medial preoptic area (Champagne et al., 2001). This epigenetic regulation persists into adulthood and, in female offspring, can account

for the intergenerational transmission of maternal behaviors (Maestripieri et al., 2007). Furthermore, the levels of OXT receptors in the central nucleus of the amygdala are significantly greater in females that receive more maternal care, regardless of their reproductive status (Francis et al., 2000).

Moreover, other studies of animal models (whose results have not yet been verified in humans) showed that rats who had only brief separations during early infancy had higher expression of OXT receptors than rats that received poor maternal care or more extended separations as pups (Lukas et al., 2010). Moreover, early maternal separation interferes with the healthy development of OXT receptors in specific forebrain regions, such as the agranular cortex (juveniles and adolescents), the lateral septum (adults), the caudate putamen (adults), but increased the level of OXT receptors in the ventromedial hypothalamus (adults) (Lukas et al., 2010). Maternal separation also increased or had little effect on OXT-immunoreactivity in the paraventricular nucleus of males (Tsuda et al., 2011), but led to a decreased OXT-immunoreactivity in the paraventricular nucleus of lactating and non-lactating adult females (Veenema et al., 2007).

Similarly, human studies showed that after a period of contact with infants, salivary OXT levels were only greater among mothers who had highly affectionate contact and among fathers who had highly stimulatory contact (Feldman et al., 2010). This led to the conclusion that higher levels of parental OXT are linked with higher levels of parental care. Thus, greater maternal contact soon after the birth of an infant leads to greater maternal production of OXT in experimental animals and humans (Francis et al., 2000; Feldman et al., 2010).

Administration of exogenous OXT after maternal separation or a stressful experience may provide protective effects and increase the resilience of mothers and infants (Barrett et al., 2015). More specifically, administration of OXT into the central amygdala increased the social competence of newborn rats after separation, and also reversed the effects of early prenatal stress (Lee et al., 2007). Another rat study demonstrated that maternal separation induced depressive-like behaviors in adult male mice, and that these behaviors were associated with abnormal mitochondrial function and immune-inflammatory responses in the hippocampus (Amini-Khoei et al., 2017). However, activation of the OXT system by OXT injection into the brain protected against the negative effects of maternal separation by altering the brain and behavior (Amini-Khoei et al., 2017).

Thus, these many studies suggest that regulation of the OXT system mediates the negative effects of an atypical early social environment (such as early maternal separation), and promotes pro-social behaviors. Additional human studies are necessary to improve our understanding of the impact of upregulation of the OXT system, after exogenous administration or endogenous production, on an infant's brain and social behaviors the context of maternal contact.

Role of OXT in Modulation of Pain

The role of OXT in pain modulation is now well-established, and it has putative action at almost every level of the pain pathway, including the peripheral, spinal, and supra-spinal systems. However, a recent review of clinical studies found that OXT reduced pain in adult subjects in only about half of all studies (Boll et al., 2018). Among the positive results, OXT reduced low back pain after intrathecal infusion (Yang, 1994), reduced visceral pain symptoms in patients with irritable bowel syndrome after intravenous injection (Louvel et al., 1996), and reduced headache in a dose-dependent manner after intranasal administration (Wang et al., 2013). Additional human studies of cortical integration in regions associated with the emotional dimension of pain expression using functional magnetic resonance imaging (fMRI) found that OXT modulated certain socio-emotional tasks (Herpertz and Bertsch, 2015; Wigton et al., 2015). These observations and others led to the recent proposal that OXT modulates several dimensions of pain expression, and had strong effects on emotional output, attentional processes, and social interactions (Tracy et al., 2015). However, there is still no direct and unambiguous link between pain expression and OXT. The few available imaging studies indicated that intranasal administration of OXT to humans reduced negative emotions related to pain from heat stimulation, and positively modulated responses related to empathy when viewing an emotional picture (Singer et al., 2008; Zunhammer et al., 2015, 2016). This result corresponds with the hemodynamic responses in emotion-processing brain structures, such as the amygdala (Zunhammer et al., 2015). In contrast to these human studies, there is unambiguous evidence that OXT has analgesic effects in many animal models of pain (Rash et al., 2014), and these studies have led to characterization of the underlying molecular and cellular mechanisms.

Mechanisms of Central Analgesia

Central nervous system analgesia first relies on axonal projections, which originate from the oxytocinergic hypothalamic neurons (in paraventricular, supraoptic, and accessory nuclei) and innervate many pain processing structures (Poisbeau et al., 2018). This includes the spinal cord, where OXT release onto second-order neurons selectively inhibits "pain messages" carried by nociceptive-specific C and Aδ-type sensory neurons in animal models (Rojas-Piloni et al., 2007, 2010; Eliava et al., 2016). In agreement, intrathecal or intracerebroventricular injections of OXT or a selective agonist of the OXT receptor substantially reduced the symptoms of pain in several animal models of inflammatory and neuropathic pain (Miranda-Cardenas et al., 2006; Russo et al., 2012; Eliava et al., 2016).

This likely results from an increased inhibition mediated by $GABA_A$ receptors, and from an overall reduction in the excitability of spinal neurons that express the OXT receptor (Breton et al., 2008, 2009). Compared to the spinal cord, there are limited data regarding the effect of OXT on pain modulation in supraspinal structures although it is generally also associated with increases in GABAergic inhibition.

Interestingly, OXT modulation of $GABA_A$ receptors is likely mediated by changes in intracellular phosphorylation (Brussaard and Koksma, 2003; Vergnano et al., 2007) and by establishment of an optimal chloride transmembrane gradient, because the action of OXT is mediated by chloride-permeable $GABA_A$ receptor-channels. Recent studies indicated that OXT

receptor signaling directly regulated the expression of the potassium-chloride transporter KCC2, which maintains low intracellular chloride concentrations in neurons, and ensures optimal $GABA_A$ receptor-mediated inhibition (Tyzio et al., 2006; Leonzino et al., 2016). This discovery is of fundamental importance, because there is evidence that altered OXT levels and impaired chloride-mediated inhibitory control contribute to several neurodevelopmental disorders (Lefevre and Sirigu, 2016) and are responsible for the appearance and maintenance of neuropathic and inflammatory pain in adults (Price et al., 2009). Recent research indicated that separation of neonatal rats from their mothers leads to reduced OXT signaling, and this accounted for the development of nociceptive hypersensitivity and a failure of stress-induced analgesia during the postnatal development of pups and of mature rats (Melchior et al., 2018).

Mechanisms of Peripheral Analgesia

Several lines of evidence support the interpretation that OXT provides analgesia by acting on the peripheral nervous system, although some contradictory results indicate that vasopressin receptors might explain this effect (Schorscher-Petcu et al., 2010; Qiu et al., 2014). Juif and Poisbeau (2013) administered intravenous bolus injections of OXT and vasopressin to rats, and demonstrated that low physiological concentrations of both neurohormones reduced the number of action potentials carried by C-type nociceptors. However, simultaneous intravenous injection of an OXT receptor antagonist abolished these anti-nociceptive effects. This result is consistent with the results of optogenetic experiments which showed that selective stimulation of hypothalamic neurons increased the release of OXT into the bloodstream (Eliava et al., 2016). In agreement, release of OXT (but not vasopressin) into the blood of rats after an acute swim stress had an analgesic effect (Wotjak et al., 1998; Juif and Poisbeau, 2013). Despite some discrepancies, there is a broad consensus that OXT has analgesic effects on the peripheral nervous system. The target structures are unknown in most cases, although some recent results have suggested novel mechanisms.

Oxytocin can act on several different peripheral targets, including the skin. Skin cells express the OXT receptor and keratinocytes can produce and release OXT (Denda et al., 2012). In line with this observation, recent research showed that subcutaneous administration of OXT decreased neuronal firing of Aδ/C fibers (Gonzalez-Hernandez et al., 2017). This result could be explained by the expression of OXT receptors in non-peptidergic C-fiber cell bodies (Wrobel et al., 2011; Moreno-Lopez et al., 2013). The presence of OXT receptors in peripheral terminal axons of the skin (Gonzalez-Hernandez et al., 2017) is of particular interest, because touch-evoked OXT release by keratinocytes could explain the analgesia induced by stimulation of C tactile afferents (Walker et al., 2017) that occurs when newborns receive hand massages or perform sucking responses (Matthiesen et al., 2001). One possible mechanism could be membrane hyperpolarization of the sensory afferents, followed by increased intracellular calcium (Gong et al., 2015).

The most recent surprising results were from Nersesyan et al. (2017), who described the agonistic effect of OXT on TRPV1 channels, which are expressed by a subset of C nociceptors and are well-known for their role in responses to heat stimulation. These researchers identified the binding site for OXT on TRPV1 channels, and demonstrated that OXT blocks their function, thus explaining the analgesic effect of OXT.

To conclude, there is strong evidence that OXT has analgesic effects in the vast majority of animal models of pain (Rash et al., 2014). However, the evidence for this effect in humans needs further investigation, even if the recent over mentioned studies are encouraging.

Even though recent studies support the analgesic effects of OXT in humans, further studies of this topic are needed. Studies of PTIs indicated that OXT influences multiple psychological dimensions that impact the experience of pain, such as selective attention to pain, negatively valenced emotions, and social support. All of these are associated with neuronal activities in brain regions that are modulated by OXT administration and have roles in socio-emotional tasks.

ROLE OF OXT IN EARLY CONTACT AND PAINFUL PROCEDURES

Effects of Parental Presence on Pain Management

Optimal pain management requires careful assessment and a combination of prevention and treatment by pharmacological and non-pharmacological methods. Parental interaction is an important non-pharmacological method for reducing pain in PTIs. Furthermore, some forms of interaction, such as SSC, breastfeeding, and EVC with familiar voices, must occur in an appropriate socio-emotional context and can be performed only by the mother and the father (except for breastfeeding). The efficacy of breast feeding and SSC are well established (Cignacco et al., 2007; Pillai Riddell et al., 2015). Thus, SSC alleviates pain responses to single painful procedures (such as a heel stick) (Mooncey et al., 1997; Johnston et al., 2017) and reduces cortical pain responses after venipuncture in PTIs (Olsson et al., 2016). The analgesic effect of SSC increases when it is given in combination with sweet solutions (Johnston et al., 2017). The optimal duration of SSC, the long-term impact of repeated SSC, and its interactions with other interventions require further investigation (Johnston et al., 2017). Breastfeeding is effective in diminishing mild procedural pain in neonates (Carbajal et al., 2003; Codipietro et al., 2008; Shah et al., 2012), and breast milk seems to be as effective as sweet solutions in relieving pain in full-term neonates (Watterberg et al., 2016). The odor of mother's milk also appears to reduce pain from a heel stick in full-term neonates (Nishitani et al., 2009) and in PTIs (Baudesson de Chanville et al., 2017). Thus, parents, especially the mother, can help a neonate remain calm during painful procedures and to recover more rapidly by delivering appropriate sensory cues to the infant. Moreover, parental presence appears to be associated with reduced pre-procedural pain (Carbajal et al., 2008). In particular, the large EPIPPAIN 2 study reported that parental presence was associated with lower pain scores (DAN pain score <3) following venipuncture (Courtois et al., 2016a). The

same study also reported that parental absence before a heel-stick was associated with a lack of pre-procedural analgesia (Courtois et al., 2016b). Although parental presence reduces procedural pain of infants, parents may need help in to developing coping strategies that reduce distress related to their infant's pain (Franck et al., 2004, 2005).

Developmental care programs can help to reduce stress in the parents of PTIs. Infant- and family-centered developmental care programs aim to adapt the sensory environment of vulnerable newborns to their sensory abilities and expectations, and to integrate parents as the primary caregivers so they can better support infant well-being and neurodevelopment, and the bonding process. Infant pain management is an important component of infant- and family-centered developmental care, and has documented short- and long-term benefits (Montirosso et al., 2012b, 2016a,b). This holistic approach can support infant pain management through an architectural NICU design that supports parental presence, close observation of the infant, high involvement of parents as primary caregivers, and coordinated use of non-pharmacological methods for pain relief. Previous research has examined the impact of parental presence and NICU architectural design on pain management by comparing PTIs cared for in single family rooms or open-bay NICUs (Lester et al., 2014). PTIs in single family rooms received fewer medical procedures, received more parental care, and had less neonatal pain (based on Preterm Infant Pain Profile scores) as determined by the nursing staff of each shift. A precise and individualized evaluation of the signs of withdrawal and approach of each infant using Newborn Individual Developmental Care Program (NIDCAP) observations allow individualization of care procedures, with adjustment according to the tolerance of each child. Previous researchers have used NIDCAP cues to evaluate pain and have integrated them into different pain scores (Holsti et al., 2004; Holsti and Grunau, 2007; Lundqvist et al., 2014). The primary goals of infant- and family-centered developmental care are to reduce systematic and unnecessary procedures, and to support continuous parental involvement in the care and the evaluation of the infant. These programs also promote grasping opportunities and hand-to-mouth interactions to support the autonomy of the infant. These developmental care strategies can effectively reduce pain during and after routine care procedures (Sizun et al., 2002; Catelin et al., 2005). The newly proposed EValuation of INtervention scale allows evaluation of the use of non-pharmacological strategies to reduce pain and stress in the NICU (Warren et al., 2016). This scale helps caregivers to record different evidence-based best practices implemented before, during, and after routine care or painful interventions, and can potentially allow further evaluation of the impact of infant- and family-centered developmental care on infant pain management.

Individualized developmental care programs are also effective. For example, a prospective observational study in Netherlands indicated that implementation of NIDCAP-based stress reduction strategies was temporally associated with a significant decline in the mean number of painful interventions per NICU patient and per day (Roofthooft et al., 2014). Another study reported that the NIDCAP program decreased stress, pain-related behaviors, physiologic stress responses, and the use of sedatives and opioids (Westrup et al., 2007). A randomized clinical study reported that NIDCAP decreased stress responses due to painful procedures and the requirement for sedation (Heller et al., 1997). A randomized controlled trial of 36 PTI receiving 68 eye examinations reported that a NIDCAP-based intervention did not decrease pain responses, but led to faster recovery, as determined by lower salivary cortisol levels at 60 min after the examination (Kleberg et al., 2008).

Taken together, these studies support the benefits of parental presence, ideally *via* an infant- and family-centered developmental care program such as the NIDCAP, on infant pain management in the NICU.

Maternal Contact and Regulation of Endogenous OXT

The most striking effect of OXT is its promotion of pro-social behaviors, and this is indirectly related to its neuroprotective effect during infant development. Two pioneering studies performed several decades ago (Pedersen et al., 1982; Fahrbach et al., 1984) first reported that maternal OXT is a crucial hormone for the regulation of prototypical social behaviors, such maternal behaviors.

A strong parent-infant bond supports the infant's development and protects the infant from danger and stress. During the bonding process, there is an increase of typical maternal behaviors, such as affective and synchronized vocalizations, gazing, and touching. These early maternal behaviors shape the infant and are shaped by the infant. The first reciprocal interactions of the mother and infant have important effects on the infant's brain structure and development, especially on the infant's social, emotional, and cognitive competences, and can provide long-term protection against stress and pain (Meaney, 2001; Feldman and Eidelman, 2003, 2007).

Maternal behaviors in the bonding process are intuitive (Papoušek and Papoušek, 2002) and are tuned to the infant's needs and requests. The bonding process is not a unilateral action, but is a reciprocal and bidirectional; infants are directed and shaped by maternal behaviors, and they actively engage mothers during their interactions. This mutual responsiveness between mothers and infants leads to mother-infant bonding in humans and animals (Nagasawa et al., 2012). Most mammalian infants produce a variety of cues to the mother, such as olfactory or auditory signals (Lévy et al., 2004; Ehret, 2005), that stimulate a range of maternal behaviors. For example, rodents mothers search for and retrieve their pups using vocalizations (Branchi et al., 2001).

Maternal separation induces changes in the reciprocal responsiveness of the mother and infant, and these are mediated by the OXT system. The intracerebral release of OXT, among other mechanisms, may mediate this response.

Association of OXT Level With Maternal Behaviors

The plasma OXT level of a pregnant woman is initially relatively stable, and then gradually increases as pregnancy progresses. The elevated plasma OXT level of the woman is associated with the expression of maternal behaviors soon after birth

(Feldman et al., 2007; Levine et al., 2007). More specifically, an increase of the plasma OXT level between the first and the second trimester correlates with mother-infant bonding, and higher plasma and salivary levels of OXT occur in mothers who have more affectionate contact with their infants (Feldman et al., 2010). Similar to its role in other mammals, OXT supports bond formation in humans. In particular, OXT has roles in micro-level processes of parent-infant synchrony, in a parent's attachments to his or her partner and infant, and in the parenting role and the parent-infant interaction.

Provision of appropriate maternal care increases OXT levels in the infant, and this affects brain organization early in life (Meaney, 2001). In particular, these behaviors increase OXT receptor binding in brain areas central to parenting, and the reward parents derive from their infants (Ross and Young, 2009). Caring behaviors are associated with OXT regulation, and there are increased levels of OXT in mothers and infants when the mothers provide comfort to their babies (Chisholm et al., 2005). Different types of maternal care (licking and grooming behaviors) are associated with increased levels of OXT receptors in brain regions previously known to regulate the expression of maternal behaviors in rats (Francis et al., 2002).

On the contrary, the intracerebroventricular infusion of a selective OXT antagonist into female rats disrupts the development of specific maternal behaviors, such as pup licking and adoption of the crouching posture used during nursing (Pedersen and Boccia, 2003). Rats reared a mother who expresses few maternal behaviors become anxious as adults (Caldji et al., 1998), and this is associated with hyperactivity of the HPA axis (Liu et al., 1997). Modulation of maternal behavior may also have a conditioning effect on the OXT system of progeny. These animal studies suggest that promotion of the OXT system may be an excellent strategy to prevent the impaired neurodevelopment from early and prolonged exposure to stress and pain.

Parental Contact During Routine Painful Procedures Provides Protection by Stimulating the OXT System

Early social experiences can affect social behaviors during adulthood by modifying the OXT system (Meaney, 2001). In particular experiences of early contact or separation have long-term effects – even transgenerational effects – by modulating the OXT system.

In parallel, early and repeated painful experiences (especially in PTIs) induce long-term over-sensitization to pain and stress, and have significant consequences on infant social and emotional competencies. As with maternal separation, the OXT system also plays a crucial role in repairing and reconstructing the infant's resilience in response to painful stimuli. Thus, clinical and maternal care can act by increasing the endogenous activation of the OXT system.

These results suggest that the care of PTIs should consider establishment of an appropriate ecological niche to promote infant development (Browne, 2017) and administration of individualized care (Als et al., 2004). A positive social environment, with experiences of the social interactions of daily life, continuously activates the OXT system. Interventions that sustain social engagement, especially when there is diminished mother-infant contact due to infant prematurity or postpartum depression (Feldman et al., 2010), can have a positive impact on the OXT systems of the infant and mother and on subsequent social and emotional competencies.

Effect of Early Vocal Contact on Stress and Pain of Neonates

Recent research has shown that non-pharmacological analgesic interventions, such as SSC, can diminish the adverse outcomes associated with neonatal pain and reduced maternal care. In addition, Seltzer et al. (2010) demonstrated that infant-directed speech ("motherese") led to increased peripheral OXT release in 6 year-old children who were exposed to a social stressor. Thus, vocalizations may be as important as skin-to-skin contact for the neuroendocrine regulation of social bonding in humans.

Recent animal studies have also identified the effect of social vocalizations on OXT regulation and social behaviors (Tops et al., 2011; Theofanopoulou et al., 2017). Interestingly, electrophysiological studies in mice reported activation of the mother's auditory cortex in response to pup ultrasonic vocalizations (USVs), but no such activation in females not exposed to these USVs (Liu and Schreiner, 2007; Cohen et al., 2011). Two species of "singing mice" (*Scotinomys teguina* and *Scotinomys xerampelinus*), which have a complex vocal repertoire, exhibit high OXT receptor binding in brain regions related to social memory, including the hippocampus and medial amygdala (Campbell et al., 2009). Moreover, OXT null mutant mice were less vocal than wild-type controls during separations from the mother and peers (Winslow et al., 2000). Remarkably, OXT also mediates the response to acoustic social stimuli (Marlin et al., 2015). Furthermore, the injection of OXT into the hypothalamus increases the rate and duration of USVs by female hamsters, suggesting that OXT controls these USVs as a crucial component in the initiation or maintenance of social contact (Floody et al., 1998).

Given that a rat's OXT receptors are very active in the auditory cortex of the mother, and are activated by USVs, it is plausible that reciprocal vocalizations or calls play a fundamental role in the mother-infant bonding, possibly by activating a dopaminergic response and activation of OXT receptors. Studies of 2 week-old *Octodon degus* rodents reported an increased density of the NMDA receptors in limbic brain areas at 3 days after 6 episodes of brief parental deprivation and exposure to an unfamiliar environment, and that parental vocalizations during the separation period suppressed this response (Ziabreva et al., 2003).

Moreover, behavioral observations indicated that parental vocalizations suppress the exploratory activity of rat pups, most likely through its "anxiolytic" effect (Braun et al., 2003). There is also evidence that parental vocal communications regulate the pup's physical development (Poeggel and Braun, 1996; Braun and Scheich, 1997) and behavior, and presumably protect the pup from exposure to frightening situations and reduce the level of anxiety during stressful experiences, such separation or

pain. During and after exposure to pain, maternal protective and consolatory vocal behaviors are essential for emotional recovery of offspring, even though these behaviors do not directly impact the origin of pain. OXT plays a crucial role in these consolatory behaviors (Burkett et al., 2016).

Potential Effects of Early Vocal Contact on OXT Regulation

In light of these previous studies, early vocal contact (EVC) in the form of live maternal speech and songs, can be an effective method for reducing pain in infants who are undergoing medical procedures. EVC is an early family-based intervention with a high degree of contact, in which mothers and fathers speak and sing intimately with their preterm infant (Filippa et al., 2017b). This increases the PTI's emotional and autonomic stability (Filippa et al., 2013) and reduces maternal anxiety (Arnon et al., 2014). The support from a music therapist can allow the PTI to engage in communicative musicality (Haslbeck, 2014) when they hear specific songs of kin ("lullabies") (Loewy, 2015).

Moreover, EVC, as a form of live and dynamic musical contact, decreases an infant's sensitivity to painful stimuli. Maternal singing is one of the most widespread forms of intuitive and nurturing music experiences among humans. This ubiquitous form of communication provides early social and communicative cues to the infant. It is finely tuned to the infant's needs and expectancies. The infant is not merely a passive receiver, but experiences an active "call" for participation in a reciprocal musical play. Music can affect social interactions among humans, and Chanda and Levitin (2013) proposed that the OXT system plays a crucial role in this response. EVC is also an effective method because it is a social vocalization involving emotions.

OXT and Recognition of Vocal Emotions

It is well known that emotional prosody can affect socialization and the capacity of humans to infer the mental states of others, either implicitly, or explicitly (Grandjean et al., 2006). Many studies found that OXT plays a crucial role in improving recognition of emotions from vocalizations. For example, intranasal administration of OXT improves the recognition of emotions associated with different facial expressions (Domes et al., 2010; Shahrestani et al., 2013) and body postures (Bernaerts et al., 2016). Tops et al. (2011) suggested that individuals who have a specific OXT receptor polymorphism (GG genotype, rs53576), which presumably has stronger binding to OXT, have increased sensitivity to social processing and fewer difficulties in hearing and understanding people in the presence of background noise.

Similarly, Hovey et al. (2018) showed that activation of the OXT pathway, specifically the aryl hydrocarbon receptor nuclear translocator 2 (ARNT2) gene, is significantly associated with the ability to recognize audio-visual emotions. Other research showed that nasal administration of OXT specifically enhanced the ability to discern the emotional states of others, but not with inferring their beliefs. In particular, Aoki et al. (2014) performed a clinical double-blind, placebo-controlled, within-subject crossover trial of subjects with autism spectrum disorders, and found that intranasal OXT administration increased the rate of correctly inferring the social emotions of others, but not inferring their beliefs. Their imaging analysis also indicated that the right anterior insula, which was initially negatively correlated with difficulties in emotion inferences in these subjects, is significantly increased and correlated with the enhanced ability to infer the emotions of others following OXT administration. Furthermore, Hollander et al. (2007) reported improved recognition of emotion in vocalizations following OXT administration to patients with autism spectrum disorders. These findings thus establish relationships of vocal communication, social processing, and OXT level.

OXT AS A NEUROPROTECTIVE FACTOR IN THE DEVELOPMENT OF PRETERM INFANTS

In addition to the potential effect of EVC and OXT on reducing pain and stress in infants, there is also evidence that OXT acts as a direct neuroprotective factor during development of the infant brain, and that OXT has different mechanisms and potential molecular targets in this process.

OXT and the GABA Switch in Early Life

The neonate's brain is particularly vulnerable to excitotoxic damage, necessitating a balance between excitatory and inhibitory neurotransmission. OXT is responsible for the "developmental switch" in GABA polarity, in that it provides critical neuroprotective and analgesic effects that counteract postnatal excitotoxic damage (Ben-Ari, 2018). More specifically, $GABA_A$ receptors are ligand-gated Cl^- channels, and the postsynaptic GABAergic signal regulates intracellular Cl^- concentration. OXT influences the intra-neuronal Cl^- level during the perinatal period by regulating the expression NKCC1 and KCC2 transporters. Down-regulation of NKCC1 and up-regulation of KCC2 initiates the postsynaptic GABAergic shift from depolarizing to hyperpolarizing just after birth.

Interestingly, the postnatal GABAergic shift is incomplete or delayed in several animal models of autism spectrum disorder (Deidda et al., 2015) and after gestational immune challenges that exacerbate symptoms of autism in animal models (Corradini et al., 2018). These findings led to the development of "neuro-archeology" by Y Ben-Ari during the last decade, which posits that the neurodevelopmental consequences of prematurity and the high vulnerability of the premature brain could be caused by the reduced or delayed GABA shift caused by an effect of OXT.

OXT and Inflammation in Intrauterine Growth Restriction and Prematurity

The OXT neurons of the adult rat exist as a small population of about 30 parvocellular neurons in the paraventricular nucleus of the hypothalamus, and these coordinate the peripheral and spinal release of OXT, and limit the symptoms of inflammatory pain (Eliava et al., 2016).

The OXT-mediated prevention of inflammatory pain may be extended to the entire central nervous system, and may

be particularly relevant to intrauterine growth restriction and premature birth. Indeed, inflammation in the central nervous system plays a crucial role in the pathophysiology of perinatal brain damage in animal models and human neonates (Hagberg et al., 2012). Abnormal microglial activation induces white matter damage, neurocognitive disabilities, and neuropsychiatric disorders in children and adults (Rezaie and Dean, 2002; Leviton et al., 2005). A recent study used a double-hit rat model of perinatal brain injury induced by a low protein gestational diet and potentiated by postnatal injections of sub-threshold doses of IL1β (Mairesse et al., 2018). The results showed that systemic postnatal administration of carbetocin (a selective, long lasting, and brain diffusible OXT receptor agonist) reduced microglial activation at the transcriptional and cellulat levels, and provided long-lasting neuroprotection. Carbetocin treatment also had beneficial effects on myelination, long-term intrinsic brain connectivity, and behavior. Thus, targeting OXT signaling in the developing brain may be an effective approach to prevent neuroinflammation-induced brain damage that originates during the perinatal period.

CONCLUSION AND PERSPECTIVES

Oxytocin is a neuropeptide hormone that functions in the physiological responses to pain and stress (Neumann et al., 2000) and promotes prosocial behaviors (Carter, 1998). In particular, during the early period after birth, OXT regulates maternal behaviors (Pedersen, 1997) by promotion of social interactions and positive emotions (Uvnas-Moberg, 1998). Inhibition of OXT receptors or a decrease in OXT production, such as following separation of the mother and infant or stress during the critical neonatal period, correlate with poor maternal behaviors, and this has long-term negative consequences on the prosocial behaviors of mothers and infants. Animal studies have documented the protective effect of OXT administration, in that a single dose can reverse the effects of maternal separation and the many adverse sequelae in rodent pups (Lee et al., 2007). PTI often experience early maternal separation and painful events or procedures in the NICU, and these two risk factors are often simultaneous and appear to interact synergistically.

Positive social interactions can suppress internal physiologic systems that are activated by stress, and stimulate other internal systems that attenuate stress. OXT plays a crucial role in the attenuation of stress by enhancing the buffering effect of social support on stress responsiveness (Heinrichs et al., 2003). The cumulative effects of early maternal contact and an increased level of OXT can protect PTIs against many sequelae of early maternal separation and their painful experiences during their first weeks of life in the NICU. In this context, the maternal voice can have positive effect on infant recovery from stressful events (Seltzer et al., 2010).

Creating an environment that decreases the negative effects associated with preterm birth is one of the main aims of individualized developmental care in the NICU. Implementation of a series of protective actions during the different stages of painful procedures, mediated by the OXT system, can reduce the impact of these procedures on PTIs during their time in the NICU (**Figure 1**).

In light of the many studies reviewed here, we suggest the following protective actions for pain management in the NICU:

- Active involvement of parents with the infant during all phases of painful procedures in the NICU, including the

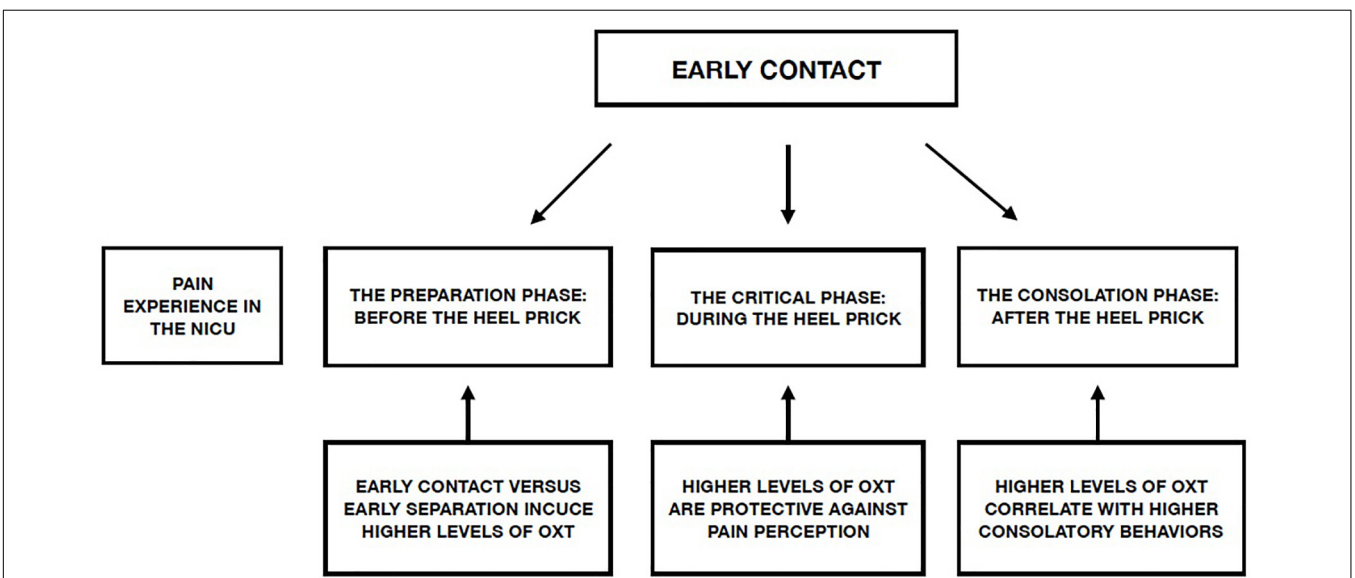

FIGURE 1 | Possible effect of early contact on the different phases of a preterm infant's experience of pain in the NICU and role of the OXT system.

preparation phase, the phase of acute pain, and the consolatory/reunion phase;
- Active involvement of nursing staff in supporting parental involvement with their infants during the preparation and consolatory phases of painful NICU procedures;
- Use of EVC as a non-pharmacological intervention to encourage contact between parents and PTIs, by use of live and directed speech and songs directed to the PTIs.

A limitation of this review is the lack of human studies, especially on the impact of pain and early maternal separation on the OXT system. Moreover, additional human studies are necessary to improve our understanding of the impact of exogenous administration – or endogenous production – of OXT on an infant's brain and social behaviors.

Finally, further research is needed to investigate the impact of early contact between parents and infants in the NICU on regulation of the OXT system. Identification of the role of the OXT system during stressful conditions, such as painful procedures in the NICU, seems to be a particularly promising topic for future research.

AUTHOR CONTRIBUTIONS

MF and PK contributed to conception and design of the review. MF wrote the first draft of the manuscript. PP, JM, PH, MM, OB, and PK critically revised the manuscript for important intellectual content. PP, JM, DG, and PK wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Darwin's Other Dilemmas and the Theoretical Roots of Emotional Connection

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Modern scientific theories of emotional behavior, almost without exception, trace their origin to Charles Darwin, and his publications *On the Origin of Species* (1859) and *The Expression of the Emotions in Man and Animals* (1872). The most famous dilemma Darwin acknowledged as a challenge to his theory of evolution through natural selection was the incomplete Sub-Cambrian fossil record. However, Darwin struggled with two other rarely referenced theoretical and scientific dilemmas that confounded his theories about emotional behavior. These included (1) the origin of social instincts (e.g., altruism, empathy, reciprocity and cooperation) and the reasons for their conservation in evolution and (2) the peripheral control of heart rate vis-à-vis emotional behavior outside of consciousness. Darwin acknowledged that social instincts are critical to the survival of some species, but had difficulty aligning them with his theory of natural selection in humans. Darwin eventually proposed that heart rate and emotions are controlled via one's intellect and cortical mechanisms, and that instinctive behavior is genetically programmed and inherited. Despite ongoing efforts, these two theoretical dilemmas are debated to this day. Simple testable hypotheses have yet to emerge for the biological mechanisms underlying instinctive behavior or the way heart rate is controlled in infants. In this paper, we review attempts to resolve these issues over the past 160 years. We posit that research and theories that supported Darwin's individualistic brain-centric and genetic model have become an "orthodox" Western view of emotional behavior, one that produced the prevailing behavioral construct of attachment as developed by John Bowlby. We trace research and theories that challenged this orthodoxy at various times, and show how these challenges were repeatedly overlooked, rejected, or misinterpreted. We review two new testable theories, *emotional connection theory* and *calming cycle theory*, which we argue resolve the two dilemmas We show emerging scientific evidence from physiology and a wide variety of other fields, as well from clinical trials among prematurely born infants, that supports the two theories. Clinical implications of the new theories and possible new ways to assess risk and intervene in emotional, behavioral and developmental disorders are discussed.

Keywords: vagal tone, attachment theory, instinct, calming cycle theory, autonomic conditioning

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INTRODUCTION

The modern scientific study of emotions began with Darwin's comparative studies of animals and humans and soon inspired the fields of psychology and physiology, among many others. To this day, however, Darwin's assumptions about emotional behavior persist as a kind of "orthodoxy" throughout science and society, one that has been remarkably resistant to change or challenge over the ensuing 160 years.

Great breakthroughs and advances have been made in science and medicine since Darwin. We can coax a body's own immune system to attack a tumor, eradicate viral and bacterial infection, fix heart defects, identify and correct for genetic disorders, and overcome the most debilitating injuries. We can also extend the life span and save infants born at twenty-two weeks of gestation. However, when it comes to understanding and treating emotional and behavioral disorders that arise in infancy and early childhood, science has made relatively little progress since Darwin (Dong et al., 2012; Lean et al., 2017). Despite exponential increases in research over the last 40 years, there is little evidence that science is any closer to fully understanding autism spectrum disorder (ASD), let alone curing it (French and Kennedy, 2018). The same can be said about Attention Deficit Hyperactivity Disorder (ADHD) (Catala-Lopez et al., 2017), school-age child developmental disorders (Kingston and Tough, 2014), post-traumatic stress disorder (de Graaff et al., 2018), and a host of other psychiatric and neurodegenerative disorders. Where signs of success in the treatment and prevention of emotional and behavioral disorders have appeared (Moore et al., 2016; Moreno-Peral et al., 2017), the lack of scientific theories that can explain their efficacy have hampered efforts to change standard medical care. All in all, emotional and behavioral disorders in infants and young children continue to be daunting challenges to science (Cameron et al., 2017), and among the greatest burdens to modern society (Younger, 2016).

It is indeed perplexing that our relative lack of progress has failed to prompt a fundamental rethinking of the way we view human emotions and behavior. In most scientific fields, longstanding failure to explain anomalies and solve challenges typically prompts a rethinking of the theoretical underpinnings of the discipline (Frontiers Collection, 2015). Where would we be, for instance, if 100 years ago Einstein had not challenged conventional assumptions in order to explain dilemmas that Newtonian physics could not explain? We suggest that it is time to question the underlying assumptions and beliefs about human emotion as a means to finding new ways to treat emotional and behavioral disorders.

In this review, we historically track efforts to resolve two rarely referenced dilemmas that challenged Darwin's assumptions about emotional behavior. These dilemmas are separate from Darwin's well-known, self-acknowledged inability to explain the incomplete fossil record below the Cambrian explosion at the time (Conway, 2006). One dilemma for Darwin was that he could not explain instinctive (innate) behaviors that emerge in the perinatal period of development. What could account for the behaviors of a mother and baby soon after birth, especially the instincts of empathy and altruism? Still another dilemma was that he could not explain peripheral control of heart rate. Darwin pondered if, as his theory posited, emotional behavior is controlled via the central nervous system, how can heart rate be inhibited separately by peripheral influences? What is the mechanism and function of peripheral inhibition of heart rate? Darwin believed that these two dilemmas needed to be answered in order to fully explain human emotions.

The two dilemmas raised heated political arguments about the origin and nature of emotions that took on ideological dimensions. To "compete and dominate" vs. to "empathize and care" foreshadowed the great 20th century ideological and political split, pitting Capitalism, with *Natural Selection* as its raison d'etre (Spencer, 1984), against Communism, which made *Cooperation and Mutual Aid* its sources of inspiration (Adams, 2016). The problem of how heart rate is controlled was equally controversial, with psychologists favoring cortical control pitted against physiologists presenting data that challenged that view.

Darwin considered the problems of instinctive behavior and heart rate control to be connected. Through discussing them in the new evolutionary light of natural selection, he questioned why heart rate and emotions, positive and negative, can be influenced both by conscious will and unconscious physiological mechanisms. The idea that man's emotions *might not* be controlled by higher order consciousness – his God-given superior human intellect and will – *the very things that were believed to separate him from inferior species* – was blasphemous and unthinkable to religious Victorian England. Darwin did his best to walk the line between what he believed, what his data showed, and what his audience did not want to hear. And, he left it to his followers to sort out the reality.

While this dichotomy had a profound effect upon political debate, we limit our review to the theoretical scientific story, which we follow separately and chronologically for each dilemma. In Part 1, we present Darwin's thoughts on instincts, along with competing scientific evidence that emerged from the study of instincts over the ensuing 160 years. In Part 2, we follow Darwin's discussions on peripheral control of heart rate and review the various competing theories and evidence that emerged through the present. In Part 3, we first critically review John Bowlby's prevailing behavioral construct of attachment and discuss why the construct is not useful in fully understanding, assessing and treating emotional behavior. Following this, we critically review evolutionary game theory and discuss why it does not fully address fundamental problems identified by Darwin. We explain why polyvagal theory provides a partial explanation for the problem of peripheral control of rate, but does not provide an adequate mechanism or theory of change. We also review the authors' calming cycle theory, which provides a novel explanation for how socioemotional behavior is governed in an interpersonal co-regulatory manner via bottom-up sub-cortical *Pavlovian conditioning* and *visceral/autonomic learning* mechanisms, along with evidence that supports this new theory. Finally, we review the authors' theoretical construct of *emotional connection* and explain why it is different from attachment, why it has more predictive value and is more useful in assessing and treating behaviors of infants and mothers, and the data from recent clinical trials that supports the construct.

PART 1: DARWIN'S SOCIAL INSTINCT DILEMMA

Overview

Throughout his writings, Darwin searched for a rationale to explain the evolution of "social instincts" in light of his *Principle of Natural Selection* (Darwin, 1859). He argued that humans with superior intellect, who exhibit competitive characteristics attuned to and advantageous for their specific environment, will most likely survive, reproduce, and pass on their genes. Because of this, subsequent generations are more likely to possess those advantageous characteristics. Herbert Spencer popularized this idea with the phrase "survival of the fittest," partly based on the Lamarckian belief that struggle for survival led to traits that could be inherited.

In his first book, Darwin used the term instinct or innate behavior 152 times and acknowledged that instincts presented a challenge to his theory. He asked, "Can *instincts* be acquired and modified through natural selection?" (Darwin, 1859). Without offering a definition for instinct, he acknowledged:

> *An action, which we ourselves should require experience to enable us to perform, when performed by an animal, more especially by a very young one, without any experience, and when performed by many individuals in the same way, without their knowing for what purpose it is performed, is usually said to be instinctive* (Darwin, 1859).

An action repeated over and over can become a habit that is performed without thinking, but Darwin argued that such action (habit) is different from an instinct. Citing multiple examples from the animal world, Darwin concluded:

> " *. . . metaphysicians have compared instinct with habit. This comparison gives, I think, a remarkably accurate notion of the frame of mind under which an instinctive action is performed, but not of its origin*" (Darwin, 1859).

A decade later and with evolved thinking, Darwin distanced himself from Spencer's "*survival of the fittest*" sentiments in *The Descent of Man* (Darwin, 1871) by acknowledging that widely exhibited traits like cooperation, empathy, reciprocity and altruism are sometimes crucial to a species' ability to survive. Such traits, he mused, could be an after-effect of a group of behaviors underlying instincts. "The aid which we feel impelled to give to the helpless," he wrote, "is mainly an incidental result of *the instinct of sympathy*, which was originally acquired as part of *the social instincts*, but [this instinct was] subsequently rendered . . . more tender and more widely diffused" (Darwin, 1871).

Nonetheless, this reasoning did not resolve the dilemma for Darwin. He wrote, "It is extremely doubtful whether the offspring of the more sympathetic and benevolent parents, or of those which were the most faithful to their comrades, would be reared in greater number than the children of selfish and treacherous parents of the same tribe" (Darwin, 1871). Darwin argued that genes of cooperative altruistic humans, if passed on, would ultimately weaken and degrade the species. To support his argument, he pointed to the practice of animal husbandry, which had over thousands of years improved the quality of livestock by culling out inferior and weak individuals, without regard to their suffering. Only humans, he complained, protect and nurture their weak and infirm.

Darwin wrote extensively about emotional expression in both humans and animals. He incorporated contemporary insights from natural observations to frame the problem of instincts within his first two principles of emotional expression and behavioral habits (Darwin, 1872). His description of emotional instinct was closely tied to observations in the postnatal period and was partially inspired by the behavior of his own first-born child. He concluded that behaviors could not be the result of habits, noting, "The far greater number of the movements of expression, and all the more important ones, are, as we have seen, innate or inherited; *and such cannot be said to depend on the will* [i.e., cognition] of the individual" (Darwin, 1872).

Darwin summarized all emotional expressions in animals and humans with three general principles [reviewed in-depth by Hess and Thibault (2009)]. First, *the principle of serviceable habits* stated that certain useful habitual emotional expressions or "instincts" are acquired over time through experience by the species and are genetically inherited by offspring [a view previously developed by Lamarck (1809)]. Darwin insisted, however, that emotional behaviors had evolved, as many emotional behaviors that were functional in the past or characteristic of lower animals were no longer present in humans. Second, *the principle of antithesis* asserted that some emotional expressions are simply the opposite of serviceable ones and that these become traits, in addition to physiological and psychological states. Over time, certain emotional expressions became habitualized or ritualized in order to act out an emotion, as occurs in courtship or fighting. Third, *the principle of the direct action of the excited nervous system on the body* stated that some emotional expressions are universal and are directly associated with an underlying emotional state. For example, Darwin described laughter as quasi-convulsive movement that discharges an overflow of nervous energy induced by either physical or psychological tension.

These three principles laid a groundwork for the scientific investigation into emotions and emotional behavior that influenced multiple new scientific fields; genetics, electrophysiology, cognitive neuroscience, neurology, psychology, ethology, endocrinology and computer science, to name a few (**Figure 1**) (Pagel, 2009). Darwin's insights and theories permitted researchers to make new inferences about human behaviors, such as learning mechanisms, memory, emotions, and social interactions, based on animal and human observations and experiments. In this respect, Darwin's collective writings, rather than resolving the dilemma of instincts, have served as source documents that have encouraged ongoing scientific investigations into emotions and instincts.

Attempts to Resolve the Social Instinct Dilemma

Prior to Darwin, instinctive behavior was largely explained through the nativism teachings of Plato and Descartes, which assumed that a God or a similar being, or a process, placed innate ideas and principles in the human mind. In the decades following Darwin, such behavior was being studied by a rapidly growing

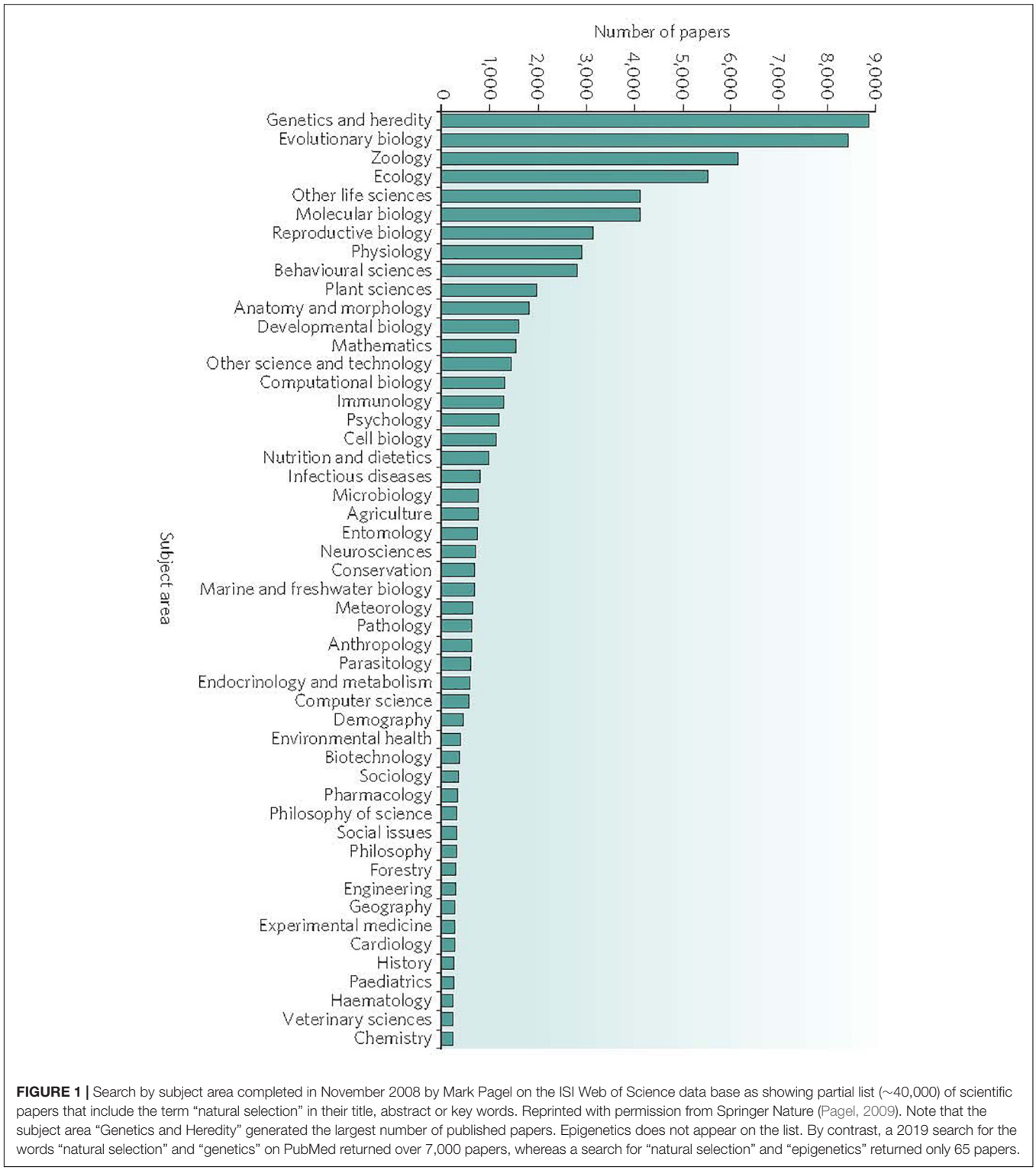

FIGURE 1 | Search by subject area completed in November 2008 by Mark Pagel on the ISI Web of Science data base as showing partial list (∼40,000) of scientific papers that include the term "natural selection" in their title, abstract or key words. Reprinted with permission from Springer Nature (Pagel, 2009). Note that the subject area "Genetics and Heredity" generated the largest number of published papers. Epigenetics does not appear on the list. By contrast, a 2019 search for the words "natural selection" and "genetics" on PubMed returned over 7,000 papers, whereas a search for "natural selection" and "epigenetics" returned only 65 papers.

number of entomologists and ethologists who were cataloging instinctive behaviors.

Entomologist Jean Henri Fabre (1823–1915) spent his life observing the behavior of insects. He considered instinct to be any behavior that did not require cognition or consciousness to perform. The American amateur zoologist and ethologist Charles O. Whitman (1842–1910) revolutionized thinking about the physiological mechanisms by which hormones influence behavior and launched the field of behavioral endocrinology (Marler, 2005). But it was C. Lloyd Morgan (1852–1936), more

than any other theorist, who tried to resolve the confusion over natural selection and inherited habit (Morgan, 1896).

Morgan drew heavily on the experimental work of physiologist Charles S. Sherrington (1857–1952), who demonstrated that complex reflex responses could still be elicited in animals with their cerebrum removed. Morgan argued strenuously that instincts at birth are subconscious reflexes that lay in the inherited arrangement of neural connections found in the *lower brain centers* (Morgan, 1912). However, under intense resistance from researchers who were focused on reflexes that require higher-order consciousness, Morgan agreed to a definition of reflex that included both subconscious *and* conscious actions. Thus, Morgan's ultimate explanation, which formed the theoretical foundation of Neo-Darwinism in the twentieth century, left the definition of reflex broad enough to be confusing and therefore Darwin's dilemma of instincts unresolved (Richards, 1977).

Two other researchers who focused on conscious behaviors were German physician and physiologist Wilhelm Maximilian Wundt (1832–1920) and psychologist Edward Titchener (1867–1927), who helped found a new independent field of *Experimental Psychology*. Together, they developed the concept of *Structuralism* to analyze the adult mind as the total sum of experience from birth to the present. Titchener's method for analysis focused on introspection via self-reports of sensations, views, feelings and emotions to establish the simplest definable emotional components of one's life, so that they may be reconstructed and more fully understood (Gozli and Deng, 2018).

Among the researchers invigorated by Darwin's ideas about natural selection and cognition was the German neurologist Ernst Haeckel (1834–1919), whose "biogenetic" principle played a major role in the thinking of Sigmund Freud (1856–1939), both in the formation of psychoanalytic theory in general, and its metapsychology in particular (Makari, 2008).

Freud incorporated aspects of Darwin's ideas about survival and emotional behavior into his concepts of psychoanalysis. For instance, Darwin's first principle of the expression of emotions, especially his definition of instinct as "inherited learning," had a clear continuation in Freud's psychoanalytic theory. It posits that a specific state of mind is associated with a habit or movement, and can be repressed by will. This repression of instinctive or innate feelings, mind over body, is a hallmark of psychoanalytic theory and psychopathology.

Freud accepted that natural selection is genetic in nature, yet postulated that instinct and emotion can be understood by examining the mind. Thus, Freud failed to address the philosophical dilemmas that natural selection posed for Darwin. Freud adopted much of Haeckel's theory and the ideas and methods of Titchener, which emphasized introspection, self-reflections and hypnosis as the gateways to understanding emotional behavior (Ritvo, 1974).

At the turn of the twentieth century the argument was largely dominated by Neo-Darwinists, who accepted Darwin's evolutionary theory of natural selection without resolving the theoretical dilemmas it posed. Ethologists, meanwhile, were busy cataloging the developmental aspects of perinatal parent/infant instinctive emotional behaviors in animals and insects. Aspects of behavior in humans were being explored by psychologists, who were split between those who were influenced by the psychodynamic approach of Freud and those following a new school of *Behaviorism*. Both groups were increasingly interested in abnormal emotional behaviors that deviated from normal instinctive behaviors soon after birth and the relationship between these early abnormal behaviors and emotional problems later in life.

Meanwhile, Freud's proposed genetic basis for instinctive behavior was generally accepted in the field of psychology as the key to understanding neuroses. The scientific climate in the late 1800's formed a strong base for Freud's theoretical thinking and for the growth of psychoanalysis, but advances in biology were beginning to challenge the basis for this synthesis at the beginning of the twentieth century (Hofer, 2014). Calls for scientifically testable hypotheses in psychology began to grow. Even Freud recognized the problem, writing in 1925, "There is no more urgent need in psychology than for a securely founded theory of the instincts" (Freud, 1925).

This search was already well underway. V. M. Bekhterev (1857–1927) was the first to employ the use of experimental methods to study the central nervous system in psychology. His research on associated responses became highly influential in a new branch of psychology called *behaviorism* (later the basis of *Reflexology*, *Gestalt Psychology*). John B. Watson (1878–1958), an early proponent of behaviorism, attempted to make psychology scientifically relevant and acceptable, adding his voice to a growing chorus of basic researchers criticizing Freud's nebulous and metaphoric ideas about "consciousness."

> "*The position is taken here that the behavior of man and the behavior of animals must be considered on the same plane; as being equally essential to a general understanding of behavior. It can dispense with consciousness in a psychological sense. . .the findings of psychology become the functional correlates of structure and lend themselves to explanation in physico-chemical terms*" (Watson, 1913).

Parting completely from Darwin and Freud, Watson proposed that *nothing is instinctual in the infant*. Rather, Watson stated that everything is built into a child through the interaction with his environment. Impressed with Pavlov's early work on learning, he incorporated a highly simplified version of Pavlov's principles of the conditional reflex and autonomic conditioning into his behaviorism concepts and experiments.

By the 1940s, however, two other American behaviorists, B. F. Skinner (1904–1990) and Edward Lee Thorndike (1874–1949), were modifying the ideas of Watson and embracing Konorski's cognitive learning mechanism. Skinner believed that Pavlovian conditioning reflex was too simplistic to explain complex human behavior. He looked for the causes of an action and its consequences, calling his cognitive learning approach *operant conditioning* (i.e., changing behavior by following a desired response with reinforcement) (Skinner, 1988). This method followed Thorndike's *Law of Effect,* which states behavior that is reinforced tends to be repeated and, that behavior not reinforced tends to be extinguished.

Despite the growing emphasis on a cognitive and mechanistic view of human behavior among psychologists, ethologists continued to study instinctive behavior in the animal world between 1900 and 1950. American experimental psychologist

and behavior scientist Wallace Craig (1876–1954) studied the way innate and learned emotional behavioral tendencies are integrated with evolutionary, motivational, experiential, social and ecological degrees of freedom, and how vocal and social behaviors are organized (Craig, 1922). The work of Dutch biologist Nikolaas Tinbergen (1907–1988) (Tinbergen, 1951) and Austrian biologists Konrad Lorenz (1903–1989) and Karl von Frisch (1888–1982) combined laboratory and field science with other disciplines, such as neuroanatomy, ecology, and evolutionary biology, to study aggression, "nurture" and "bonding" in animals. Tinbergen did not venture into theoretical matters, but instead focused his efforts on standardizing the study of instincts in the field. His methods continue to be the gold standard for fully explaining behavior (Bateson and Laland, 2013).

Lorenz, on the other hand, theorized prolifically. He gained acclaim and popularity for his theory on the evolution of instinctive animal communication, based on his observations of fixed action patterns of geese and their ability to bond to humans after birth. Lorenz popularized the notion of *imprinting*, whereby a newly hatched chick forms what he termed an "attachment" to the person caring for it and follows it around. From his observations, Lorenz concluded that the attachment phenomenon provided compelling evidence that social instincts are not learned through experience. Rather, he concluded disturbingly, they are inherited, genetic and *decadent* (Kalikow, 1983).

The research of ethologist Lorenz and the beliefs of Freudian psychologists produced a similar consensus that instincts are inherited, genetic in origin, and more or less fixed at birth. These theories and ideas collectively formed the foundational principles of the new interdisciplinary field of *neuroscience*, which was bringing together the fields of Physiology, Behavioral Psychology and Genetics, among others. A general excitement abounded at the mid-point of the twentieth century that Darwin's dilemmas could finally be explained and resolved through increasingly reductive brain and gene research.

It was at this time and in this theoretical climate that British psychiatrist and researcher, John Bowlby (1907–1990) set out to find a testable hypothesis to Freud's ideas on instincts. He did so by compiling theories and research that prevailed at the time. Mary Ainsworth, Bowlby's protégé, explained his goal in 1969:

> "[Bowlby] *proposes to replace Freudian instinct theory with a set of propositions, testable through research, more closely in line with present-day knowledge, while at the same time respecting the many psychoanalytic contributions to understanding human experience and behavior which are not tied inextricably to an antiquated instinct model*" (Ainsworth, 1969).

It is difficult to follow Bowlby's theory and the arguments supporting it, mainly because the theory is not a tightly articulated or consistent theory but a wide-ranging and sometimes contradictory amalgamation of theories from various fields. Nonetheless, in Part 2 of his first book (Bowlby, 1969), Bowlby claims that he has created a new comprehensive explanation for instinctive behavior:

> "*A long-awaited theoretical breakthrough has been achieved by analytical biology and control theory, which together have elucidated the basic principles that underlie adaptive, goal-directed behavior. Exploiting this breakthrough have been three empirically based sciences: ethology, experimental psychology, and neurophysiology.*"

Bowlby stated that his new *Attachment Theory* was built upon the Neo-Darwinist genetic theory of natural selection and Freud's drive theory. Citing Williams (1972), Weiner (1948), Skinner (1938), Lorenz (1937), Hinde (1970) and various cognitive scientists and their research, Bowlby proposed that behavioral systems are individual and self-controlled through feedback loops in a hierarchical top-down manner in ways that promote and satisfy the individual's needs and survival instincts. Thus, Bowlby places his work solidly within the Darwinian orthodox theoretical mainstream without offering any new insights on the two dilemmas discussed here.

Bowlby did part slightly from Freud on instinctive behavior, postulating that rather than inheriting the instinct itself one inherits a "potential" for the instinct. In the end, however, Bowlby, like Darwin before him, did not offer a definition of instinctive behavior, but rather offered a description of it. Bowlby's ideas about early infancy and early childhood, as well as the research they stimulated, were soon criticized as lacking rigorous scientific validation (Mercer, 2011) or theoretical rigor (Lehrman, 1953). Despite these shortcomings, however, Bowlby's description of behavior associated with the Attachment construct and Ainsworth's behavioral coding system (Crittenden, 2017) remain the standards by which psychologists assess and treat behavior.

In the late 1960's and 1970's, influenced by a group of child development researchers at Harvard University, Colwyn Trevarthen (born 1931) developed the theory of "innate intersubjectivity" (Trevarthen and Aitken, 2001) to explain the emergence and development of active "self-and-other" awareness in infancy. Intersubjectivity theory proposes that "the infant is born with awareness specifically receptive to subjective states in other persons," and that a human individual "grows in active engagement with an environment of human factors – *organic at first*, then psychological or inter-mental." The terms "innate" and "organic" are not clearly defined and no pre-natal learning mechanism is proposed. Thus, intersubjectivity theory focuses on the psychological mechanisms associated with social development following birth, leaving the dilemma of instincts unresolved.

Contemporaneous with, yet completely separate from Bowlby's work in the 1970's, American psychiatrist Martha G. Welch reported anecdotal evidence that contradicted accepted theories on instinctive behavior. Welch treated autistic children and their families with an intervention that involved regular and repeated physical "co-calming" sessions, primarily between mothers and children (Welch, 1988). The hallmark of the intervention was that the treatment method led to profound phenotypical changes, from maladaptive avoidant behavior to adaptive approach behavior in both the child

and mother, sometimes following a single calming session (Welch et al., 2006).

Welch's work came to the attention of Nobel Laureate Niko Tinbergen, who had called in his Nobel speech for young clinicians and scientists to take a new look at autism. After visiting Welch's treatment center and observing phenotypical behavioral changes in autistic children and their mothers, Tinbergen became convinced that Welch had discovered a therapeutic breakthrough. For the last ten years of his life, he and his wife helped promote Welch's discovery (Welch, 1983). But, by the late 1990's, it became clear that Welch's insights were not going to change the treatment of emotional disorders without providing rigorous scientific evidence to support her method, including a biological mechanism and a theory of change to support the behavioral changes observed using her treatment method.

From the late 1940s to the present, various *developmental psychobiologists* sought scientific explanations for ontological questions surrounding prenatal, perinatal and early childhood development and emotional behavior by combining biological psychology, neuroscience and many other areas of biology. Of this group, Myron A. Hofer, was one of the most influential. An early proponent of Bowlby, Hofer came to realize that it was not possible to generate testable hypotheses within Bowlby's theoretical construct of attachment as a unique motivational system (Hofer, 2006). Instead, Hofer and his colleagues focused their research on the basic biological components underlying mother/infant behaviors, such as sleep, feeding, thermoregulation, attention, and on Michael M. Myers' work on the specific relationship between behavior and cardiovascular function (Myers, 1992).

Hofer posited that within normal mother–infant interactions there are three main categories of "hidden regulators" associated with the caregiver: behavioral-sensorimotor, thermal-metabolic and nutrient-interoceptive (Hofer, 1994). Using animal models to study specific maternal stimulations, he found that warmth, milk and touch had immediate regulatory effects on various physiological activity, including heart rate (Hofer, 2006). Hofer theorized that these hidden regulators form the biological basis of Bowlby's "internal working model" of attachment. These regulatory interactions become associated with physical and psychological events (Hofer, 2006), an idea similar to Darwin's first principle on the expression of emotions (see above). Hofer's data pointed to autonomic regulation embedded in social interactions between mother and infant, with mother regulating infant and, in turn, infant regulating mother, especially with regard to eliciting maternal behaviors. In experiments involving what he termed "social entrainment of biologic rhythms," Hofer suggested *Pavlovian learning* could be at work (Hofer, 1984). He postulated that disruption of this mother-infant regulation through social separation can have profound lasting negative impact on various functions of the autonomic nervous system, including heart rate, and that such events can negatively impact physiology throughout the life span.

Yet another group of scientists, *evolutionary biologists* (Payne and Wagner, 2018), were beginning to reveal intriguing new statistical facts about Darwin's social instincts. Applying new mathematical models in the 1960's, William Donald Hamilton (1936–2000) proposed a genetic basis for cooperation and altruism that supported Darwin's theories, based upon the concept of inclusive fitness. Also building on new mathematical breakthroughs, evolutionary game theory originated in 1973, when John Maynard Smith (1920–2004), and George R. Price (1992–1975) proposed mathematical criteria that can be used to analyze and predict contests and competing strategies first identified in animals by ethologists Lorenz and Tinbergen (see above) (Maynard-Smith and Price, 1973). The theory proposed a basis for social instincts in Darwinian evolution by applying game theory to evolving populations in biology (Nowak and Sigmund, 2005).

Today, evolutionary game theory is of interest to economists, anthropologists, and philosophers and to sociobiologists, such as E. O. Wilson (see above). An offshoot of evolutionary game theory, indirect reciprocity theory proposes an explanation of altruistic, cooperative interactions in human populations (Nowak and Sigmund, 2005). This theoretical work has advanced the understanding of certain aspects of human behavior by going beyond the rational, touching on the subconscious and proposing various cognition-based mechanisms that drive emotions, especially with respect to cooperation (Wang et al., 2017; Xia et al., 2017). In that sense, evolutionary game theory ends up supporting Darwin's theories of natural selection and instincts and falls in line with traditional views on the origin of emotions. While solving certain aspects of social instincts, evolutionary game theory falls short of producing a simple testable hypothesis that can account for epigenetic factors influencing instinctive behavior in the perinatal period. See more on this point in Part 3 below.

About the same time, however, ethologists were accumulating new evidence that suggested evolution might be occurring at the group level, and that social instincts play a key role. Gradually, a new group calling themselves *sociobiologists* (Wilson, 2008b) began to challenge Darwin's natural selection theory. In 1966, Suzanne Batra (born 1937) introduced the term *eusocial* (meaning "good" social) to describe the nesting behavior of certain species of bees. Batra observed that these bees, males and females alike, displayed social stratification that included cooperative, altruistic behavior during various brood raising tasks within the colony, and that these behaviors gave the group an advantage over competing groups. About the same time, E. O. Wilson (1929) mounted a formidable challenge to the prevailing belief that all behavior was genetic, based on observed altruistic behavior of ants. Wilson theorized that evolution actually tends to favor epigenetic environmental factors and traits, such as empathy, altruism and cooperation, over individual traits. Starting in 1994, E. O. Wilson, together with David Sloan Wilson (born 1949) and Elliot Sober (born 1948), began proposing that *amplification* of social instincts through small mutations in polymorphisms or alleles (*which importantly have to do with how the young are cared for*) has given certain species, including homo sapiens, a phenotypical competitive survival advantage (Sober and Wilson, 1998; Wilson, 2008a).

Due largely to advances in research technology, there was tremendous growth in scientific knowledge about the structure

and function of genes during the second half of the twentieth century, thus promising new ways to test genetic theories. Solving the structure of DNA in 1953 by James Watson (1928) and Francis Crick (1916–2004), opened a new era in gene research, and with it predictions that breakthrough treatments of emotional disorders were imminent. This excitement gained even more intensity in the early 1960's, when the description of nerve signals and synaptic transmission by Julius Axelrod led to a rapid acceleration of biochemical brain research on the effects of psychotropic agents on brain chemistry. Then, with development of DNA sequencing in 1977 by Frederick Sanger (1918–2013), excitement spread to the business world, which quickly monetized these discoveries. By 2000, however, the search for simple genetic explanations and/or brain mechanisms for emotional disorders, such as autism, began to stall as genetic makeup by itself could not account for behavioral variance and anomalies. The stall was driven in large part by a growing body of science that showed epigenetic factors, such as plasticity, played an important role in the etiology and regulation of emotional disorders (Agrawal, 2001; Gardener et al., 2011; Turecki and Meaney, 2016).

Aside from the excitement generated by the new genetic findings, research was revealing ways that genes can be modified behaviorally through experience. In his 1972 essay titled "Ethology," Tinbergen summarized a large number of studies that demonstrate ways in which innate behavior that is not changed by cognitive learning interacts with environmental experience to produce structured patterns of instinctive behavior (Tinbergen, 1972). Gilbert Gottlieb (1929–2006) showed that transgenerationally recurring prenatal sensory experiences are critical to learning during the prenatal and postnatal periods of development (Blumberg, 2017; Lickliter, 2017). Gottlieb's theory of *probabilistic epigenesis,* which states that there is no predetermined path to trait development, was the first serious challenge to both the Darwinian and Neo-Darwinian theories of natural selection (Gottlieb, 2002).

The first two decades of the current century have witnessed a sea change in the science of behavior, how the body communicates with and influences cognition and how the brain processes communications from the body to produce behaviors. Neuroscientist Joseph LeDoux popularized the term "Social Brain" (LeDoux, 1996) and identified two integrated sensory pathways to the amygdala, one fast and subcortical and the other slow and cortical (LeDoux and Brown, 2017). Neurobiologist Antonio Damasio mapped the neural systems that underlie emotion, decision-making, memory, language and consciousness. His somatic marker hypothesis (Damasio, 1996) describes how the biological underpinnings of emotions influence both positive and negative decision-making, often subconsciously. Many have worked to merge the latest findings in evolutionary biology as first noted by Darwin, with developmental biology (Kutschera and Niklas, 2004), as well as attachment theory (Lickliter, 2008). This new "evo-devo" field builds on work of SG Mivart (1827–1900), who was one of the first to recognize the limited role of natural selection (Chhetri, 2014). To explain how species evolved from one another, Mivart ceded natural selection as an environmental and efficient cause, but he proposed that instincts within the individual organisms are a formal "cause."

The even newer field of eco-evo-devo adds ecological considerations. This field examines the developmental processes of two organisms in symbiotic relationship to each other to uncover the ways that animal bodies evolved and function (**Figure 2**). This new field is studying genetic and environmental mechanisms that underlie the development of social and cognitive competencies, as well as the epigenetic (gene–environment interactions) processes that adapt these competencies to local conditions (Lickliter, 2017) (**Figure 3**).

Another relatively recent scientific breakthrough that has raised appreciation for the power of body functions to shape emotional behavior and cognition is the mapping of the human microbiome. Gut microbiota have been found to have significant effects on cardiac modulation (Polsinelli et al., 2017), mood regulation (Vitetta et al., 2014), immunologic, hormonal and metabolic homeostasis (Lach et al., 2018) and early infant development (de Weerth, 2017). At the same time, gut-brain vagal stimulation was introduced to remediate a wide range of medical, psychiatric and emotional disorders, including autism (Engineer et al., 2017) and G.I. disorders (Bonaz et al., 2018). In animal models of sepsis, the vagus nerve has been proposed to play a crucial role in the regulation of the immune response (Matteoli and Boeckxstaens, 2013). Gut vagal afferents differentially modulate learned fear and innate anxiety, adding further weight to theories emphasizing an important role of afferent visceral signals in the regulation of emotional behavior (Klarer et al., 2014). While microbiome research has shifted attention of science and the public to peripheral influences on behavior and health, for the most part microbiome and vagal signaling findings are still being interpreted within the orthodox theoretical brain-centric self-regulation paradigm that has prevailed since Darwin (Foster et al., 2017).

Since 2000, the ability to examine instincts, or innate behaviors, in humans has grown rapidly, due to several converging factors. Rising premature birth rates coupled with improved survival, now account for one out of ten births in the United States (Ambrose et al., 2015). The maternal separation required by intensive hospitalized care, along with the long-term emotional, behavioral and developmental consequences of these early births (Agrawal et al., 2018), have created opportunities for researchers to test theories about perinatal mother-infant innate behavioral mechanisms in a highly controlled environment. Yet, despite recent growth and diversity in scientific findings, the main medical assumptions that determine how socioemotional behavior is viewed or treated have evolved very little over the past 160 years. Innate behaviors are still regarded as individual and genetic in origin and therefore resistant to change. When symptomatic emotional behavior arises, it is often regarded as psychological in origin and is assessed within Bowlby's attachment construct and theory (Perry et al., 2017; Mikulincer and Shaver, 2018). The strategies for remediating symptomatic behavior involve various cognitive learning therapies and/or drug therapies targeting the brain. This current thinking and strategy, however, is increasingly criticized as not meeting growing worldwide mental health problems (Patel et al., 2018).

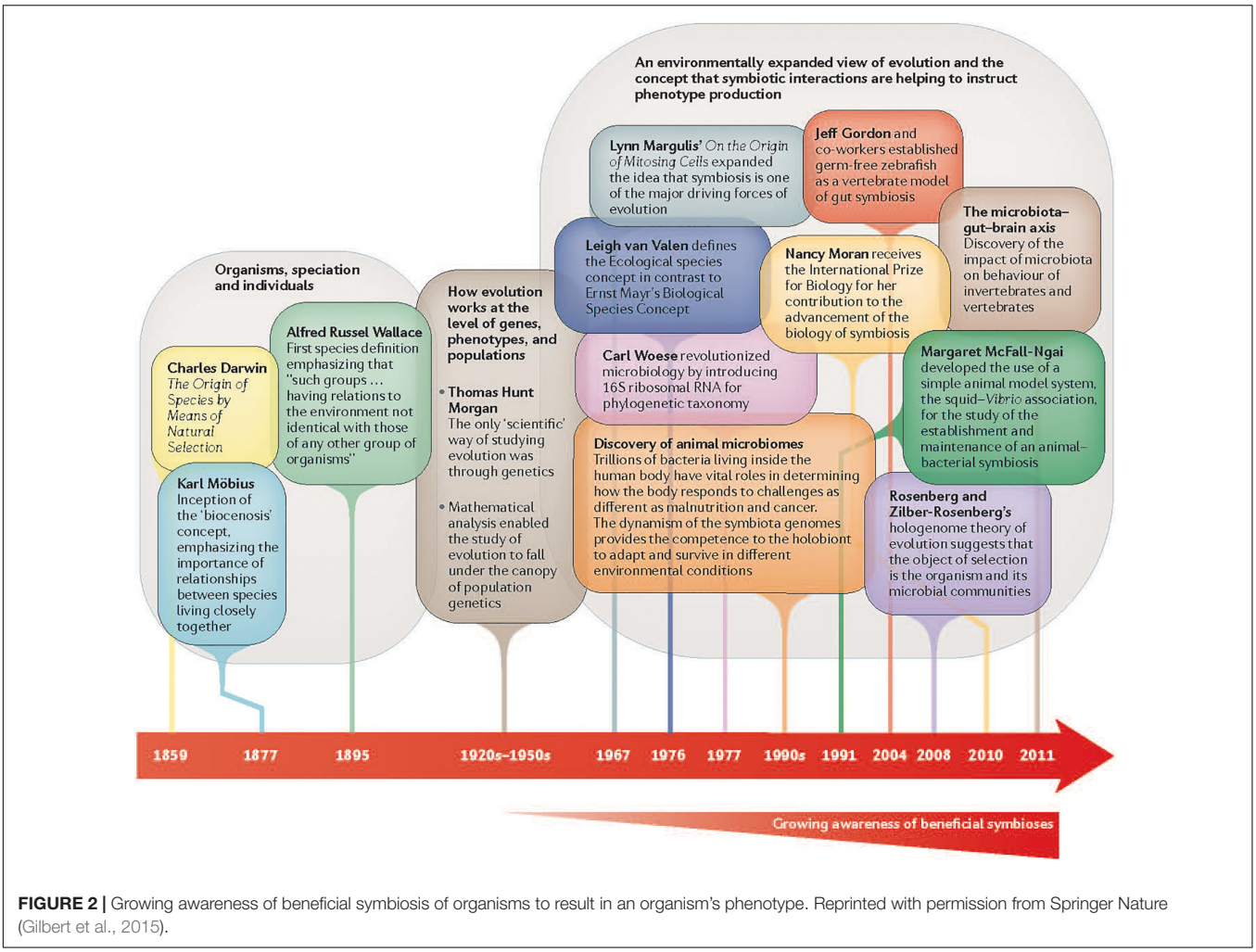

FIGURE 2 | Growing awareness of beneficial symbiosis of organisms to result in an organism's phenotype. Reprinted with permission from Springer Nature (Gilbert et al., 2015).

Attempts to link Attachment phenotypes to physiology have failed thus far (Barazzone et al., 2018). And, attempts to exploit brain mechanisms using pharmaceuticals and conventional behavioral interventions have proved costly and/or when effective, not scalable (Timlin et al., 2014; Feliu-Soler et al., 2018).

To summarize, there has been a vast amount of research in the past century increasing scientific knowledge about the brain and genes, accompanied by many medical and scientific "reductionist" breakthroughs. Multiple attempts to revise, repackage or reinterpret Darwin's original conclusions about instincts, based upon the assumptions that he himself was not entirely satisfied with, have yet to produce a simple testable hypothesis when it comes to the emotional behaviors that arise between mother and infant in the perinatal period.

PART 2: DARWIN'S CONTROL OF HEART RATE DILEMMA

Overview

While it was known since antiquity that heart rate could be inhibited or even stopped by stimulating the peripheral pneumogastric or vagus nerve without conscious input (Hoff, 1940), the phenomenon was a curiosity to physicians and physiologists. Descartes was among the many prominent scientists who offered theories on peripheral and central inhibition of the heart (Stuart et al., 2014), but the mechanism(s) were not yet understood (Hoff, 1936). This presented a particular problem for Darwin's theory of natural selection, which favors the behaviors of intellectually superior individuals. If evolution is influenced by conscious mechanisms, what is the role of peripheral and unconscious mechanisms? Darwin was greatly puzzled by the power of the unconscious actions of social instincts, especially in the newborn infant. He stated:

> "*Reflex actions*, in the strict sense of the term, are due *to* the excitement of a peripheral nerve, which transmits its influence to certain nerve cells, and these in their turn excite certain muscles or glands into action; *and all this may take place without any sensation or consciousness on our part …*" (Darwin, 1872).

The idea that something other than the individual's higher cognitive mechanisms could influence *behavior*, however, more or less preoccupied Darwin's thinking. He wrote, "*when movements, associated through habit with certain states of the*

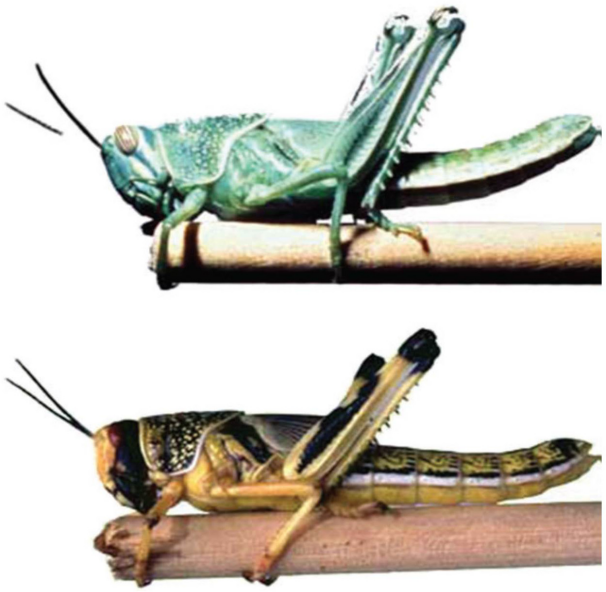

FIGURE 3 | Solitary and gregarious morphs of the desert locust. This dramatic and rapidly produced difference in both appearance and behavior is triggered by increased social stimulation. Reprinted with permission from AAAS (Anstey et al., 2009).

mind, are partially repressed by the will, the strictly involuntary muscles, as well as those which are least under the separate control of the will, are liable still to act" (Darwin, 1872).

Darwin could not provide answers to the dilemma of peripheral control of heart rate with any certainty and concluded his thinking with a challenge to physiologists. "*From these several causes, we may conclude that the philosophy of our subject* [i.e., the expression of emotions in man and animals] *has well deserved the attention which it has already received from several excellent observers, and that it deserves still further attention, especially from any able physiologist* (Darwin, 1872).

Attempts to Resolve Control of Heart Rate Dilemma

Prior to the publishing of Darwin's theory of natural selection in 1859, the understanding of heart rate control had advanced significantly, particularly with the work on *vagal inhibition* by the Weber brothers (Ernst, Wilhelm, and Eduard) and the discovery of *vagal arrest* by Claude Bernard (Hoff, 1940) in the 1840's. As a result, by the time Darwin published his theory, a new generation of physicians, physiologists and neurologists were beginning to dissect the influence of the central nervous system on the heart.

By 1850 early work had begun to reveal the workings of the central nervous system and encourage speculation on emotional behavior. In the 1860's Russian physiologist Ivan Sechenov (1829–1905) pioneered the use of electrophysiology, demonstrating that the brain is linked to cardiac inhibition through electric currents (Stuart et al., 2014). In 1864, John Hughlings Jackson (1835–1911) proposed that the nervous system is organized in a hierarchical manner, with the cortex at the top (Jackson, 1958). Neural functions that appear first in evolution, he argued, are the ones that appear first in development and the last to disappear in disease. New neural functions are layered onto the earlier functions over the lifespan through learning. These disappear through disease, trauma and aging in a last-in-first-out manner. Jackson called his process dissolution, which he borrowed from Herbert Spencer (Berrios, 2001). Jackson showed that lesions in higher brain centers inhibited the lower centers and caused negative symptoms whereas positive symptoms were caused by emergence of the lower, subconscious centers (i.e., brain stem and visceral relays).

William James (1842–1910), among the most influential thinkers of the late 19th century, differed with Jackson's conclusions, but like Jackson advocated for a *functional approach* to the study of emotions (James and Lange, 1922). Influenced by Darwin, James' theory of emotions stated that emotions are the mind's perception of physiological conditions that result from some stimulus, often internal. Danish neurologist Carl Lange (1834–1900) independently arrived at a similar theory but took the more radical position that vasomotor changes are actually *synonymous* with emotions (James and Lange, 1922). Echoing Darwin's first and second principles of emotional expression, the James–Lange theory held that emotions arise in physiologic conditions of the body and become encoded as emotional memory by the brain.

By the dawn of the twentieth century, Darwin's dilemma of *heart rate control* remained unresolved, with arguments falling into two distinct camps. One group of scientists (James–Lange, Pavlov) were focused on visceral control of emotional behavior and heart rate. The other group (Morgan, Wundt, Titchener, Jackson, Sherrington, Freud) favored theories that promoted cognitive control that were mostly in concert with Darwin. The field of Psychology was split between James and Lange's idea that emotional behavior stemmed from internal signaling to the brain and Freud's brain-based psychoanalytic theory. The Western and Russian schools of Physiology and Neurology were also split on the subject. Western researchers, influenced by Jackson and Sherrington, focused on the central nervous system and central excitation and inhibition. The Russian school, under Pavlov's influence, focused on the autonomic nervous system and peripheral inhibition.

The work of Ivan Pavlov (1849–1936) took a viscera-centric "*subconscious*" approach to the study of emotional behavior that was increasingly at odds with Western schools of thought. Pavlov and other Russian scientists openly criticized many of the scientific methods and arguments being used and promoted in Western Psychology as being subjective, vague or confused (Pavlov, 1932; Gantt, 1960). This theoretical and methodological conflict was exacerbated by the growing political and ideological divide between Russia and the West. As a result, Russian research on autonomic conditioning was increasingly ignored and portrayed as "old school," simplistic and obsolete (Rescorla, 1988).

Pavlov is best known for discovering his eponymous subconscious reflex conditioning mechanism, and for demonstrating the effects of that mechanism on salivation

and the digestive system. Less known is Pavlov's work on the study of conditional neurotic behavior in dogs, and the effects of Pavlovian conditioning on the autonomic nervous system and peripheral inhibition of heart rate (Pavlov et al., 1928; Gantt, 1965). Sherrington recognized that the spinal reflex is composed of integrated actions of the central nervous system involving the excitation and inhibition of many nerves, but Pavlov postulated that the origin of neurotic disturbances is principally through a collision or conflict between cortical excitation and subcortical inhibition. Pavlov, in concert with Carl Lange's theory of emotion (see above), came to believe that subcortical reflexes and social instincts are one and the same (i.e., that the subconscious learning mechanism underlying the cardiac conditional reflex is the same learning mechanism that underlies instinctive behaviors) (Pavlov et al., 1928).

The term "conditional reflex" as used here was defined by Pavlov (Gantt, 1968). Pavlov always employed the adjective modifier "conditional" to describe the response to a stimulus, never the past participle "conditioned," which implies being fixed. Conditional refers to the quality of the response and indicates that the response always varies with conditions (Gantt, 1968). With extreme clarity, Pavlov showed that under certain conditions the autonomic nervous system can play a dominant role in regulating emotions and inhibiting heart rate, through what he termed the "cardiac" or "social" reflex. He was the first to describe a phenomenon whereby the heart rate in dogs undergoing an emotional challenge can be significantly reduced by the physical presence of and contact with the dog's trusted handler. Pavlov termed this phenomenon "effect of person" (Pavlov et al., 1928).

In 1927, physiologists Walter B. Cannon (1871–1945) and Philip Bard (1898–1977) dramatically altered the study of emotions with their *Thalamic theory of emotions*, which they claimed overturned the viscera-centric James–Lange theory of emotions (Cannon, 1987). The James–Lange theory proposed that emotions are generated by the physiological conditions of the body and that emotional behavior results from two-way communication between the gut and brain (Welch and Ruggiero, 2005). The new Cannon–Bard theory proposed that emotions are patterns generated in the newly discovered thalamus and that emotional behavior results *entirely* from signals transmitted from the brain to the viscera (Welch and Ruggiero, 2005). This idea and most of Cannon–Bard's arguments were soon disputed. Nonetheless, Western science became increasingly focused on the central nervous system and higher brain function as the keys to understanding emotional disorders to the exclusion of peripheral and autonomic nervous systems.

The Russian school of physiology, under the leadership and influence of Pavlov, continued to examine conditional reflexes in the viscera and peripheral inhibitory influences on heart rate (Klimov, 1986). At this same time, Western physiologists were elucidating reflexes that evoke vagal discharge, most importantly change in blood pressure within the aorta and carotid sinus (Hoff, 1940). Neurophysiologist Sherrington described neuronal synaptic communication and demonstrated the importance of peripheral inhibition to the central nervous system. In his 1932 Nobel speech, *Inhibition as a coordinative factor*, Sherrington emphasized the problem of *peripheral* cardiac inhibition.

> "*The role of inhibition in the working of the central nervous system has proved to be more and more extensive and more and more fundamental as experiment has advanced in examining it. . .In the working of the central nervous machinery, [peripheral] inhibition seems as ubiquitous and as frequent as is excitation itself*" (Nobelstiftelsen, 1965).

Western scientists were turning their attention to the new more exciting "cortical" learning mechanism discovered in the late 1920's by two Polish medical students, Jerzy Konorski (1903–1973) and Stefan Miller (1903–1940) (Miller and Konorski, 1928), who argued that this mechanism was separate and distinct from Pavlovian conditioning (Miller and Konorski, 1928).

Skinner and Thorndike's operant conditioning and Law of Effect (aforementioned) and support after two influential publications in 1948 that reinforced a growing consensus in Western science about Darwin's puzzling over control of heart rate. First, Konorski published his monograph, *Conditioned Reflexes and Neuron Organization* (Konorski, 1948), that provided a theoretical cortical learning mechanism to control emotions through executive brain function. Second, Norbert Wiener (1894–1964) published his revolutionary book, *Cybernetics: Or Control and Communication in the Animal and the Machine* (Weiner, 1948), which reinforced John Hughlings Jackson's argument for a hierarchical nervous system, with the cortical brain in control at the top. Weiner's Control Theory essentially eliminated the boundary between man and machine, proposing that there is virtually no difference between the way machines and animals decide and control their actions. Both are determined by circular, causal chains, or feedback loops *within a closed system*. Behavior in humans, Weiner stated, moves from *action* to *sensing* to *comparison* with desired goal, and then to *corrective action*. The theories of Konorski and Weiner dovetailed with those of Skinner and Thorndike, and added to a postwar enthusiasm for all things cognitive, mechanistic and non-emotional. A breakthrough came in the understanding of heart rate when it was linked to respiratory rhythms by Hering in 1910 (Hering, 2013), however, the significance of this phenomenon was not clear.

By the 1940s, however, two other American behaviorists, B. F. Skinner (1904–1990) and Edward Lee Thorndike (1874–1949), were modifying the ideas of Watson and embracing Konorski's cognitive learning mechanism. Skinner believed that Pavlovian conditioning reflex was too simplistic to explain complex human behavior. He looked for the causes of an action and its consequences, calling his cognitive learning approach *operant conditioning* (i.e., changing behavior by following a desired response with reinforcement) (Skinner, 1938). This method followed Thorndike's *Law of Effect,* which states behavior that is reinforced tends to be repeated and behavior that is not reinforced tends to die out or be extinguished.

While many Western scientists continued to believe that Cannon and Bard had overturned James–Lange's visceral theory of emotions, physiological evidence was accumulating that

challenged many, if not all, of their arguments [e.g., that the viscera are too insensitive, uniform and slow to offer a satisfactory means of influencing emotions (Lang, 1994; Pessoa and Adolphs, 2010)]. At the same time, Pavlov's ideas about autonomic conditioning and studies on experimental neuroses in dogs had gained a foothold in the West. They were continued and advanced through the efforts of physiologist W. Horsely Gantt (1892–1980) at his Pavlovian research lab at Johns Hopkins Medical Center. Gantt had studied with Pavlov in Russia for many years and was translating Pavlov's work into English. In 1948, the same year Konorski and Weiner were helping build a scientific consensus around cortical learning and self-regulation, respectively, Gantt published a paper entitled *Physiological Psychology*. Sensing that the tide of argument was shifting toward the central nervous system, Gantt argued strenuously that, "*The role of the subcortical and segmental nervous system in mammals remains unsettled*" (Gantt, 1948). He argued that psychologists should maintain a holistic approach to the study of behavior by examining the physiology of the entire organism.

By 1950, the debate over whether the brain or the body controls our emotions had consolidated into an "orthodox" view that *emotional behavior* is self-controlled by higher brain regions within a closed neural system of feed-back signaling loops. Neo-Darwinism reached its pinnacle of expression in the *Modern Synthesis* of evolutionary biology by Julian Huxley (1887–1975) and others. The synthesis stated their position succinctly in three unequivocal tenets (Kutschera and Niklas, 2004): (1) Instructions for building organisms reside in genes; (2) Genes are the exclusive means by which these instructions are faithfully transmitted from one generation to the next; (3) There is no meaningful feedback from the environment or the experience of the organism to its genes.

Not all scientists, however, especially physiologists, agreed with this consensus. Horsley Gantt and his colleagues continued their experiments on peripheral inhibition of heart rate and the *effect of person* phenomenon (Gantt, 1972). Notable psychologists remained unconvinced, as well. Some were taking up Freud's challenge to seek biological mechanisms for his subconscious theories in fields other than Psychology. A new group of researchers, calling themselves developmental psychobiologists, echoing William James, believed that psychological processes have biological or physiological correlates. These researchers criticized behaviorism for focusing on externally observable behavior and not taking into account subconscious internal influences on behavior. Influenced by the work of Tinbergen, which codified the methods for studying instinctive behavior in the natural habitat (Tinbergen, 1951), psychobiologists such as Daniel S. Lehrman (1919–1972) were beginning systematic studies of the physiology underlying instinctive behaviors of the mother and infant in the early postnatal period (Silver and Rosenblatt, 1987).

The last half of the 20th century saw major advances in the understanding of the autonomic nervous system and control of heart rate. Whereas, psychophysiological research on autonomic cardiac inhibition associated with emotional behavior was previously tied to and influenced by sympathetic activity (Duffy, 1957), new research was beginning to challenge this idea, especially new discoveries into the function of heart rate variability and respiratory sinus arrhythmia. There was increased interest in "slow breathing" techniques that were practiced in Eastern disciplines to control heart rate (Zaccaro et al., 2018). New anatomical discoveries and new phylogenetic and functional insights on the vagus in emotional behaviors gradually emerged between the 1960 and 1995 (Sokov, 1963; Lacey and Lacey, 1978). These found their synthesis in the *Polyvagal Theory* of Porges (1995, 2011).

Polyvagal Theory emphasized that humans utilize two distinct vagal systems to control heart rate in response to threat or fear; a phylogenetically older reptilian system, and a more newly evolved mammalian vagal system. "The behavioral derivative of the two branches of the vagus," Porges wrote, "suggests a typology in which one branch of the vagus deals with unconscious reflexive functions and the other is involved in more conscious, voluntary, flexible, and often social activities" (Porges, 2011). The two neural pathways are theorized to regulate autonomic state and the expression of emotional and social behavior through a closed system of feed-back loops, with the newer mammalian vagus controlled via the cortex and the older vagus controlled via autonomic state (Porges and Kolacz, 2018; Porges and Lewis, 2018).

Importantly, Porges pointed out that strong emotion can "instantly" affect heart rate, independent of the spinal cord and the sympathetic nervous system, which in turn can affect the brain and behavior through afferent feedback signaling. In this respect, Polyvagal Theory advanced ideas first developed by Darwin and later by William James (Colzato et al., 2017). However, the theory does not fully resolve Darwin's dilemmas. The theory, for example, does not provide a mechanism to account for learned "unconscious reflexive functions." Nor does it provide a definition of instinctive behavior. Rather than fully resolving these questions, Polyvagal Theory provides a new expanded evolutionary framework within which to view heart rate and the role of the autonomic nervous system, and within which intriguing questions can be asked. For instance, can the "instant" direct heart rate phenomenon described by Porges be related to Pavlov's *effect of person* phenomenon?

Pavlov's *effect of person* phenomenon, as mentioned above, was being separately and extensively studied throughout the post-war years into the 1980's. Horsley Gantt and his collaborators at Johns Hopkins found the effect within and between multiple species, including humans (Lynch and Gantt, 1968; Gantt et al., 1991). Gantt tried to convince his peers that the "effect of person" phenomenon could have enormous implications for the treatment of emotional disorders (Gantt, 1972). He argued the phenomenon had particular relevance to heart rate control, since his research had shown conclusively that contact with a trusted other person can have profound "instant" inhibitory effects on the spike in heart rate in the face of emotional challenge.

Gantt's findings pointed to a radically different view of heart rate control. In matters of deep emotional challenge, Gantt's team showed that heart rate is not solely controlled by conscious control within an individual closed system. Rather, heart rate is subject to a subconscious conditional reflex formed between an individual and a trusted other. Gantt came to believe that

heart rate of the individual is subject to a subconscious calming reflex during physical contact with a trusted other. Interestingly, Darwin was familiar with the *effect of person* phenomenon. He related a story told to him by his father, a physician, about the exceptional calming effect that he himself had on a patient with heart disease. The patient complained that his pulse was habitually irregular to an extreme degree, *except when Darwin's father entered the room*, whereupon he was calmed and his heart rate invariably became regular (Darwin, 1872).

The growing field of epigenetics is beginning to challenge some of the prevailing ideas about instincts and evolution. New insights from sociobiology are expanding understanding of complex behavior systems in animals and insects, and discovering that Darwin's social instincts – altruism and cooperation – actually provide an advantage for the survival of some groups. While intriguing, these advances in knowledge raise as many questions as they do answers. In the past two decades, advances in computer science, technology and data analysis have made it possible to examine body-wide developmental systems and the physiological relationships between mother and infant in ways never before possible, bringing us closer to the ability to understand how peripheral control of heart rate relates to innate behavior or to the mechanisms involved.

PART 3: PROPOSED RESOLUTION OF DARWIN'S DILEMMAS

Whether the aforementioned theorists agreed with Darwin's theory of emotion or not, most sought to understand emotional behavior and provide a biological mechanism to account for it, especially during the perinatal period. Most tried to account for so-called phenotypical variation that occurs in infant and early childhood behavior. Why does one infant or child display pro-social behavior and another anti-social behavior? Is such phenotypical behavior fixed, or can it change? If phenotypical behavior can be changed after birth, what biological mechanism(s) account for the change?

We now critically review three currently influential theories on emotional behavior and review two theories that offer a new resolution to Darwin's instinct and control of heart rate dilemmas.

Attachment Theory

It was in response to the questions outlined above that Bowlby developed his attachment construct in response to government and social service organizations' struggle to find a practical solution to antisocial behaviors displayed by large numbers of children who had been separated from their families during WWII. The construct was designed to describe a system of behavior that could be acted upon in order to change antisocial to social behavior. As pointed out above, Bowlby believed that he had created a new way to describe the behavior of infants and children that was in line with biological science at the time. As Mary Ainsworth put it,

The great strength of attachment theory in guiding research is that it focuses on a basic system of behavior, the attachment behavioral system, that is biologically rooted and thus species-characteristic. This implies a search for basic processes of functioning that are universal in human nature, despite differences attributable to genetic constitution, cultural influences, and individual experience (Ainsworth, 1989).

Bowlby, however, was not a basic scientist. He relied upon scientific theories and evidence of others at the time to provide the mechanism underlying attachment behaviors. The practical problem of finding a way to identify and measure the attachment behavioral system fell to Ainsworth (1969), a developmental psychologist, who created a system of identification. Ainsworth originally described three attachment types, *Secure, Insecure-Avoidant* and *Insure-resistant*, to which *Insecure-disorganized* was later added by others (Benoit, 2004) (see **Table 1**).

Today, Bowlby's attachment construct and Ainsworth's system for coding attachment behavior are the accepted method by which the majority of research psychologists categorize the behavior of infants and children. Bowlby's attachment construct describes an individual system of behavior separate from the mother. It states that the mother/child relationship does not have to be reciprocal and that the mother should serve as a secure base from which the child moves off according to the child's priorities (Bowlby, 1969). However, the attachment field has struggled to associate the categories with physiological correlates in order to provide a simple explanation of the differences of the four attachment behaviors. Mechanisms, when they are proposed are apt to be behavioral (Moretti et al., 2015), or cognitive-based and complicated (Laurita et al., 2017).

While there have been notable recent attempts to revise attachment theory to align with growing evidence that challenges it (Schore, 2005; Lickliter, 2008; Feldman, 2017), the attachment construct remains firmly grounded in the assumptions that emotional behavior is controlled within the individual organism through self-regulation and subject to cortical control. Brain imaging studies are seeking to associate brain function with attachment style (Pratt et al., 2018), or by dropping the heralded four types of attachment and simply dividing subjects between secure and insecure attachment (Saunders et al., 2015). Interestingly, researchers looking at mechanisms in the neonatal period have begun focusing on the association between cardiac function and attachment (Sancho-Rossignol et al., 2018). Nevertheless, the attachment construct has failed to deliver a practical tool for clinicians (Flores et al., 2018; Valikhani et al., 2018). Attachment coding methods require substantial training, are time-consuming and costly to administer, and are not scalable as a clinical tool.

TABLE 1 | Attachment styles and their origin.

	Secure	**Insecure**		
		Avoidant	**Resistant**	**Disorganized**
Caregiver's Style	Loving	Rejecting	Inconsistent	Atypical
	↓	↓	↓	↓
Offspring's Coping Strategy	Organized	Organized	Organized	Disorganized

Whether one agrees or disagrees with this critical assessment of attachment theory, one must conclude that the attachment construct has not been the breakthrough on instinct that Bowlby heralded it would be in 1960. Nor has Bowlby's theory fulfilled his promise to answer Freud's call for a mechanism underlying emotional behavior. Importantly, as Hofer and others noted, attachment as a unique motivational system has not generated a simple testable hypothesis (Hofer, 2006). In any case, Bowlby's reliance on analytical biology, gene theory and control theory has produced an increasingly complex and opaque view of the basic principles that govern mother-infant emotional behavior.

In **Figure 4**, on the left side, we trace the theoretical path that led from Darwin to the attachment construct. Note that this path is dominated by *psychology and research focused on the central nervous system*, leading to the attachment construct. In contrast, on the right side, we trace the theoretical path to the emotional connection construct and calming cycle theory, a path dominated by *physiology and research focused on the autonomic nervous system* (see **Table 2**).

Evolutionary Game Theory

Evolutionary game theory provides an explanation for Darwin's social instincts, by identifying how issues of cooperation and defection can profoundly affect strategies of survival. This epigenetic process played out over multiple generations is theorized to favor kin relationship and, therefore, introduces the idea of inclusive fitness, which includes an individual's offspring as well as any offspring equivalents found in kin. In this way, the genetic makeup of the species is determined by repeated games of competition for survival, which happen to include the traits of altruism and cooperation usually found within kinship.

We see several limitations of evolutionary game theory and indirect reciprocity regarding the theoretical problems posed by Darwin's writings on emotional behavior. First, the application of equations from mathematics and physics to problems in biology *per se* is subject to limitations of reductionist theories in general. Gyorgy Szabo summarizes this limitation at the end of his impressive and exhaustive review of evolutionary game theory (Szabo and Fath, 2007), "Unfortunately, systematic investigations are strongly limited by the wide variety of microscopic rules and the large number of parameters." Reducing biological processes to mathematics and equations is an activity no doubt valid for some applications. But, at a certain point, the process fails to have practical relevance to infants.

In the context of our review, evolutionary game theory fails to solve the most fundamental question that baffled Darwin regarding perinatal social instincts. Where do instincts come from and how are they preserved evolutionarily? Aside from considerations of evolution, these are questions that have special current relevance and urgency in the field of neonatology, where prematurely born infants suffer disproportionately from emotional and behavioral disorders.

Evolutionary game theory purports to consider behavioral and biological mechanisms, but to our knowledge the field is mostly concerned with how populations evolve at the genetic, individual and group levels, by investigating the strategic mechanisms involved in various competitions for resources between organisms. The theory is less concerned with how to intervene in a particular "competition" in order to change the outcome. On this matter, the theory is agnostic.

We in no way mean to demean or slight evolutionary game theory or indirect reciprocity. There are many fields and applications that have benefited and will continue to benefit from the theories. Even the fields of human behavior and emotions have been enriched by the theories. However, all the theories on emotions from Darwin to present, including evolutionary game theory, have failed to produce a simple testable hypothesis for a biological mechanism that can account for the origin of social instincts in the perinatal period, or to provide an intervention that can overcome maladaptive behavior when it arises.

Polyvagal Theory

Polyvagal theory, originally conceptualized by Stephen Porges, has increased understanding of vagal inhibition of heart rate and advanced new ways of assessing the role of the autonomic nervous system in emotional behavior, and provided a system to measure autonomic health in the individual (Kirby et al., 2017; Mulkey and du Plessis, 2019).

Importantly, Porges' theory is supported by recent clinical innovations that take advantage of a *vagal mechanism* that utilizes an *inflammatory reflex* (Tracey, 2002), building on Pavlov's research on the cardiac reflex. The growing field of vagal nerve stimulation is drawing attention to the powerful influences of the autonomic nervous system on the control of inflammation and hormone release (Reyes-Lagos et al., 2018), including oxytocin (Kenkel et al., 2014).

In our effort to fully explain instinctive behavior and control of heart rate, we are guided by ethologist Niko Tinbergen. To understand any behavior fully, Tinbergen proposed that we need to answer four questions (Tinbergen, 1963):

(1) What is the *evolution or phylogeny* of the behavior, i.e., how might it have arisen?
(2) What is the *adaptive value* of the behavior, i.e., how does it impact the animal's chances of survival and reproduction?
(3) What is the *causation or mechanism* of the behavior, i.e., what stimuli elicit the response, and how is it modified by learning?
(4) What is the *development or ontogeny* of the behavior, i.e., how does it change based on genetic and epigenetic factors?

Polyvagal theory answers question 1, how the mammalian polyvagal system evolved to modulate emotional behavior and control of heart rate (Porges, 1995), and question 2, what the adaptive value of the vagal system is (Porges, 2011).

As Porges points out, modulation of heart rate and the *social engagement system* matures before birth during the third trimester of gestation, when the myelination of the fetal vagal system matures.

When vagal tone, through myelinated vagal pathways, to the [cardiac] pacemaker is high, the vagus acts as a restraint, or brake, limiting the rate at which the heart can beat and functionally calming the individual. When vagal tone to the pacemaker is low,

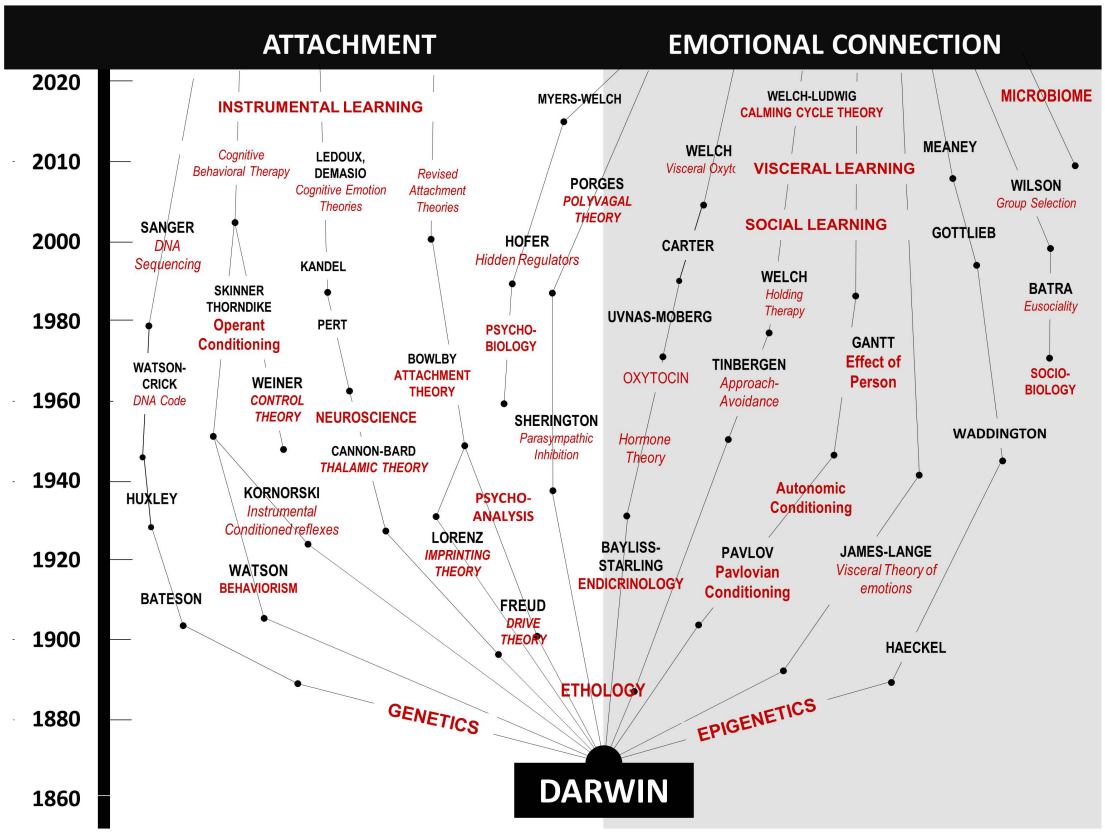

FIGURE 4 | A diagram showing the contrasting development of theories related to the dilemmas of instincts and control of heart rate since Darwin. On the left, are those theories leading to the behavioral construct of attachment. On the right are theories leading to the behavioral construct of emotional connection. The two sides highlight scientific arguments and theoretical conflicts in multiple fields, as outlined in **Table 2**.

TABLE 2 | Comparison of theories supporting attachment and emotional connection by category.

Area of Interest	Attachment	Emotional Connection
Behavior	Psychiatry, neuroscience, ethology *Freud, Bowlby*	Physiology, endocrinology, sociobiology, microbiology, ethology *Pavlov, Porges, Welch*
Cell signaling	Central nervous system *Sechenov, Sherrington, Kandel*	Autonomic nervous system *Sherrington, Porges, Welch*
Learning mechanism	Operant conditioning *Konorski, Skinner, Thorndike*	Pavlovian conditioning *Pavlov, Gantt, Welch-Ludwig*
Evolution	Genetic *Bateson, Sanger*	Epigenetic *Haeckel, Gottlieb, Meaney*
	Natural selection *Darwin, Smith*	Group selection *Batra, Sober and Wilson*
Control of Emotions	Self-regulation, Cognitive *Canon-Bard, Skinner, Thorndike, Weiner, Damasio, Ledoux*	Co-regulation, Visceral *James–Lange, Pavlov, Gantt, Welch*
Instincts	Genetic, Inherited *Freud, Bowlby*	Environmentally Learned, epigenetics *Waddington, Tinbergen, Gottlieb, Meaney*
Control of Heart Rate	Cortical – central nervous system *Sechenov, Sherrington, Damasio, Ledoux*	Sub-cortical – parasympathetic nervous system *Morgan, Pavlov, Porges*

there is little or no inhibition of the pacemaker, and the heart rate increases. The vagal brake construct may be used to describe functional modulation of heart rate by myelinated vagal efferent pathways (Porges, 2011).

Vagal tone can be used to determine health. Heart rate and the amplitude of respiratory sinus arrhythmia (RSA) are subject to direct vagal mechanisms. However, there are situations in which the measures appear to reflect independent sources of neural control. This paradox in the data remains the subject of debate among physiologists. The paradox is described thus: Increased vagal tone can produce neurogenic bradycardia. Decreased vagal tone produces suppression of RSA. However, bradycardia can occur during periods of suppressed RSA.

This vagal paradox presented by the physiologists' data had broad clinical significance. If vagal tone is a positive indicator of fetal or neonatal health when monitored with RSA, then why is vagal tone a negative indicator of health when it includes bradycardia? This paradox provided the stimulus for the development of Porges/polyvagal theory, which identifies the relationship between visceral experiences and parasympathetic vagal control of the heart (Porges, 1995).

Porges lists five explanations for the vagal paradox, all based upon the assumption that there is a single common source of cardiac vagal tone:

(1) RSA and average heart rate (during sympathetic blockade) reflect different dimensions of vagal activity.
(2) RSA is being confounded by respiratory frequency and tidal volume.
(3) Variation in quantification methods may contribute to the divergence between RSA and heart rate.
(4) RSA does not reliably measure parasympathetic tone because it decreases with baroreflex stimulation.
(5) Average heart rate is influenced by a complex and dynamic interaction between sympathetic and vagal systems, making it difficult to extract a vagal tone dimension.

Alternative explanations proposed by polyvagal theory, also based upon a single source, suggest that cardiac vagal tone may vary from person to person and according to varying conditions. However, a convincing explanation for the vagal paradox has not emerged based on the assumption that cardiac vagal tone derives from a single source.

Calming Cycle Theory

Having reviewed efforts to resolve Darwin's dilemmas of instincts and control of heart rate with the assumption that emotional behavior is self-regulated in a top-down fashion within a closed system of feed-back loops, we will now propose a resolution based upon a new set of assumptions. This work came out of Welch's aforementioned clinical insights in the 1970s through the 1990's, and summarizes concepts that emerged from basic and clinical research in the BrainGut Initiative and the Nurture Science Program at Columbia University Medical Center over the past twenty years.

Calming cycle theory is first and foremost a *theory of change*. It provides the answers to Tinbergen's question 3 by explaining the causation and mechanism of so-called instinctive behavior in the perinatal period and how the vagal system works mechanistically on heart rate to produce instinctive behaviors (Welch, 2016; Welch and Ludwig, 2017a). Calming cycle theory also provides the answer to Tinbergen's question 4 by explaining the development or ontogeny of instinctive behavior and how phenotypical maladaptive behaviors can be changed, based on facilitated interactions between mother and infant (Welch and Ludwig, 2017b).

Calming cycle theory advances understanding of the vagal system in humans. For instance, the theory proposes a novel explanation for the *vagal paradox* by proposing a second and separate source of regulation of infant cardiac vagal tone; *the mother*. According to calming cycle theory, vagal tone and heart rate are *co-modulated* within an *open co-regulatory feedback system* between mother and fetus/infant (Welch, 2016). In a normal and healthy gestation, an autonomic reflex is created during gestation that co-regulates, among other physiological processes, heart rate. Autonomic co-conditioning continues after birth, producing a co-regulatory parasympathetic cardiac calming reflex. Importantly, perinatal physiological co-regulation is conditional upon emotional connection (see below), which ensures that mother and infant seek proximity to one another after birth.

Our proposed solution to Darwin's heart rate dilemma is consistent with the large amount of evidence accumulated by Pavlov and Gantt documenting the cardiac or social reflex and the *effect of person,* as cited above. Animal research has shown that normal mother–infant interactions are associated with autonomic regulation. For instance, physiological co-regulation has long been viewed as inherent to optimal mother–infant interactions (Winberg, 2005). Other researchers, such as Uvnas-Moberg et al. (1987) and Hofer (2006), found that stimuli emanating from the infant also serve as regulators of maternal physiology. Prior vagal research may also support this model. For instance, Porges et al. (1996) demonstrated that infants with difficulty decreasing vagal tone (the vagal brake) during social/attention tasks at 9 months had more behavioral problems at 3 years of age.

In healthy relationships, proximity of mother and infant activates a parasympathetic calming reflex that results in higher vagal tone (Field and Diego, 2008). These proximity-seeking behaviors are what have traditionally been associated with instinctive or innate behaviors. We hypothesize these mother/infant proximity-seeking behaviors are the result of Pavlovian co-conditioning of the cardiac calming reflex that occurs in utero. Accordingly, instincts are not inherited, but are environmentally shaped in utero and continue to be environmentally shaped postnatally.

According to calming cycle theory, the ability to suppress vagal tone to attend to one another (social/attention tasks) is conditional on emotional connection or the lack of connection. Lack of emotional connection (i.e., through physical or emotional separation) produces various *self-regulatory* mechanisms of mother and infant and devolves into dysregulation. According

to calming cycle theory, the vagal paradox data reflects a break in co-regulation, which produces a *dysregulated autonomic state*. Such a dysregulated state can be resolved through co-regulating contact with the mother. This hypothesis that can be easily tested, as shown below.

According to calming cycle theory, the mechanism underlying instinctive or innate behavior can be summarized with these four postulates:

Postulate 1. Subcortical Pavlovian co-conditioning of the autonomic nervous system of mother and infant during gestation leads to a perinatal calming reflex and to emotional connection.

Postulate 2. Autonomic calming reflexes between mother and infant are activated upon contact and result in positive socio-emotional behaviors (Welch and Ludwig, 2017a).

Postulate 3. The autonomic calming reflex can be broken or interrupted by physical and/or emotional separations between mother and infant during the perinatal period, producing emotional disconnection and negative socio-emotional behavior.

Postulate 4. Disrupted or maladaptive autonomic behavioral reflexes can be counter co-conditioned through regular and repeated mother–infant calming interactions, or calming cycles.

We do not mean to say that there are no cortical influences on these processes. Sensory processing by the cortex, amygdala, and many brain circuits are inherently involved in certain aspects of behavioral regulation. As Gantt showed, however, the cognitive and autonomic systems function separately (Gantt, 1953). We posit that anomalies in behavioral (and vagal) characteristics seen during the perinatal period, including those viewed as pathological and often attributed to disorders of cortical/cognitive mechanisms, are in fact disruptions of subconscious, autonomic mechanisms.

Recent evidence supports our proposed calming cycle theory. Randomized controlled trials of Family Nurture Intervention (FNI) in the neonatal intensive care unit (NICU) tested a theory of change based on calming cycle and emotional connection theories (see **Figure 5**). In the trials, infants born at 26–34 weeks postmenstrual age were randomly assigned to one of two groups; standard care alone or standard care plus FNI (Welch and Myers, 2016). FNI is narrowly focused on re-establishing an emotional connection between the mother and infant after traumatic separation caused by premature birth and NICU isolation, while the infant is still in the hospital. This strategy hypothesizes that repeated facilitated calming sessions between mother and infant in the NICU aimed at emotional connection will co-condition an adaptive autonomic reflex in both the mother and infant in response to contact with one another (see **Figure 6**).

The first FNI-NICU trial and calming cycle study (clinicaltrials.gov, NCT01439269) subsequent ongoing multi-site trial (clinicaltrials.gov, NCT02710474) and an effectiveness trial (clinicaltrials.gov, NCT02710474) are important for two main reasons. First, they have provided evidence that supports calming cycle and emotional connection theories, i.e., significant short and long-term outcomes in infant/child and mother across multiple domains (Welch et al., 2014b, 2015, 2016; Hane et al., 2015). Second, they are providing the opportunity to test our theories about instinctive behavior and control of heart rate in a human population at high risk for life-long socioemotional disorders. These hospital-based trials are arguably the most rigorous and comprehensive studies of any intervention between mother and infants in the NICU to date. Analyses of data through 5-years follow-up in the first trial are ongoing.

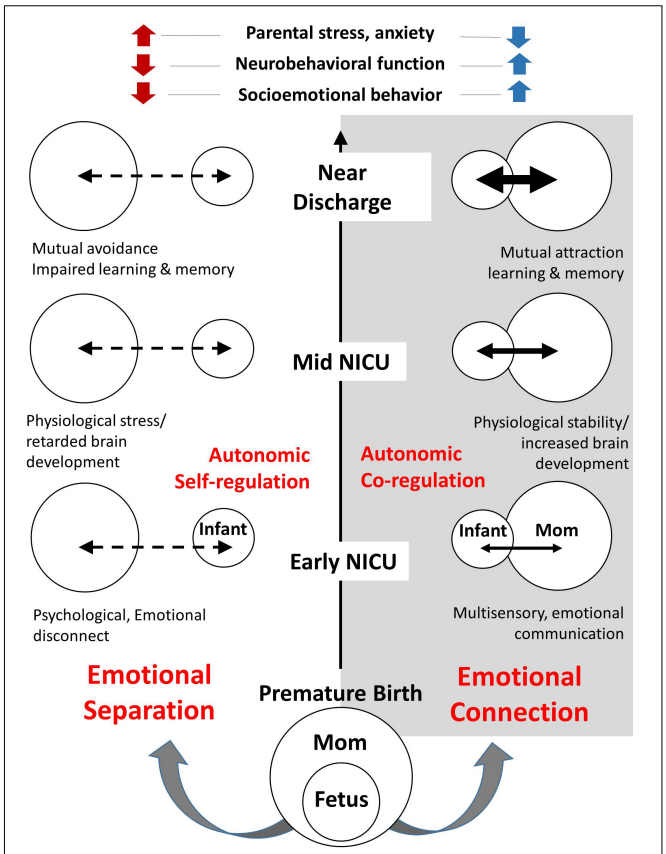

FIGURE 5 | Schema showing the positive effects of facilitating and maintaining emotional connection between mother and infant in the NICU (SC = standard care; FNI = standard care plus Family Nurture Intervention).

In collaboration with Porges et al. (2018), we tested the hypothesis that facilitating repeated calming sessions will lead to improved autonomic function in the infant. Analyses of the FNI-NICU trial data showed that FNI significantly enhances infant autonomic regulation after 2 to 6 weeks of FNI in the hospital (Porges et al., 2018), compared to infants receiving standard care. In the study, electrocardiograms (ECG) were collected for approximately 1 h during sleep at two time points, approximately 35 and 41 weeks postmenstrual age. Heart rate and RSA were quantified from the ECG. Across the two time points, the FNI group exhibited greater increases in RSA and slope between RSA and heart rate, a measure of vagal efficiency. FNI infants demonstrated enhanced autonomic regulation consistent with greater maturation of cardiac function (**Figure 7**).

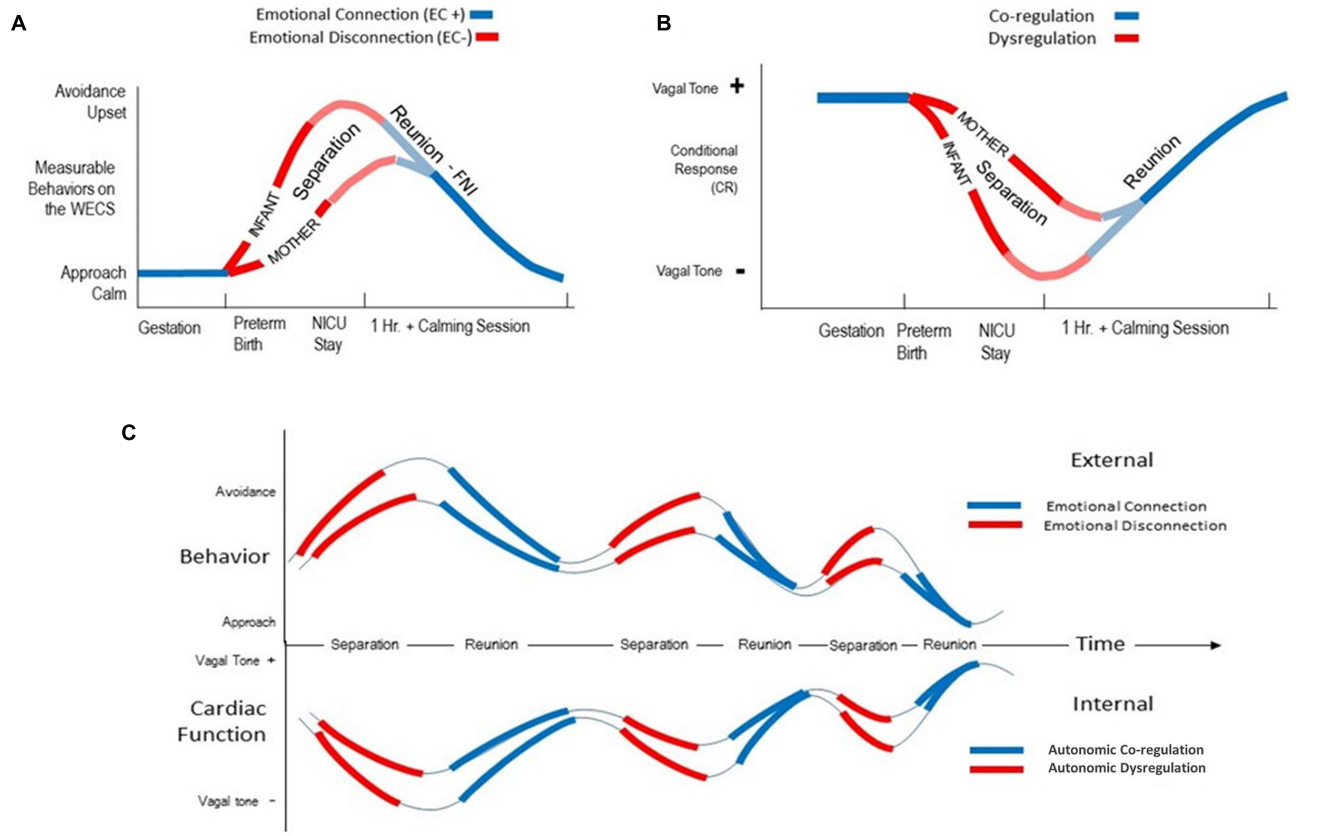

FIGURE 6 | Hypothesized relationship between perinatal behavior and cardiac function. Panel **A** depicts dyadic behavior over time in the NICU. Traumatic separation results in a break in emotional connection. Calming session restores dyadic calming. Panel **B** depicts the hypothesized autonomic cardiac function over time in NICU. Panel **C** depicts behavior and cardiac function together. The hypothesized calming cycle over time conditions the positive cardiac reflex and leads to emotional connection.

These cardiac findings are significant for several reasons. Prematurely born infants have maturational delays in several neurobehavioral systems, which negatively impact long term emotional, behavioral and developmental outcomes (Baron et al., 2012). The fact that a significant change in infant autonomic regulation occurred following a relatively small amount of intervention, an average of 6 h of facilitated calming sessions over an average 7 weeks, is remarkable. It suggests that FNI is acting directly upon a powerful biological mechanism.

In another analysis of FNI-NICU data, we tested the hypothesis that the emotional connection between mother and infant in the NICU intervention would lead to improved socioemotional behaviors between mother and infant at 4 months using the still face experiment, first developed by Tronick et al. (1978). Beebe et al. (2018), performed microanalysis on FNI trial data and provided evidence that supports the hypothesis. FNI dyads displayed more attraction behaviors and more sensitivity, compared with controls. In addition, the data supports the proposed mother-infant subcortical co-regulatory mechanism. The study found that at 4 months, importantly, the millisecond reaction-times of eye contact between mother and infant strongly suggest that the behaviors are not cortical in origin. The timing of the reactions indicate that they may be the result of LeDoux's faster sub-cortical signaling network (LeDoux and Brown, 2017), as predicted by calming cycle theory (Welch, 2016; Welch and Ludwig, 2017a,b), as well as by Polyvagal Theory (Porges, 1995; Porges et al., 1996).

Apart from clinical trials, we have conducted basic research based on calming cycle theory. The translational work of the BrainGut Initiative and the Nurture Science Program has elucidated the function of oxytocin in the gut, which has been consistent with the proposed neuroendocrine co-conditioning mechanism. Oxytocin has long been associated with perinatal physiology of both mother and infant (Welch and Ludwig, 2017a). We were the first to demonstrate the presence and functions of the oxytocin receptor in the gut epithelium and in the neurons of the enteric nervous system, in both of which the oxytocin receptor is developmentally regulated (Welch et al., 2014a). Welch also showed the efficacy of treating peripheral inflammation with "peptides of nurture," a combination of oxytocin and secretin (Welch et al., 2010) and showed the downstream effects of oxytocin/oxytocin receptor signaling in gut and brainstem (Klein et al., 2017,

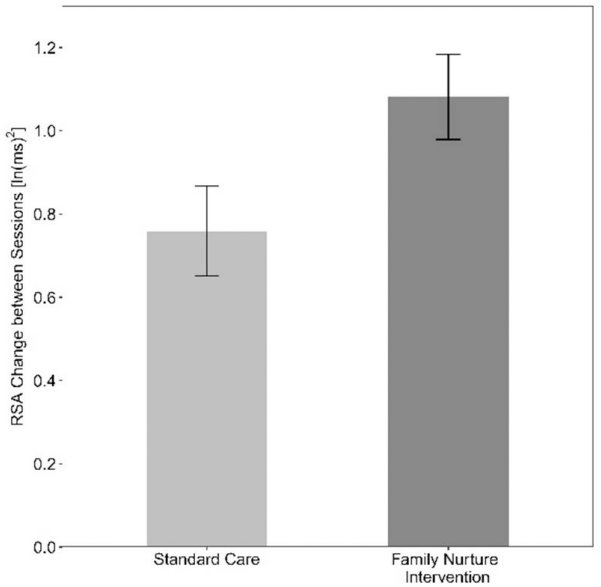

FIGURE 7 | As the infants approached term, increases in RSA were significantly greater in infants receiving FNI compared to the SC group ($p = 0.031$; Cohen's $d = 0.38$; error bars represent standard error).

2018). In addition to its many other effects, oxytocin has been shown to enhance approach behaviors (Spengler et al., 2017). Also, gastric neurohormones have been shown to affect vagal function (Chu et al., 2013; de Lartigue, 2014). Taken together with the literature, this body of work points to a complex mechanism by which mother-infant calming may be co-regulated by visceral/autonomic function, neurohormones in dyadic interactions.

Emotional Connection Theory

We have proposed a new behavioral construct – *Emotional Connection* – that is distinct from other currently accepted constructs. The construct describes emotional behavior between mothers and infants/children (Welch, 2016; Welch and Ludwig, 2017a,b). We have demonstrated that the emotional connection construct is quickly measured and immediately actionable (Hane et al., 2019). Analogous to the force between two magnets, emotional connection describes the force that attracts two individuals to one another and keeps them together. A polar force, absence of emotional connection, holds the two apart.

Emotional connection is distinct among current behavioral constructs in that it is a two-way phenomenon in which reciprocity is an absolute requisite. Emotional connection is also behaviorally and physiologically distinct in that it describes an *open feedback system* of behaviors and physiology between mother and infant. The eco-evo-devo examination of symbiosis (see **Figure 2** above) supports our view that symbiotic emotional connection and autonomic co-regulation between mother and infant determines phenotype. By changing a mother/infant relationship from weak emotional connection to strong emotional connection, the phenotype can be changed through repeated calming sessions, as described above.

The emotional connection construct has long been is supported by ethologist's observations of approach and avoidance behaviors in animals (Tinbergen, 1951). Schneirla and Rosenblatt (1961) further researched and reported on the phenomenon. It was Tinbergen's work on approach-avoidance that inspired Welch's clinical insights. The *Welch Emotional Connection Screen (WECS)* was developed to assess emotional connection (Hane et al., 2019). Behaviors displayed during close physical face-to-face proximity, i.e., eye contact or gaze aversion, physical attraction or avoidance, vocal cooing and soothing or distress, reciprocal responsiveness, can be used to determine mutual or impaired emotional connection between a mother and infant.

The emotional connection construct provides a new and simple way to view the mother/infant–child relationship and understand the mechanisms that underlie it. It has practical relevance to neonatologists, pediatricians, therapists and others in the healthcare field. Together with calming cycle theory, the emotional connection construct is actionable in that it points to ways to improve outcomes for infants, children and families. The calming cycle interventions provide an effective parenting tool for families to promote adaptive socioemotional development and optimal behavior throughout childhood within the family structure.

For researchers, the advantage of the emotional connection construct is that emotional behaviors assessed on the WECS are associated with measureable autonomic states and cardiac function that are directly associated with autonomic state (see **Figure 6**) (Hane et al., 2019). In this sense, the construct provides a clear window to the physiology underlying emotional behavior that is simple and measureable.

Prior to the ability to study fetal growth in prematurely born infants Darwin and many of his followers concluded that instinctive behavior must be genetic and inherited. However, evidence has been accumulating for the last century that the intrauterine environment profoundly influences fetal learning. As early as 1907, physician and surgeon Byron Robinson showed that fetal learning does not necessitate adult-like brain organization or even cortex (Robinson, 1907), a fact more recently elucidated by our collaborator, Michael D. Gershon, in his book *The Second Brain* (Gershon, 1999).

For instance, it has been proposed that the fetus encodes memory via subcortical networks and a mechanism that controls reflex-type reactions and the early emotional behavior of the infant (Dirix et al., 2009), and that fetal memory may have adaptive value (Hepper, 1996). Chemosensory stimuli occurring during gestation can promote the acquisition of long-term memories, which can affect behavior in the adult. These findings suggest that some likes and dislikes (e.g., a preference for licorice) expressed in adulthood can result from exposure (conditioning) in utero (Gruest et al., 2004). Finally, recent evidence suggests that innate anxiety and learned fear are both subject to modulation through abdominal vagal afferents (Klarer et al., 2014), adding further weight to theories emphasizing an

important role of afferent visceral signaling in the regulation of emotional behavior.

Using data from the FNI trial, the Welch Emotional Connection Screen (WECS) was validated by Hane et al. (2019). In this study, WECS maternal scores were positively associated with maternal sensitivity and quality of vocal contact at 36 weeks (caregiving) and maternal positivity at 4 months (face-to-face). WECS infant scores of face-to-face interaction videos at 4 months positively correlated with infant social engagement and maternal positivity, when compared to scores obtained with observed behavior tracked in real time with frame-by-frame analysis. Coding the same videos with a separate set of blinded coders, infants from dyads assessed not emotionally connected displayed autonomic dysregulation and less approach-seeking behavior toward mother during interactive/play sessions of the still-face paradigm, when compared to dyads assessed emotionally connected. In a study of 6-month old full term infants, emotional connection assessed by the WECS predicted 3-year old behavior as measured on the Child Behavior Checklist (Frosch et al., 2019). Taken together, these data provide validation of the emotional connection construct and support the theoretical foundation of the WECS as a valid instrument for assessing emotional connection in preterm mother–infant dyads. The results also strongly suggest that emotional connection is a behavioral mirror of the dyad's co-regulatory autonomic response to contact with one another (**Figure 5**).

The WECS assessment tool is the first instrument to correlate behaviors with the internal autonomic state. Importantly, in the Hane et al. (2019) study only some of the behaviors coded by the Ainsworth coding system, such as attraction and sensitivity, mapped onto behaviors coded by the WECS. The majority of the Ainsworth scales measured behaviors associated with higher order/cortical function. As aforementioned, Gantt showed that cortical and autonomic behaviors are distinct and separate (Gantt, 1953; Dykman and Gantt, 1997).

Together, the FNI-NICU trial data support the emotional connection and calming cycle theories. They also support a new theory of change of instinctive behavior in the NICU to optimize postnatal development of autonomic regulation and neurobehavioral outcomes in preterm infants.

CONCLUSION

Although research continues to unwind the mysteries surrounding perinatal mother/infant emotional behavior, most theories on emotional behavior are still built upon the very same assumptions Darwin made in his theories of natural selection and emotions. Arguably, these assumptions have permeated Western religion and society for millennia and undergird much of today's society, schools, scientific research agenda and popular culture. This has resulted in a resistance to or inability to reconsider the belief systems that underlie today's science. As William Schoenfeld aptly quoted in his presidential address at the 12th annual meeting of the Pavlovian Society in 1972, *Problems of Modern Behavior Theory* (Cornfold, 1931), "If we look beneath the surface of philosophical discussion, we find that its course is largely governed by assumptions that are seldom, or never, mentioned."

The new theories presented at the end of this review stem from a theoretical path quite different from the one that leads to the current thinking and practice in behavioral science today. One might conclude that the divergence in paths outlined in **Figure 4** stems from a differential emphasis on brain and body. But, this is a false dichotomy. The real dichotomy is between *I* and *we*.

To understand social instincts from the new perspective we present here, one must change several wide-spread assumptions about the origin, development *and control* of perinatal emotional behavior; from a self-regulatory to a co-regulatory system, and from a top-down to a bottom-up perspective. This new perspective engenders practicable and scalable interventions that increase mother-infant emotional connection and autonomic co-regulation, in order to overcome a devastating and fast-growing burden of emotional, behavioral and developmental disorders that threaten the educational and care systems underpinning human society.

Emotional connection and calming cycle theories are based on new assumptions that cast the emotional behaviors between mother and infant in new light. Emotional connection theory assumes that the mother/infant emotional relationship is measureable in a way that is predictive of future health and adaptability. Calming cycle theory assumes that the relationship that emerges between mother and infant in the perinatal period is not fixed, and that maladaptive behaviors can be changed to adaptive. The significant theoretical advance of these theories is that both theories generate simple testable hypotheses.

In closing, with Schoenfeld's admonition in mind, we disclose a final assumption that contrasts with Darwin's. We assume that the social instincts of *sympathy, empathy, altruism and co-operation,* rather than being traits that will degrade and doom our species, as Darwin feared, are the ones that will actually save it.

AUTHOR CONTRIBUTIONS

RL contributed to theory, literature research and writing. MW contributed to theoretical concepts, writing and editing.

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Demographic and Parental Factors Associated With Developmental Outcomes in Children With Intellectual Disabilities

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The aim of the study was to examine the relation between demographic variables, parental characteristics, and cognitive, language and motor skills development in children with intellectual disabilities (ID). A sample of 89 children with ID, aged 20–47 months, completed the Bayley Scales of Infant Development to measure cognitive, motor, and linguistic development. Parents were administered questionnaires about demographic information and parental anxiety, depression, parental stress, conjugality and familial functioning. Parenting behaviors (affection, responsiveness, encouragement, and teaching) were observed using the Spanish version of PICCOLO (Parenting Interactions with Children: Checklist of Observations Linked to Outcomes). A bivariate analysis showed that cognitive development in infants was significantly related to the mother's and father's responsiveness, and to the father's teaching scores. Infant language development was related to a variety of maternal factors (educational level, anxiety, depression, maternal responsiveness) and to the father's teaching scores. None of the factors were statistically related to child motor development. A multivariate regression analysis indicated that children's cognitive development can be predicted by a linear combination of maternal responsiveness and paternal teaching scores. Language development can be predicted by a linear combination of maternal anxiety and responsiveness, and paternal teaching scores. The present study provides evidence of the importance of paternal involvement for cognitive and language development in children with intellectual disabilities, and contributes to the increasing literature about fathering. Gaining knowledge about parental contributions to children's development is relevant for improving positive parenting in early intervention programs.

Keywords: children with intellectual disabilities, child development, parental anxiety, parental depression, parental stress, family functioning, positive parenting

INTRODUCTION

The influence of family context on children's development has received increasing attention in recent years (Cabrera et al., 2011; Velasco et al., 2014; Chiang et al., 2015; Barreto et al., 2017), but understanding and explaining how this effect is exerted is a complex task.

In children with intellectual disability (ID), the slower rate of development affects their learning and their interaction and communication with others. Risk factors identified in children with ID include lower IQ, poorer communication skills, more medical illness, presence in some cases, of autistic symptomatology and difficulties in social interaction or behavior problems (Hauser-Cram et al., 2001).

Sociodemographic Factors and Children's Developmental Outcomes

Among the familial and demographic variables related to developmental outcomes, socioeconomic status (SES) has received considerable attention, usually in families with children of normative development (Sohr-Preston et al., 2013; Roubinov and Boyce, 2017). Among other variables, SES includes family income and parents' educational level. In this respect, higher levels of parental education have been consistently associated with better developmental outcomes in children (Steinberg, 2001; Tamis-LeMonda et al., 2009). In contrast, a lower family SES is associated with a higher risk of delayed childhood development (Cheng, 2003; Emerson et al., 2006; Emerson and Hatton, 2007). A precarious economic situation and lower levels of parenting education (Zheng et al., 2012) may affect the development of children with IDs by limiting access to the resources they need (Saunders et al., 2015) and restricting their use of care services (Wu et al., 2003).

Parental Factors: Parents and Family Well-Being

Research has suggested that having a child with ID may produce negative reactions in the family, and may make family members reluctant to foster the child's development (Byrne and Cunningham, 1985; Hauser-Cram et al., 2001; Blacher and Baker, 2002; Hastings, 2003). Many parents caring for children and toddlers with a disability report high levels of anxiety, depression, and stress (Dyson, 1997; Keller and Honig, 2004; Al-Qaisy, 2012). It has been found that parents of children with ID generally have higher levels of anxiety, depression, and stress than parents of typically developing children (Olsson and Hwang, 2002; Eisenhower et al., 2005; Oelofsen and Richardson, 2006; Singer, 2006; Baker et al., 2010; Hayes and Watson, 2013). However, not all family members respond in the same way to being a relative of a child with ID. Most research in this area has focused on the well-being of mothers, but very little has been published about the well-being of fathers (Glidden and Natcher, 2009; MacDonald et al., 2010).

Many families with children with disabilities also report lower levels of marital satisfaction (Brobst et al., 2009) and Family Quality of Life (FQoL) (Hu et al., 2012). The literature has shown that social support, especially spousal support, is a protective factor against stress and depression among mothers caring for typically developing children (Boyd, 2002; Holloway et al., 2005; Suzuki et al., 2009; Manuel et al., 2012; Skipstein et al., 2012). Spousal emotional support is also very important for mothers who care for children with ID (Dunst et al., 1986; Glidden and Schoolcraft, 2007). Cohen et al. (2013, 2016) showed the benefits of spousal support for mothers with children with disabilities in relation to child behavior problems. Despite the move toward "family-centered" service models, in the majority of cases it is mothers who spend the largest amount of time caring for the needs of a child with a disability. Mothers of children with disabilities typically tend to be more involved in caregiving and housekeeping than fathers (Pozo, 2010). This caregiving role was found to generate both positive and negative perceptions among mothers of children with ID (Vilaseca et al., 2014). These latter authors established that mothers presented higher levels of anxiety and depression than did fathers from the same family, similar to other studies on families with children with disabilities (Hastings, 2003; Saloviita et al., 2003; Hastings et al., 2005a,b), and they found a significant relationship between the degrees of maternal anxiety and depression and those of fathers (Vilaseca et al., 2014).

Few studies have directly examined the relationship between familial emotional well-being and children's developmental outcomes. Most have studied the relation between parents' well-being and children's behavioral problems (Hauser-Cram et al., 2001; Hastings, 2003; Eisenhower et al., 2005; Cohen et al., 2013, 2016). The increasing understanding of environmental influences on child developmental outcomes stresses the importance of a good environment (Kingston et al., 2012). Some factors of this environment may be less beneficial for infant development, for example, maternal distress or anxiety or depression levels in parents. The detrimental effect of maternal depression after childbirth on emotional, cognitive, and language development in normal developing children is well documented (Cornish et al., 2005; Sohr-Preston and Scaramella, 2006). Some studies have also suggested that maternal anxiety in the postpartum period is associated with lower cognitive and language development scores in typically developing children (Reilly et al., 2006; Glasheen et al., 2010). Another aspect to consider is that maternal distress and parents' psychological disorders could negatively affect the mother–child interaction, which, in turn, might have an influence on child cognitive and linguistic development (Dilworth-Bart et al., 2007; Feldman and Eidelman, 2009; Edwards and Hans, 2015). However, not all parents of children with disabilities will engage in non-optimal parenting. Several studies have analyzed mothers and fathers of children with disability in different contexts, and have found that parents with positive perceptions of their child with disability expressed more feelings of happiness, family togetherness and personal growth than those with negative perceptions. Increasing parents' knowledge about disability and developing more coping strategies and positive perceptions led to an increase in family well-being, which helped foster their children's development. Families who view their relationship with their infant with a disability as something positive experience less parental stress and perceive

themselves as more competent parents (Glenn et al., 2009; Ferrer et al., 2016).

Parental Factors: Parenting

A responsive environment that includes a positive interaction between parents and children, is predictive of better children's developmental outcomes for infants with ID (Spiker et al., 2002; Innocenti et al., 2013). A wide range of different outcomes may be affected, including neurological development, linguistic, socioemotional, motor, cognitive, and behavioral development, psychopathology and school adjustment (Totsika et al., 2014). Although a large number of studies support the relationship between positive parental practices and child development for typically developing children (Love et al., 2005), fewer studies have examined this association for families with a child with developmental disabilities (for a review, see Dyches et al., 2012). However, this is a very important area of research due to the many stressors these parents experience, and their possible effect on the quality of the parent–child interactions (Gray, 2006; Kersh et al., 2006; Dabrowska and Pisula, 2010).

"Positive parenting" refers to the adult behaviors that promote development in face-to-face interactions in daily routines (Roggman et al., 2013a). Parenting that supports children's early development can help children with ID to improve their development of language and communication, their cognitive development and their autonomy. Parenting behaviors cover the domains of warmth, responsiveness, encouragement and cognitive stimulation or teaching (Bernier et al., 2010; Roggman et al., 2013a).

Emotional warmth, or affection, refers to the expression of positive emotions, positive evaluation of the child, and positive regard (Roggman et al., 2013a). Emotional warmth and affection are relevant for the construction of secure attachment, which is related in turn to the child's general development, both for typically developing children and for children with disabilities (Pipp et al., 1992; Kochanska, 2001; Sanders et al., 2004). Several studies have shown that parental affection is associated with less antisocial behavior, and better cognitive abilities and readiness for school (Laible et al., 2000; Zhou et al., 2002; Caspi et al., 2004). Maselko et al. (2011) found that parental affection in the early stages of life also had a positive impact during adulthood.

Responsiveness is another key dimension in the definition of positive parenting. In the context of child–adult interactions, parental behaviors are considered responsive when the adult responds to the signals of the child quickly (immediately) and contingently (in a way linked to the child's action), adjusting to the child's initiative and interests, as well to his/her focus of attention and/or action (Spiker et al., 2002; Roggman et al., 2013a; Tamis-LeMonda et al., 2014). Responsiveness has been linked to secure attachment, linguistic development, executive function and general cognition, self-regulation, empathy, and socially appropriate behavior (Osofsky and Thompson, 2000; Landry et al., 2001, 2006; Tamis-LeMonda et al., 2001, 2014; Crouter and Head, 2002; Davidov and Grusec, 2006; Hirsh-Pasek and Burchinal, 2006; Bernier et al., 2010). Both mothers and fathers can be sensitive and supportive to their children (Cabrera et al., 2007). Many studies have demonstrated that fathers who interact with their children in a positive manner and are attentive and responsive promote cognitive, linguistic, social and emotional development in their children, complementing the effects of mothers' parenting (Shannon et al., 2002; Tamis-LeMonda et al., 2004; Shimpi and Huttenlocher, 2007). Landry et al. (2001) pointed out that parental responsiveness is particularly relevant during early developmental stages.

Parental responsiveness has also been shown to be predictive of developmental outcomes in children with disabilities (Warren and Brady, 2007). In the case of children with intellectual disabilities (ID), maternal responsiveness has been positively related to the child's linguistic development (Yoder and Warren, 1998, 2000, 2001; Hauser-Cram et al., 2001), cognitive development (Waserman et al., 1985), and social development (Girolametto et al., 1994; Mahoney and Perales, 2003).

Encouragement is another relevant factor defining parental interactions that promote the child's development. Encouragement is identified in parental behaviors that promote a degree of autonomy, are adjusted to the child's competencies, set limits and demand maturity according to age, and it predicts adaptive child development and low levels of externalizing problem behavior (Barber, 1996; Hart et al., 2003). Encouraging and supporting children's efforts, initiative and exploration, while offering appropriate guidance without being intrusive, enhances children's willingness to take on challenging tasks and develops their executive function, sustained attention and emotion regulation, social adjustment, and adjustment to school (Landry et al., 1997; Hubbs-Tait et al., 2002; Joussemet et al., 2005; Bernier et al., 2010, 2012; Graziano et al., 2011; Roggman et al., 2013a; Fay-Stammbach et al., 2014). In contrast, intrusive parenting has been related to poor social abilities (Rubin et al., 2002; Degnan et al., 2008). Some studies have pointed out the relationships between parental behavior regulation and the developmental outcomes of children with disabilities. Hughes and Kasari (2000) found that children with Down syndrome expressed less pride when they completed a task when their mothers were directive. Maternal directiveness is negatively associated with developmental delays in communicative behaviors (Girolametto and Tannock, 1994). On the other hand, encouraging and supporting the child's efforts increases their play with objects, the quality and complexity of play, intentional communication, and vocalizations (Cielinski et al., 1995; Roach et al., 1998). Even though these studies have been conducted with mothers, other researchers focusing on fatherhood have shown a relation between the father's support of the child's autonomy and the child's level of executive functioning during the preschool years (Meuwissen and Carlson, 2015). These results are similar to those of previous research with mothers (Sethi et al., 2000; Bernier et al., 2010, 2012).

Finally, cognitive and linguistic stimulation (e.g., explanations, asking the child questions, using a rich vocabulary, joint attention, promoting the child's participation in adult–child conversation) has been related to cognitive, linguistic, and socioemotional development as well as to emergent literacy skills in typically developing children (Tamis-LeMonda et al., 2001; Hubbs-Tait et al., 2002; Kim-Cohen et al., 2004; Bingham, 2007; Farah et al., 2008). Cognitive and linguistic stimulation focuses

on children's early learning of vocabulary and is predictive of long-term academic success (Cook et al., 2011). In this respect, the father's role is also relevant for a child's development (Tamis-LeMonda et al., 2004; Cabrera et al., 2011). In children with disabilities or in children at risk, a developing parenting approach through home visits is useful to engage parents by using specific strategies to promote the child's language and cognitive development (Peterson et al., 2013; Roggman et al., 2016a,b). Home visiting practices have shown experimental evidence of increased language development and other enhanced developmental outcomes in children with disabilities or with developmental delay using different intervention programs such as the bookmaking intervention, which includes observations of shared conversation and play and cognitive and linguistic stimulation (Boyce et al., 2010a,b, 2017). For families with a child with a disability, achieving good parenting and positive parent–child interactions, such as those described above, can be a real challenge. Children with ID may provide less salient cues, be less responsive or have behavioral problems compared to typically developing children (Innocenti et al., 2013); they may show less emotional expressiveness, and have difficulties in joint attention, language and communication, and behavioral problems, all of which may contribute to difficulties in establishing good interaction patterns (Biringen et al., 2005; Spiker et al., 2005; Salisbury and Copeland, 2013).

Few studies have addressed the relation between diverse variables related to parents and family (demographic variables, quality of parental interactions or parenting, parents' emotional well-being, family functioning and conjugality) and infant cognitive, linguistic, and motor development, particularly in children with ID. We agree that the transactional model offers a theoretical framework for understanding the impact of parenting on cognitive and language development (Sameroff and Chandler, 1975; Sameroff and Fiese, 2000), considering mothers, fathers and children as units of a family system, with interconnected patterns of actions and relationships. As Cabrera et al. (2011) suggested maternal and paternal parental behaviors are linked to a child's developmental outcomes, implying a complementary system of parenting, with both commonalities and differences between mothers and fathers within a systemic framework. This is why we consider it is necessary to look at both mothers and fathers when analyzing parenting and other variables in the familial context with respect to child development. Both the child and the parents affect each other in reciprocal ways and the results of this interaction pave the way for subsequent development (Warren and Walker, 2005).

More research in this area is clearly needed, especially because the results could be used to design strategies for families with children with IDs. Our study is clearly exploratory in nature, aiming to examine the relation between family-related demographic variables (e.g., parents' educational level, family income, and so on), parental factors, and cognitive, linguistic and motor development in young children with IDs. Under "parental factors" we include both the parents' and family's well-being (anxiety, depression, parental stress, conjugality, family functioning) and parenting, defined in terms of affection, responsiveness, encouragement, and teaching.

MATERIALS AND METHODS

Participants

Participants were recruited from several Early Intervention Centers (EIC) in Spain. The following criteria were used for inclusion of children in the study: (a) children aged between 20 and 47 months; (b) with an ID (associated or not with another type of disability) diagnosed at least 6 months before carrying out the study.

The study sample comprised 89 children, 61 males (68%) and 28 females (32%), aged from 20 to 47 months (M = 33.4, SD = 6.8). Fifty-six percent of children were younger than 3 years old (20–35 months), and 44% were 3 years old or over (36–47 months). The degree of ID was mild (from 33 to 64%) in 44%, moderate (from 65 to 74%) in 48% and severe (>75%) in 8%. In Spain, the assessment of the percentage of disability is a standardized process carried out by a governmental agency, the Valuation and Guidance Services for People with Disabilities (CAD). In the case of ID, it is graded as mild, moderate and severe. The centers carry out the assessment and establish the degree of disability. **Table 1** contains additional demographic information on the participants.

Instruments

A brief *sociodemographic questionnaire* (see Appendix in **Supplementary Data Sheet**) was produced to collect data from participants (mother, father, and children).

The Spanish version (Caro and Ibáñez, 1992) of the *Hospital Anxiety and Depression Scale* (HADS; Zigmond and Snaith, 1983) was used to assess anxiety and depression symptoms in mothers and fathers. The HADS is a self-reporting screening questionnaire composed of 14 items scored on a four-point Likert-type scale (0–3). Seven items assess depression and seven assess anxiety. Previous research on members of families with children with ID has shown that the HADS is a reliable instrument, with Cronbach's alphas of at least 0.80 for both anxiety and depression for mothers and fathers (Hastings, 2003). In the Spanish version of the HADS, a factor analysis showed a clear two-factor structure for all groups, and the results demonstrated the internal consistency and reliability of the questionnaire (Quintana et al., 2003). With regard to validity, the correlations with related constructs are acceptable or highly acceptable (Terol-Cantero et al., 2015). In our sample, the HADS Cronbach's α value for mother's and father's anxiety were 0.88 and 0.77, respectively, and 0.80 and 0.78 for mother's and father's depression.

The Spanish version (Oronoz et al., 2007) of the *Parental Stress Scale* (PSS; Berry and Jones, 1995) was used to evaluate the degree of stress of the mothers and fathers. The PSS was designed to measure the degree to which situations in one's life are appraised as stressful. It is a self-report scale, composed of 12 items scored on a five-point Likert scale, from 1 (total disagreement) to 5 (full agreement). The items describe feelings and perceptions about the experience of being a parent. The Spanish version of the PSS demonstrated adequate psychometric properties with high reliability coefficients (internal consistency, α = 0.81, and

TABLE 1 | Sociodemographic characteristics of the participants.

Characteristic	*N*	%	Characteristic	*N*	%
Child age (20–47 months): *M* (*SD*)	33.4	6.8	Child gender (male)	61	68.0
Mother's age (27–45 years): *M* (*SD*)	37.0	4.1	Father's age (26–60 years): *M* (*SD*)	38.9	4.9
Mother's civil status			Father's civil status		
Married or cohabiting	79	90.8	Married or cohabiting	78	96.3
Single/divorced/separated/widowed	8	9.2	Single/divorced/separated/widowed	3	3.7
Mother's educational level			Father's educational level		
Elementary schooling	16	18.4	Elementary schooling	21	26.6
High school	35	40.2	High school	29	36.7
University degree	36	41.4	University degree	29	36.7
Mother's employment			Father's employment		
Full-time job	46	52.9	Full-time job	72	88.9
Partial-time job	24	27.6	Partial-time job	2	2.5
Unemployed or housework	17	19.5	Unemployed or housework	7	8.6
Monthly family income			Received help at home in the care of the children (yes)	47	52.8
Less than €1.314	24	27.9	Satisfaction (0–10) with the services received by their child: *M* (*SD*)	8.7	1.5
€1.314 – €2.450*	25	29.1			
More than €2.450	37	43.0			

**Considered an average income in Spain (INE-Instituto Nacional de Estadística, 2017).*

test–retest, $r = 0.73$), and good validity. In our sample, Cronbach's α values for both mothers and fathers were 0.82.

The Conjugality subscale on the *Basic Family Relations Inventory* (BFRI; Ibáñez et al., 2012) was used to score the quality of the couple's relationships. This subscale contains 14 items, which are answered separately by the mother and the father, and refer to how each person perceives the quality of the interaction with his/her partner and the support received from him/her. Items are scored on a five-point Likert scale, from 1 (never) to 5 (always). Conjugality is considered to consist of two poles: harmonious (seven items) and non-harmonious (seven items). As for the subscale's psychometric properties, the 14-item scores have shown high internal consistency ($\alpha = 0.95$), reflecting good reliability (Ibáñez et al., 2012). In the present study, the α coefficients were 0.91 for mothers and 0.90 for fathers.

The Spanish version (Fernández-Ballesteros and Sierra, 1989) of the *Family Environment Scale* (Moos and Moos, 1981) was used to measure perceived family interactions. For this study, we used only the Relationships dimension of the FES which consists of 27 true/false items. This dimension is assessed through three subscales: Cohesion (help and support between family members), Expressivity (expression of feelings), and Conflict (openly expressed in the family). The Spanish adaptation of the scale has revealed sufficient rates of reliability and validity (Fernández-Ballesteros and Sierra, 1989). In this study, alpha reliability coefficients were 0.78 for Cohesion, 0.44 for Expressivity, and 0.55 for Conflict. As in previous studies (Adam et al., 2010) the two last subscales yielded values below the desirable levels.

The *Parenting Interactions with Children: Checklist of Observations Linked to Outcomes* (PICCOLO; Roggman et al., 2013b) was used to assess parent–children interactions. PICCOLO is an observational measure of parenting interactions composed by 29 items, scored according to their frequency as 0 (absent), 1 (barely: brief, minor or emerging behavior) and 2 (clearly: definite, strong or frequent behavior). These items measure four interaction dimensions: (a) Affection (expression of affection, positive emotions, positive evaluation of the child and positive regard); (b) Responsiveness (reacting in a sensitive manner to a child's cues, expressions of needs or interests and behaviors); (c) Encouragement (parents' support of children's efforts, exploration, autonomy, choices, creativity, and initiative); and (d) Teaching (includes cognitive and linguistic stimulation, i.e., explanations about causal relations, talk about objects characteristics and questions). The instrument's reliability is good (see Roggman et al., 2013a) and it shows construct and predictive validity for parents who have a child with a disability (Innocenti et al., 2013). The Spanish validation of the PICCOLO (Vilaseca et al., 2019) found a high interrater reliability; the intraclass correlation coefficients (ICC) ranged from 0.69 for the responsiveness domain to 0.85 for the total score. With respect to internal consistency reliability, all domain and total scores showed satisfactory Cronbach's alpha coefficients (0.65 for Affection, 0.75 for Responsiveness, 0.76 for Encouragement, 0.72 for Teaching, and 0.88 for the total score). In this study, 25% of mother–child interactions and 21% of father–child interactions were coded by two trained observers; interrater reliability scores were adequate and the ICC ranged from 0.62 to 0.86. Regarding the internal consistency reliability of the total scale, Cronbach's α values were 0.89 for mothers and 0.90 for fathers. With respect to PICCOLO subscales, Cronbach's α values for mothers and for fathers were (respectively) 0.56 and 0.59 for Affection; 0.81 and 0.84 for Responsiveness; 0.80 and 0.83 for Encouragement; and 0.68 and 0.63 for Teaching.

Child development was assessed using the Spanish version of the *Bayley Scales of Infant Development-III* (BSID-III; Bayley, 2015). BSID-III scales (Bayley, 2006) are widely used to assess infant and child development between 1 and 42 months of age.

Cognitive, Language (Receptive and Expressive communication subtests), and Motor scales (the Fine and Gross Motor subtests) were applied. In the English language version (Bayley, 2006), the mean reliability coefficients were calculated using Fisher's z transformation. Across all ages, mean stability coefficients were 0.80 or higher. The mean reliability coefficients for the special groups included in the sample were all greater than 0.94. In this study, the Spanish adaptation of the Bayley-III Scales was used and the direct scores were transformed to Spanish percentile scores (Bayley, 2015). BSID-III scales include the possibility of using the instrument to assess children at ages outside the age range of the scale, for example, older children with limited abilities and low developmental age (Bayley, 2015).

Procedure

First, ethical approval was obtained from the University of Barcelona's Bioethics Commission (CBUB), in accordance with the International Ethical Guidelines for Health-related Research involving Humans prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO), and the WMA Declaration of Helsinki – Ethical Principles for Medical Research involving Human Subjects.

Secondly, in order to recruit participants we contacted the Catalan Association of Early Intervention, an association that manages the majority of Early Intervention Centers (EIC) in Catalonia. With their consent, 36 EICs in the province of Barcelona and Tarragona were contacted by letter and telephone, informed of the project and invited to participate. Twenty-three EICs accepted the invitation. These results show an acceptable level of participation, since research participation in Early Intervention in Spain promoting collaboration between families and professionals is qualified as moderate (Dalmau-Montala et al., 2017).

After agreeing to participate, the coordinators of the EICs were contacted to request their collaboration in recruiting families based on the inclusion criteria mentioned above. These coordinators informed us of families that might be suitable candidates for the study. Families were informed that their participation would be entirely voluntary and anonymous, and that they would not receive any incentive to participate in the study. To preserve confidentiality, each EIC was given documentation for the candidate families in a sealed envelope. It contained a newsletter, an informed consent form, a sociodemographic questionnaire, and the rest of the instruments cited below (in duplicate) to be completed separately by mother and father, and the FES to be answered jointly. Finally, a brief guide of how to video record the parent–child interaction was included; mothers and fathers were asked to engage separately in a video-recorded of an approximately 10-min play session with their infants at home, with the following instruction "*Interact and play with your children as you typically do.*" The parents had to play with their children using toys they had at home in a natural way, and recorded the video themselves. Seven video recordings were excluded (either because only the child appeared on the tape, because the audio was not clear enough or because they were recorded at bathtime or mealtimes). The PICCOLO (Roggman et al., 2013b) was then used to score parent–child interactions in these video recordings.

The *Bayley Scales of Infant Development-III* (Bayley, 2015) were administered at the EIC by researcher. The parents were present throughout the assessment.

Data Analysis

Data analyses were conducted in several steps. Firstly, a bivariate analysis was performed to study the relationship between each of the sociodemographic factors and the children's Bayley percentile scores. For categorical factors, mean Bayley scores were compared via Student *t*-test (for comparing two independent means) or via robust Brown–Forsythe ANOVA (for more than two independent means), followed by *post hoc* Games-Howell's test for pairwise comparisons. Relationships between continuous sociodemographic factors and Bayley's scores were analyzed via Pearson product-moment correlation coefficients.

Secondly, the relationship between parental factors and children's Bayley scores was examined using Pearson correlation coefficients. Finally, variables whose effect was found to be statistically significant ($p < 0.05$) in the previous bivariate analyses were included in a multiple linear regression model to predict child development scores. For variable selection, a stepwise criterion was applied (probability of $F \leq 0.05$ to add a predictor, and probability of $F > 0.10$ to remove it from the model).

IBM SPSS (version 24.0 for Windows) was used for all statistical analyses. Missing data were handled by pairwise deletion.

RESULTS

Sociodemographic Factors and Children's Developmental Outcomes

Table 1 shows statistical descriptives of the participants' sociodemographic characteristics. The relationship between each of the factors included in the *sociodemographic questionnaire* (see Appendix in **Supplementary Data Sheet**) and children's developmental outcomes (BSID-III percentile scores) was analyzed. In particular, the following sociodemographic factors were included in the study: child's age and gender; parents' age, civil status, educational level, employment situation, monthly family income, and degree of satisfaction with the services received by their child (from 0 to 10 points) at the Early Intervention Centers. Parents were also asked whether they received help at home with their children from other individuals or relatives.

Results showed a statistically significant effect of the mother's educational level on linguistic development [Brown-Forsythe's $F(2,56.3) = 5.46$; $p = 0.007$; $\eta^2 = 0.10$]. In this regard, the highest mean score for linguistic development was found in children whose mothers had a university degree ($M = 15.1$, $SD = 2.5$), followed by those whose mothers had only elementary schooling ($M = 8.21$, $SD = 2.0$), or had completed high school ($M = 4.5$, $SD = 0.8$). Pairwise comparisons showed higher linguistic development in children whose mothers had

a university degree than in those whose mothers had only completed high school ($p < 0.05$); no differences were found between the other categories of the variable.

Using Cohen (1988) benchmarks for interpreting effect sizes, the effect of the "mother's educational level" on linguistic development can be considered as medium ($0.06 \leq \eta^2 < 0.25$). None of the sociodemographic factors studied had a significant effect on the children's cognitive and motor skills.

Parental Factors

Different parental factors (mother's/father's anxiety, depression, stress and conjugality; and familial functioning) were assessed using self-administered questionnaires. Pearson's correlation coefficients between these parental factors and the children's scores on the Bayley scales of infant development were computed (**Table 2**). None of these parental factors was significantly related to cognitive or motor development ($p > 0.05$). However, linguistic development was negatively correlated with mothers' anxiety and depression scores. This result means that linguistic development was higher in those children whose mothers demonstrated lower anxiety and depression levels.

Parenting interactions with children were assessed using an observational measurement instrument (PICCOLO). Statistically significant Pearson's correlation coefficients were found between cognitive development scores and several PICCOLO domain scores: mother's responsiveness, father's responsiveness, and father's teaching. Likewise, linguistic development scores were positively correlated with mother's responsiveness and father's teaching (see **Table 3**).

TABLE 2 | Pearson's correlations between parental scores and children's BSID-III scores.

	BSID-III outcome					
	Cognitive		Language		Motor skill	
Parental score	***r***	**(*p*)**	***r***	**(*p*)**	***r***	**(*p*)**
HADS						
Mother anxiety	−0.193	(0.070)	−0.263	(0.013)*	−0.129	(0.284)
Father anxiety	−0.188	(0.096)	−0.210	(0.061)	−0.069	(0.588)
Mother depression	−0.090	(0.404)	−0.234	(0.028)*	−0.068	(0.577)
Father depression	−0.167	(0.140)	−0.197	(0.081)	0.040	(0.756)
PSS						
Mother stress	0.061	(0.574)	−0.088	(0.419)	−0.097	(0.420)
Father stress	0.082	(0.481)	0.032	(0.784)	−0.026	(0.842)
BFRI						
Mother conjugality	0.095	(0.389)	0.120	(0.275)	−0.006	(0.964)
Father conjugality	−0.028	(0.807)	0.049	(0.672)	0.030	(0.817)
FES						
Cohesion	0.051	(0.649)	0.059	(0.594)	0.063	(0.617)
Expressivity	0.021	(0.851)	0.115	(0.300)	0.033	(0.796)
Conflict	−0.112	(0.314)	−0.119	(0.284)	−0.167	(0.185)

*$p < 0.05$. *BSID-III, Bayley Scales of Infant Development-III; HADS, Hospital Anxiety and Depression Scale; PSS, Parental Stress Scale; BFRI, Basic Family Relations Inventory; FES, Family Environment Scale.*

TABLE 3 | Pearson's correlations between parenting scores and children's BSID-III scores.

	BSID-III outcome					
	Cognitive		Language		Motor skill	
PICCOLO score	***r***	**(*p*)**	***r***	**(*p*)**	***r***	**(*p*)**
Mother						
Affection	−0.054	(0.621)	−0.027	(0.802)	−0.125	(0.310)
Responsiveness	0.312	(0.003)**	0.318	(0.003)**	0.079	(0.522)
Encouragement	0.177	(0.104)	0.174	(0.109)	−0.031	(0.802)
Teaching	0.117	(0.284)	0.176	(0.105)	−0.006	(0.959)
Total	0.193	(0.074)	0.220	(0.042)*	−0.012	(0.921)
Father						
Affection	−0.127	(0.291)	−0.092	(0.444)	−0.071	(0.608)
Responsiveness	0.274	(0.021)*	0.221	(0.064)	0.027	(0.844)
Encouragement	0.216	(0.070)	0.198	(0.098)	0.083	(0.546)
Teaching	0.370	(0.002)**	0.369	(0.002)**	0.081	(0.557)
Total	0.261	(0.028)*	0.244	(0.041)*	0.049	(0.725)

*$p < 0.05$, **$p < 0.01$. *BSID-III, Bayley Scales of Infant Development-III; PICCOLO, Parenting Interactions with Children: Checklist of Observations Linked to Outcomes.*

Therefore, some aspects of parenting interactions seem to promote children's cognitive and linguistic development, in particular, mother's responsiveness and father's responsiveness and teaching. On the other hand, parents' affection and encouragement were not significantly related to children's cognitive, linguistic or motor development. At the same time, none of the four domains of parenting interactions was linearly related to the children's motor development ($p > 0.05$).

Regression Models on Bayley Scales of Infant Development

Demographic and parental factors whose effect was found to be statistically significant in the previous bivariate analyses ($p \leq 0.05$) were included in a multiple linear regression model to predict children's Bayley scores. In order to predict cognitive development, three potential factors were taken into account: (1) mother's responsiveness, (2) father's responsiveness, and (3) father's teaching. Two of the four potential predictors were selected by stepwise criteria for inclusion in the final model. Results (**Table 4**) indicate that high cognitive development can be predicted by a linear combination of high scores in the mother's responsiveness and father's teaching PICCOLO domains. The regression model accounts for 19.6% of the variance of the Bayley cognitive scale scores (adjusted $r^2 = 0.196$). No more variables or interactions were added to the model because their inclusion did not bring about a significant improvement in the model's predictive power ($p > 0.05$).

The regression model to predict the children's linguistic development included three of the five potential factors: (1) mother's educational level, (2) mother's anxiety score, (3) mother's depression score, (4) mother's responsiveness, and (5) father's teaching. The results (**Table 5**) indicate that high linguistic development can be predicted by a linear combination

TABLE 4 | Linear regression model on Bayley's cognitive-development score (n = 69).

Variable	*B*	*SE*	β	*t*	*p*
Intercept	−10.37	7.12			
Mother's responsiveness	1.65	0.64	0.296	2.55	0.013
Father's teaching	1.55	0.65	0.274	2.36	0.021

B, regression coefficient; SE, standard error; β, standardized regression coefficient.

TABLE 5 | Linear regression model on Bayley's linguistic-development score (n = 69).

Variable	*B*	*SE*	β	*t*	*p*
Intercept	−1.24	5.29			
Mother's anxiety score	−0.96	0.30	−0.332	−3.21	0.002
Mother's responsiveness	1.03	0.40	0.281	2.58	0.012
Father's teaching	0.88	0.41	0.236	2.14	0.036

B, regression coefficient; SE, standard error; β, standardized regression coefficient.

of a low score for mother's anxiety, and high scores for mother's responsiveness and father's teaching. The multiple linear regression model accounted for 29.5% of the variance of the Bayley linguistic development scores (adjusted r^2 = 0.295). The remainder of the potential predictors, including two-factor interactions, were excluded because the model's predictive power did not improve significantly ($p > 0.05$) when any of them was added. It should be noted that two parenting factors (mother's responsiveness and father's teaching) were common to both models. PICCOLO scores for mother's responsiveness and father's teaching were found to have a significant positive effect on both the cognitive and linguistic development of their children.

DISCUSSION

Sociodemographic Factors and Children's Developmental Outcomes

The overall aim of the study was to examine the relations between some familial variables and a child's cognitive, linguistic and motor developmental outcomes when the child has intellectual disabilities. The selected familial variables included various sociodemographic characteristics (e.g., parents' educational level, family income. . .), parents' and family's well-being (anxiety, depression, parental stress, conjugality, family functioning) and parenting, defined as the characteristics of adult–child interactions in terms of affection, responsiveness, encouragement, and teaching (Roggman et al., 2013b). Both mothers' and fathers' parenting were considered.

As mentioned in the "Introduction" section, there is a solid tradition of studies relating SES and children's developmental outcomes, most of them conducted in the general population. In our study, among all the variables included in the sociodemographic questionnaire described in the "Materials and Methods" section, only maternal educational level was related to children's development, and only for linguistic development. Our results showed higher linguistic development in children whose mothers had a university degree than in those whose mothers only completed high school ($p < 0.05$). No differences were found between mothers with only elementary school and mothers with higher educational levels, though this latter result may be due to the small size of the group of mothers with only elementary school education (18%). Undoubtedly, more significant relationships could be expected based on previous studies in the general population (Sohr-Preston et al., 2013; Roubinov and Boyce, 2017) but the characteristics of the sample, i.e., caregivers with children with ID, need to be considered. In this case, the observed relationship between maternal educational level and a child's linguistic development is interesting. When comparing mothers and fathers in our sample, we found a significant difference between mothers' and fathers' level of employment: only 53% of mothers were employed full-time, compared with 89% of fathers. Another 28% of mothers were partially employed and 19% of them cared full-time for their children and were fully responsible for housework. So, it is reasonable to assume that, in our sample, mothers were interacting daily with their children with ID for more hours than fathers. The effect of the duration of mother–child interactions on linguistic development would be mediated by the quality of these interactions, in terms of affection, responsiveness, non-intrusive behavior and linguistic stimulation, all of them aspects clearly related to maternal educational level (Davis-Kean, 2005; Bornstein and Manian, 2013) and to the children's linguistic developmental outcomes, both in typically developing children (Shimpi and Huttenlocher, 2007; Roseberry et al., 2014), slow-to-talk children (Levickis et al., 2018) and children with disabilities (Warren et al., 2010; Sterling et al., 2013; DeVeney et al., 2016). From a practical point of view, as pointed out by other authors (Sohr-Preston et al., 2013), parents with lower educational levels would require special attention in programs addressed at promoting a child's development – both those in the general population and in families with children with disabilities.

Parental Factors: Parent and Family Well-Being

Few studies have analyzed the relations between the parents' and family's well-being (anxiety, depression, parental stress, conjugality, family functioning) on the one hand, and infant cognitive, linguistic and motor development on the other hand, particularly in children with ID. Interestingly, our results showed that children's linguistic development was negatively correlated with mother's anxiety and depression scores, but not with father's anxiety. As mentioned in the introduction, mothers of children with ID report higher levels of anxiety and depression than fathers in the same family (Hastings, 2003; Hastings et al., 2005a,b; Vilaseca et al., 2014). This is a very interesting result because maternal anxiety, cognition and joint activity in the mother–child interaction emerged as the strongest predictors of infant language performance in a sample of young normal developing children and their mothers with anxiety disorders in the postpartum period (Reck et al., 2018). Those results suggested that maternal anxiety and depression may potentially affect infant language development. A hypothesis to consider

in future studies is that the effect of a mother's anxiety on a child's linguistic development could be mediated by the influence of anxiety on the quality of the mother–child interaction, and particularly on responsiveness, non-intrusive behavior and linguistic stimulation.

Other studies have analyzed the relationship between mothers' and fathers' emotional well-being and socioemotional development in typically developing children, and found that anxiety and depression in mothers but not in fathers were significantly associated with social development in children (Huhtala et al., 2014). As Dilworth-Bart et al. (2007) noted, emotional distress in parents, especially mothers, may interfere with their ability to provide the optimal parenting required to promote their children's development. Having a child with a disability, such as an ID, may cause higher distress in parents, especially in mothers in the early years, because of their prolonged concerns about the development of their child and because it is usually the mother who cares for the child for most of the time in this crucial period. So, it is evident that factors of the immediate environment such as maternal anxiety and depression may be less beneficial for infant development. Our findings illustrate that the emotional well-being of parents, especially mothers, plays a crucial role in infant development, but this is an aspect that has not been researched in depth in families of children with disabilities. In Spain, a few early intervention practitioners have become aware of the importance of responding to families' needs and of promoting children's participation in their natural environments, with a slow movement toward a Family-Centered Approach (FCA) (Mas et al., 2018; Vilaseca et al., 2018). In this regard, our results justify the use of family-centered practices in which early intervention practices should focus on the family unit (rather than solely on the child); they also underline the importance of the strengths and competences of the family, and demonstrate that it is the family that can most effectively foster development and learning through the routines and activities that are pursued in the child's natural and inclusive environments (McWilliam, 2000, 2010; Dunst et al., 2010). Moreover, our results add important information on the impact of the psychological well-being of mothers and fathers in the same family on the development of children with an established ID.

It should be noted that among all risk factors, maternal anxiety symptoms require particular attention in early intervention practices. Children with ID whose mothers experience recurrent anxiety symptomatology could face more difficulties regarding developmental outcomes. This aspect and the parenting characteristics of both mothers and fathers should be taken into account for early intervention practices. A FCA to intervene in the emotional factors, in mothers, may be a protective factor for child development and for parents' and family well-being. Early intervention approaches focusing on parenting strengths may be able to build upon and promote the positive coping strategies that parents use and reduce the anxiety generated by caring for a child with special needs, especially in mothers. Empirical evidence shows that the FCA has a positive impact on the child and the family unit; it improves parents' perceptions of their parenting skills, helps to lower the anxiety and stress that sometimes beset families, and helps parents to establish a better relationship with their children and thus promote their development (see the review by Dunst and Espe-Sherwindt, 2016).

Our findings suggest that maternal anxiety and depression symptoms may be negative factors for language development in children with ID. A family-centered model to intervene in the emotional factors, in mothers, may be a protective factor. Our results imply the need to address parental psychological well-being in families with children with ID so as to improve developmental outcomes especially in language.

Parental Factors: Parenting

As mentioned in the "Introduction" section, a considerable amount of research has documented the relations between parenting and child development, both in families with typically developing children (Love et al., 2005; Mahoney, 2009; Warren et al., 2010; Blair et al., 2014; Vargas-Rubilar and Arán-Filippetti, 2014) and those with developmental delay and disabilities (Spiker et al., 2002; Innocenti et al., 2013). Our results confirm that early parenting behaviors, such as those measured by the PICCOLO, are associated with cognitive and linguistic outcomes in children with ID, with different patterns of effect for mothers and fathers, as also found in typically developing children (Rivero et al., 2018). Our results confirm that both mothers and fathers contribute to their children's development in different ways (Cabrera et al., 2008, 2018). For mothers, our results showed that responsiveness is associated with cognitive development and linguistic development in children with ID. This is consistent with the findings of several previous studies (Warren et al., 2010; Innocenti et al., 2013; Roseberry et al., 2014; Tamis-LeMonda et al., 2014). Children whose mothers display more responsive behaviors during the early years achieve better language and cognitive development. Our findings also establish that the father's responsivity and father's teaching are related to cognitive development in children with ID. Few studies have analyzed fathers' contributions to their children's development, especially in families with children with ID. Our findings are consistent with previous studies relating fathers' teaching to cognitive and linguistic outcomes in typically developing children (Summers et al., 2006; Duursma et al., 2011; Anderson et al., 2013; Leech et al., 2013). Early positive father–child interaction is important for child development, as mentioned previously (Ramchandani et al., 2012). Programs in early intervention are increasingly recognizing the value of engaging fathers in home visits (Roggman et al., 2002; Lawrence et al., 2013), but few studies have explored the relation between positive parenting in mothers and fathers of the same family unit and developmental outcomes in children with a disability in natural settings. In this study, it was the parents themselves who auto-recorded their interactions at home, and so we believe that this is a highly valuable contribution to the existing literature.

We pay particular attention to the role of the father in teaching because our results stress its importance for promoting cognitive and linguistic development in children with ID. Teaching involves activities that include cognitive stimulation, shared conversation and play, explanations and joint attention, which are all known to be essential for promoting child development

(Tamis-LeMonda et al., 2001). However, in children with established disabilities these activities may be difficult to carry out, particularly since maintaining a high level of communication or play with a child with ID can be highly challenging. A substantial number of children with ID have unintelligible speech and difficulties with conversational discourse (Abbeduto et al., 2007). Moreover, some of them may present difficulties in social interaction or symptoms of autism (Bailey et al., 2001). In another of our studies (Vilaseca et al., 2019) teaching was the domain with the lowest score on the PICCOLO. Mothers and fathers of children with disabilities engage in more types of affective behavior (warmth, closeness. . .) and fewer teaching behaviors (conversation, play, cognitive stimulation. . .) as is the case in younger normally developing children (Roggman et al., 2013a; Peterson et al., 2014). According to our results, children with ID whose fathers display more teaching behaviors during the early years achieve better language and cognitive development. This finding is particularly relevant, especially with regard to intervention. Since fathers' support of children's development should be increased in intervention programs, the PICCOLO behaviors could help early intervention practitioners, as this measure focuses on mothers' and fathers' behaviors which can be easily recognized and incorporated in intervention plans (Roggman and Cardia, 2016). The Spanish version of the PICCOLO used in this study (Vilaseca et al., 2019) is suitable for research on parents of children with a disability (Innocenti et al., 2013).

On the other hand, parents' affection and encouragement were not shown to be related to their children's cognitive, linguistic or motor development. Although we already know that emotional warmth plays a prominent role in children's development, most previous studies have related parents' affection to securing attachment and better social behavior in children (Kochanska, 2001), variables that we did not analyze in our study. This aspect should be taken into account in future studies in families of children with disabilities. At the same time, encouraging and supporting the children's initiative and exploration has been associated with the development of attention, emotion regulation and good social abilities (Rubin et al., 2002; Roggman et al., 2013a) and also with language (Lillard et al., 2013) and cognitive development (Bernier et al., 2010, 2012). The fact that our sample included very young children, with cognitive levels below their chronological age, may explain our results. Perhaps, in older children, encouragement could have greater implications for child development. An interesting result of our study is that none of the four domains of parenting measured using the PICCOLO was related to the children's motor development. This is one of the least studied aspects in relation to parenting and child development. And although our results suggest that early parenting predicts language and cognitive developmental outcomes in children with a disability, more research is required in order to examine the contributions of different aspects of parenting to children's motor development.

Finally, the PICCOLO scores for mothers' responsiveness and fathers' teaching domain had a significant positive effect on both the cognitive and linguistic development of their children. Our findings show that cognitive and linguistic development can be predicted by the contribution of both parents, each of whom presents different but complementary profiles that promote child development (Cabrera et al., 2014, 2018). This view is consistent with transactional models of human development which have established that there are multidirectional effects on children's development beyond the additive contributions of the mother and father (Sameroff, 2010; Fitzgerald and Bradley, 2012).

Our results support the call, made previously by other authors (Cabrera et al., 2000; Cox and Paley, 2003; Lamb and Lewis, 2010) for the inclusion of fathers in research. Both mothers and fathers must be considered in this research, to enable us to look more closely at their similarities, differences and complementarities, and to gain a fuller picture of parenting. Our findings corroborate those of other authors (Meuwissen and Carlson, 2015) who have suggested that including fathers in intervention programs may be more beneficial than working with mothers alone.

Final Remarks

The present study extends the current literature on parenting and child development in families of children with ID. Nevertheless, the study has several limitations that should be considered when interpreting the results. The first is the selection of the sample. Although participants were recruited at Early Intervention Centers, the fact is that the procedure used to select the participants could have been conditioned by the willingness of families to participate (Hoffman et al., 2006); conceivably, the parents who took part were the ones who were the most informed about child development, and most aware of the importance of parental interactions, or even the most confident about their parenting skills. Similarly, it may be that the parents who were most worried about their child's development were reluctant to participate. Another limitation is the correlational design, which does not permit us to establish a clear causality. Since this was a cross-sectional study, we need to be cautious about the use of the term "predictor" in the regression analysis. We have to take into account that, in this context, "to predict" means just to estimate Bayley's scores based on the predictor variable scores (demographic and parental scores), and does not necessarily imply direct causality.

Another limitation to consider is the use of self-administered questionnaires. In future research it would be interesting to conduct semi-structured interviews with all family members (including grandmothers, grandfathers or siblings) in order to extract more information and to examine their different perspectives. It would be of interest to compare these new data with those of our current study.

Future research on mothers and fathers with a child with an ID should include recorded observations of their interactions at older ages and the analysis of the possible continuity of patterns of mother– and father–child interactions, and their links to children's developmental outcomes in the early school years. Information regarding the long-term impact of parental psychological well-being on the development of their children would also be interesting, given that parental distress decreases as the child grows older (Ferrer et al., 2017).

Finally, it would also be of value to measure both the distribution of housework between the father and the mother and the burden assumed by each parent, in order to study whether high levels of responsibility in family tasks could be related to the levels of parental anxiety and depression.

This exploratory study may contribute to the development of theoretical models to explain the mechanism of the effect of specific factors which can later be tested with larger samples.

ETHICS STATEMENT

Ethical approval was obtained from the University of Barcelona's Bioethics Commission (CBUB), according to the International Ethical Guidelines for Health-related Research Involving Humans prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO), and the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects.

AUTHOR CONTRIBUTIONS

RV, MR, RB, M-JC, EN-P, CV-V, and FF made substantial contributions to conception and design, and/or acquisition of data, and/or analysis and interpretation of data; and participated in drafting the article or revising it critically for important intellectual content; and gave final approval of the version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2019.00872/full#supplementary-material

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Type of Delivery, Neuropsychological Development and Intelligence in Twin Births

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Based on a retrospective cohort design with 6-year-old children born in twin births, the relationship between verbal, non-verbal, global neuropsychological development, general intelligence and type of delivery has been studied. To this end, the possible effect of third gestational, obstetric and neonatal variables, such as maternal age at delivery, fetal presentation, gestational age, newborn weight and Apgar at minute one, was controlled. The exposed cohort includes children born by cesarean section, and the unexposed cohort is composed of children born vaginally with or without induction. A total of 124 children were evaluated in their 1st year of primary school using the Child Neuropsychological Maturity Questionnaire, Kaufman's Intelligence Test and the medical histories of the children collected after birth. By means of binary logistic regression analysis, it has been found that the type of delivery is presented as an independent risk factor for disorders in verbal, non-verbal and global development and for the general intellectual difficulties of children born of multiple births. These results suggest the need to analyze in future prospective studies with broader samples the relationship between different types of obstetric and perinatal variables of birth type and infant neuropsychological development and general intelligence, in order to prevent possible psychological alterations from birth.

Keywords: type of delivery, neuropsychological development, general intelligence, retrospective cohort study, twin births

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INTRODUCTION

Certain obstetric conditions frequently associated with multiple pregnancies, such as maternal age, preterm birth, birth weight perinatal hypoxia, or labor complications, have been implicated in developmental delays among children (Calame et al., 2004; Jansson-Verkasalo et al., 2004; David and Dean, 2007; Lollar and Cordero, 2007; Wolke et al., 2008; Cattani et al., 2010; Dall'oglio et al., 2010; Mercier et al., 2010; Kurth and Haussmann, 2011; Bidzan and Bieleninik, 2013; González-Valenzuela et al., 2015a,b). Preterm birth and low birth weight are important determinants of psychological development, since it has been shown that the earlier the birth in terms of weeks of gestation and the lower the birth weight, the greater the delay observed in psychological development, particularly in the first few years (Jansson-Verkasalo et al., 2004; Mercier et al., 2010; Aarnoudse-Moens et al., 2012; Zwicker and Harris, 2012;

González-Valenzuela et al., 2015b). Maternal age under 18 and over 40 is also a cause of high-risk pregnancy and can affect the mother's health and the baby's development (David and Dean, 2007; Lollar and Cordero, 2007; Kurth and Haussmann, 2011; Bidzan and Bieleninik, 2013).

However, there are relatively few studies that relate type of delivery to psychological problems. Furthermore, there appears to be no consensus with regard to consideration of risk caused by cesarean birth compared to vaginal delivery in the newborn and the mother (Hogle et al., 2003; Villar et al., 2007; Guerra et al., 2011). Some studies establish that cesarean birth carries a risk for the baby and the mother, whereas others do not state the existence of any differences with regard to vaginal delivery. The type of delivery is, therefore, for many authors a topic that is still open to discussion regarding its relationship with the development of children born in single and multiple deliveries. For some, short- and long-term infant outcomes are affected by the mode of delivery (Matthew and Neena, 2012; Karlström et al., 2013). It is well known that respiratory problems during the early neonatal period increase two–threefold after an elective cesarean birth (van den Berg et al., 2001). The instances of hypoglycemia (Hägnevik et al., 1984), low temperature (Christensson et al., 1993), delayed breastfeeding, difficulties in maternal bonding with the newborn and in neurodevelopment also increased (Carlander et al., 2010; Asztalos et al., 2016; Polidano et al., 2017). Many studies have suggested that a cesarean section affects long-term offspring outcomes regarding metabolic syndrome, the immune system, dentition, malignancies and nervous system development, describing plausible biological mechanisms although still failing to prove casualty (Pasupathy and Smith, 2008; Hyde et al., 2012). On the other hand, others argue that a lower risk of asphyxia, encephalopathy, and intracranial hemorrhage was found with cesarean sections compared to vaginal delivery (Hogle et al., 2003; Villar et al., 2007).

The mode of delivery has been directly related to biochemical and structural changes in the central nervous system, the consequences of which are not well known. Thus, recent studies reveal the existence of *in vitro* biochemical brain changes related to the active work of a normal childbirth, noting some deterioration in the functional development of the hippocampus, which leads to the existence of lifelong neuropsychological dysfunctions in cases in which there was no labor due to a scheduled cesarean section (Simón-Areces et al., 2012).

The rate of multiple pregnancies has recently increased, largely due to the use of assisted reproduction techniques and the increase in maternal age at the time of birth (Mesa and Peral, 2011). These pregnancies lead to an overload for the mother and often result in preterm and low-birth weight infants as well as posing added challenges arising from the need to address 'simultaneously' the second stage of labor for two fetuses. The second infant is more vulnerable due to complications such as cord prolapse, detachment of the placenta, dystocia due to cervical spasms, or trauma from intrauterine manipulation in cases of fetal extraction due to non-cephalic presentation. This happens especially in cases of great discordance of fetal weight or extremely low fetal weight, although not when gestational age is <34 weeks in both (Asztalos et al., 2016; Girsen et al., 2016).

However, although there are international recommendations for multiple childbirth assistance, the evidence is weak and is usually based on expert opinions and some retrospective studies (MacKay et al., 2006). In fact, whereas there are studies that show an increased risk of morbidity in a vaginal birth in the second twin compared to the first twin (Wen et al., 2004a,b; Armson et al., 2006; Yang et al., 2006; González-Mesa et al., 2016), others fail to demonstrate in the second twin the short- or long-term benefits of elective cesarean-sections compared to vaginal delivery (Rabinovici et al., 1987; Greig et al., 1992; Hogle et al., 2003; Asztalos et al., 2016; Girsen et al., 2016).

In this context, the main objective of this study is to estimate the relationship between verbal, non-verbal and global neuropsychological development, general intelligence, and the type of delivery in children born from twin births by the age of six. To this end, other potentially confusing gestational, obstetric and neonatal variables were controlled: maternal age at delivery, fetal presentation, gestational age, weight of the newborn and Apgar at minute one.

MATERIALS AND METHODS

Design

An epidemiological study of retrospective cohorts was designed. The risk factor (type of delivery) preceded the result (verbal development, non-verbal development, global development, and general intelligence at 6 years of age). The cohort exposed children born to twin births by cesarean section (exposed to cesarean section) and the cohort did not expose children born to twin births by vaginal delivery (not exposed to cesarean section), from the selected random sample of all twin births at the Hospital Materno-Infantil of Málaga during the year 2005.

Participants

The study population comprised children who were born in the Materno-Infantil Hospital in Málaga in 2005, were at least 6 years of age, had started their compulsory education (1st year of primary education), and showed no signs of possible disorders in their psychological development that could be irrevocably established. The hospital is a tertiary center in the Spanish national health system, in which 7,120 children were born in 2005, of which 270 children were born in twin births. Of these twins, 64 children born at less than 32 weeks of gestation, and 14 children who were studying a lower school level were excluded from the study. Sixty-eight children could not be recruited because their mothers could not be located due to a change of residence or they did not want to participate in the study when they were located (**Figure 1**). Therefore, the inclusion criteria applied to participants in the study were as follows: they had to be 6 years of age, be in Year 1 of Primary Education, have been born after 32 weeks of gestation, and the mothers had to be locatable and be willing to participate in the research.

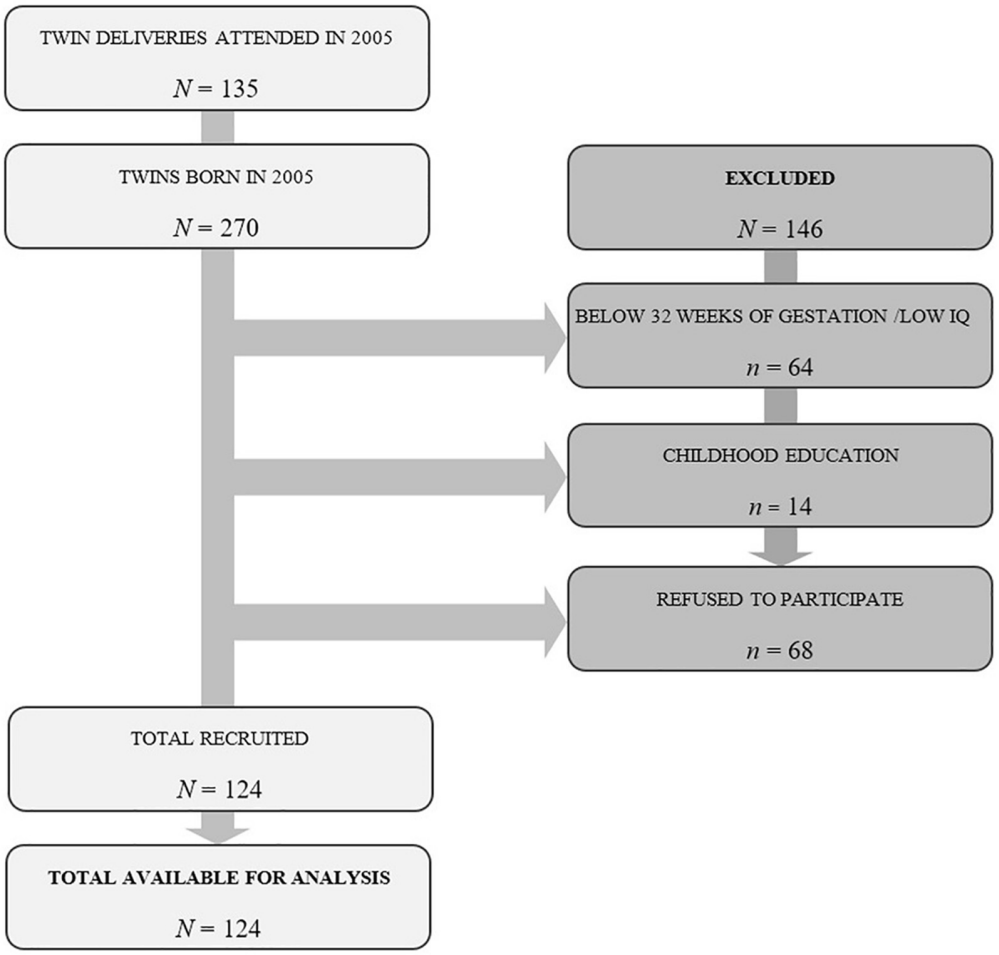

FIGURE 1 | Flow diagram of participants through each stage of the study.

Thus, the selected population is made up of 270 children born in twin births, of whom 124 children could be assessed, corresponding to 62 twin births ranging from 74 to 86 months old (M = 79.42, SD = 3.44), of whom 62 are male (50%) and 62 are female (50%). In this sample, 51 mothers (41.1%) and 58 fathers (46.8%) had a primary level of education (primary and pre-secondary studies); 38 mothers (30.6%) and 40 fathers (32.3%) had an intermediate level of education (junior high and high school, technical, and non-technical); and 35 mothers (28.2%) and 26 fathers (21%) had a higher education (college and graduate). The maternal age at the time of delivery ranged from 22 to 45 years of age (M = 33.2, SD = 4.27); the gestational age of the infants was between 32 and 41 weeks (M = 35.14, SD = 2.07); the weight of the infants was between 1179 and 3080 g (M = 2137.76, SD = 432.79), fetal presentation was cephalic in 80 deliveries (64.5%) and non-cephalic (breech and transverse) in 44 (35.5%), and the score on the Apgar 1 test ranged between 4 and 10 points (M = 8.41, SD = 1.18). Of the total sample, 84 (67.7%) were born vaginally and 40 (32.3%) were born by Cesarean section. The choice of cesarean section was indicated in 17 births (42.5%) due to maternal problems (mother's pathologies and/or non-progression in delivery) and 23 (57.5%) due to problems of fetal origin (malposition of the first twin and/or the loss of fetal wellbeing).

Instruments

To assess neuropsychological development, we used the "Cuestionario de Madurez Neuropsicológica Infantil" (CUMANIN [Child Neuropsychological Maturity Questionnaire]; Portellano et al., 2009). This questionnaire is intended to assess the degree of neuropsychological maturity reached by the subject in different areas of development and the possible presence of brain dysfunction (Portellano et al., 2009). It consists of a standardized battery of tests that assess Verbal Development (VD), Non-Verbal Development (NVD), and Global Development (GD) of children aged between 3 and 6 years. Various tests are included in the **Appendix**. The total VD and NVD score is the sum of the scores obtained in each sub-test (number of correct answers). The tests that assess GD are the same as those used for the evaluation of VD and NVD, and the total score is the sum of the scores obtained in all of the tests (total number of correct answers). Cronbach's alpha coefficient

values in all the scales ranged between 0.57 and 0.92, and in these ages, the range was between 0.83 and 0.88. The correlations between the indices of difficulty and discrimination of the items of the classical theory of the tests and the corresponding estimators of these parameters of the response to the item were high. The children were considered to have a verbal, non-verbal or global development disorder when they scored below the 25th percentile, respectively, in accordance with the evaluation criteria established for the test (Portellano et al., 2009). In other words, variables relating to neuropsychological development were defined as follows: presence of a developmental disorder if the child scored less than the 25th percentile: absence of a developmental disorder if the score was equal to or higher than the 25th percentile.

To measure general intelligence (GI), we used the Kaufman Brief Intelligence Test -K-BIT- (Kaufman and Kaufman, 2000), which assesses verbal and non-verbal intelligence at ages ranging from 4 to 90 years. It consists of two subtests: Vocabulary and Matrices. The *Vocabulary* test evaluates verbal ability related with school learning (crystallized thinking) and has two parts, Expressive Vocabulary and Definitions, which measure knowledge of words and the formation of concepts. The Matrices test assesses non-verbal skills and the ability to solve new reasoning problems through figurative and abstract visual stimuli (fluid thinking), based on the subject's ability to perceive relationships and complete analogies between objects. The total Intelligence score is the sum of the scores obtained in each of the subtests (total number of correct answers). The reliability coefficients of the scales ranged from 0.80 to 0.90. Children were considered to be at risk of presenting general intellectual difficulties when they scored below the 25th percentile, according to the evaluation criteria established for the test (Kaufman and Kaufman, 2000). In other words, the variable intelligence was defined as follows: risk of intellectual difficulties if the child scored less than the 25th percentile: no risk of intellectual difficulties if the score was equal to or higher than the 25th percentile. The rest of the variables considered in the study were assessed through the medical histories of the mothers and their children. The independent variable was the type of delivery, treated dichotomously, with the categories being vaginal delivery (induced and non-induced) and Cesarean section. Considering that the main objective of this study is to examine the effect of the type of delivery on twins' neuropsychological development and intelligence in the presence of third variables that can produce confounding and interaction phenomena (treated as control variables), we evaluated the following dichotomized gestational, obstetric, and neonatal variables (maternal age, fetal presentation, gestational age and weight of the newborn and Apgar 1). Maternal age represented the age of the mother at the time of delivery; gestational age indicated the number of weeks' gestation of the baby when born; the newborn's birth weight was expressed in grams; cephalic presentation was classified as either cephalic or non-cephalic (breech and transverse); and the Apgar-1 test measured the baby's heart rate, muscle tone and other signs to determine whether they need additional or emergency medical assistance within a range of 10 points. These variables were transformed into a categorical dichotomic scale as per González-Mesa et al. (2016), in accordance with clinical criteria. Maternal age at the time of delivery was classified as being over or under 35 years of age; fetal presentation was cephalic or non-cephalic (breech or transverse); gestational age of the newborn, with categories above or below 37 weeks, and above or below 34 weeks; the weight of the newborn was considered either above or below 1,500 g; and Apgar at 1 min, with categories of above or below 7 points.

Procedure

This study was carried out in accordance with the recommendations and approval of the Research Ethics Committee (Comité de Ética de la Investigación) of the Hospital Regional Universitario Carlos Haya in Málaga. All mothers of the subjects gave written informed consent in accordance with the Declaration of Helsinki.

After obtaining the corresponding authorization from the ethics committee, we obtained the necessary data to contact the mothers by phone. During these telephone calls, which lasted around 10 min, we explained the objectives and development of the research, and proposed an appointment for the psychological assessment of the child. This evaluation was carried out in the consultation rooms of the Hospital Materno-Infantil in Málaga. The mothers signed the informed consent form at the beginning of the assessment session, and, subsequently, the evaluators were left alone with the child in order to carry out the assessment.

The session began with the individual administration of the "Cuestionario de Madurez Neuropsicológica Infantil" [Child Neuropsychological Maturity Questionnaire] and, subsequently, the Kaufman Intelligence Test. The tests were administered by three experienced psychologists, and the estimated administration time was 30 and 15 min, respectively.

Finally, some of the authors of the study gathered data on the obstetric and perinatal variables of the selected cases of mothers who agreed to participate in the study through a review of clinical records in the hospital and using the identification number of the selected mothers after identifying them from among all the records of the mothers who gave birth in 2005.

Statistical Analysis

In accordance with the objective and design of this study, multivariate regression analysis was chosen as the main statistical technique. When selecting the most appropriate kind of regression in accordance with the properties of the data, an assessment was conducted *a priori* of the parametric assumptions required of linear regression models (linearity, normality, and homoscedasticity) by means of the analysis of scatter graphs and histograms, and the Kolmogorov–Smirnov test of normality for all variables, considering statistical significance to be proven if the probability associated with the statistic was higher than 0.05 (two-tailed). Having observed its non-fulfillment for almost all the variables, and taking into account the nature of the main independent variable studied here, binary multivariate logistic regression was chosen. In order to apply this technique correctly, all the variables that were originally quantitative, both the independent and the control variables, were dichotomized in order to improve the efficiency of the

analysis and clarity of the interpretation, in accordance with the criteria specified previously.

Before estimating the regression models, the differences between the originally quantitative variables, according to type of delivery, were explored, as well as the bivariate relationship between all the variables studied. These preliminary analyses were conducted with a view to evaluating the main relationships studied, detecting possible masking variables and selecting the most appropriate ones for the regression models. For the analysis of differences, the Mann–Whitney *U* and Student's *t*-test were applied, as applicable. For the analysis of relationships, contingency tables and Pearson's χ^2 independence tests were applied, considered statistically significant if the probability associated with these statistics was less than 0.05 (two-tailed). To quantify the strength of the association of the effect of cesarean birth on verbal, non-verbal, global and general intelligence development, as well as on each control variable, the unadjusted or crude Odds Ratio (*OR*) was estimated without adjusting for the confounding variables and their corresponding 95% confidence intervals (95% *CI*). *OR*s whose intervals did not include the null value (*OR* = 1) were considered statistically significant.

Finally, binary logistic regressions with each dependent variable of the study (verbal and non-verbal development, global development, and general intelligence) were used to evaluate the possible interaction (modification of the main effect studied) between the control variables and the independent variable type of delivery, as well as the possible confusion between the control variables and the main relationship evaluated (the effect of the type of delivery on verbal and non-verbal development, overall development and general intelligence), if statistically possible. In this case, we chose the control variables that, in the bivariate analyses, had more than 10% of cases in each cell, a probability associated with a Pearson's χ^2 statistic of less than 0.05 in the independence tests, and an *OR* whose interval of 95% confidence was statistically significant.

For the construction of the regression models for each dependent variable, the recommendations of Hosmer and Lemeshow (2000) and Kleinbaum and Klein (2001) were followed. It was based on a maximum hierarchical model, in which the statistically significant interactions and the variables implied in them would be conserved, whenever possible. Having eliminated the non-significant interactions sequentially from the model, according to the corresponding test of statistical significance, the possible confounding factors were studied, considering the possible bias on the regression coefficients, the precision (amplitude) of their intervals of confidence and their standard error, as well as non-statistical criteria, such as the change in the magnitude of the *OR*. Hence, confusion would be detected when the magnitude of the *OR*, which evaluates the strength of association between the independent and the dependent variable, changed clinically in an important way (10% between the gross and adjusted association measures) when eliminating a variable from the equation, with respect to the initial model. The variables retained would eventually be included in the construction of the most appropriate model. The goodness of fit of the selected regression models was evaluated using the Likelihood Ratio test and the Hosmer–Lemeshow test, and the Wald Chi-square test for the statistical significance of the regression coefficients. To assess the validity of the models overall, the Nagelkerke adjusted determination coefficient was used. The regression models were estimated manually by the researcher, based on the results obtained in each step. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS), version 23.

RESULTS

Following the descriptive-exploratory analysis of the data, comparisons were made between the means and the means of the ranges of originally quantitative dependent variables (verbal development, non-verbal development, global development, and general intelligence) and the control variables (maternal age, gestational age, weight of newborn, and Apgar-1), as a function of the independent variable 'type of delivery' (vaginal/cesarean). Statistically significant differences were found for verbal development, non-verbal development and global development, as well as general intelligence between the mean and the mean ranges obtained by the 84 children born by vaginal delivery and those of the 40 born by cesarean section.

And no statistically significant differences were found for maternal age, gestational age, or weight of the newborn, or for Apgar 1, between the mean ranges of the children born by vaginal delivery and those born by cesarean section (see **Table 1**).

In summary, statistically significant differences were found in all dependent variables according to the type of delivery, with higher scores in children born vaginally. On the contrary, no statistically significant differences were found in the quantitative control variables according to the type of delivery.

The analysis of the bivariate relationships between the independent variable type of delivery (vaginal/cesarean) and the control variables potentially masking the effect (maternal age, fetal presentation, gestational age, weight of the newborn, and Apgar 1), to detect interactions, is summarized in **Table 2**. There is a statistically significant relationship between type of delivery and fetal presentation [$\chi^2(2,124) = 45.53$, $p < 0.001$].

Tables 3–6 summarize the analyses of the bivariate relationships between the independent variable type of delivery (vaginal birth/cesarean section) and the dependent variables VD, NVD, GD, and GI (presence/absence of disorder) and the potentially effect-masking control variables, respectively. The frequency distributions for each category of the independent variable in relation to the categories of the other variables, along with the significance of the Pearson χ^2 statistic and the *OR* and its corresponding confidence interval, are presented.

In general, of the total sample selected, there were no alterations in VD, NVD, GD, and GI, in 92 (74.2%), 93 (75%), 93 (75%), and 91 (73.4%) children, respectively. Alterations in VD, NVD, GD, and GI were detected in 32 (25.8%), 31 (25%), 31 (25%), and 33 (26.6%) children, respectively (see **Tables 3–6**).

Analyses between the criterion variable VD, the independent variable (type of delivery) and the control variables were found to be related [$\chi^2(2, N = 124) = 6.21$, $p < 0.05$]. Out of the 40 (32.3%) children who were born by cesarean section, 16 (40%)

TABLE 1 | Descriptive and exploratory analysis of originally quantitative variables and statistical tests for means and mean ranks differences.

Variables							Type of delivery									
	N = 124						Vaginal delivery *n* = 84				Cesarean *n* = 40			Statistical test		
	M	*SD*	*Range*	*K–S*	*df*	*p*	*M*	*SD*	*Range*	*MR*	*M*	*SD*	*Range*	*MR*	*U*	*Z*	*p*
VD	21.18	3.70	10–27	0.15	124	0.000*	21.70	3.38	10–27	67.01	20.08	4.13	10–26	53.04	1301.50	−2.04	0.042
NVD	42.94	5.67	23–55	0.13	124	0.000*	43.65	5.56	23–52	68.52	41.45	5.77	29–55	49.85	1174.00	−2.71	0.007
GD	64.12	7.84	39–79	0.11	124	0.000*	65.36	7.46	39–76	69.09	61.53	8.08	45–79	48.66	1126.50	−2.96	0.003
															t	*df*	
GI	48.17	8.92	21–72	0.05	124	0.200	49.50	7.79	29–70	–	45.38	10.48	21–72	–	2.46	122	0.015
WNB	2137.76	432.79	1179–3080	0.05	124	0.200	2154.48	445.26	1310–3080	–	2102.65	408.59	1179–2905	–	0.62	122	0.535
															U	*Z*	
MA	33.24	4.27	22–45	0.09	124	0.006*	32.71	4.03	22–40	59.21	34.35	4.42	28–45	69.40	1404.00	−1.48	0.139
GA	35.14	2.08	32–41	0.08	124	0.020*	35.09	1.99	32–40	62.31	35.25	2.27	32–41	62.90	1664.00	−0.08	0.932
A1	8.41	1.18	4–10	0.43	124	0.000*	8.36	1.23	4–10	61.11	8.53	1.06	5–9	63.85	1586.00	−0.51	0.612

*VD, verbal development; NVD, non-verbal development; GD, global development; GI, general intelligence; WNB, weight of newborn (grams); MA, maternal age (years); GA, gestational age (weeks); A1 (Apgar 1); M, mean; SD, standard deviation; K–S, Kolmogorov–Smirnov test; MR, mean rank; U, U-Mann–Whitney test; t, t-Student test. *The distribution is not normally distributed at 0.05 level.*

did not pass the VD scale, whereas out of the 84 (67.7%) children who were born vaginally, 16 (19%) did not pass the VD scale. The crude *OR* indicates that cesarean section multiplied the probability of presenting alterations in VD by 2.83, *OR* = 2.83, 95% *CI* [1.23, 6.52] (see **Table 3**). Fetal presentation and VD were also significantly related [$\chi^2(2, N = 124) = 3.97, p < 0.05$]. Out of the 44 (35.5%) children with non-cephalic presentation (breech or transverse), 16 (36.4%) did not pass the VD test, and out of the 80 (64.5%) with cephalic presentation, 16 (20%) did not pass the VD test. The crude *OR* indicates that fetal presentation multiplied the probability of presenting alterations in VD by 2.28, *OR* = 2.28, 95% *CI* [1.00, 3.97]. We found no statistical significance between VD and the other control variables evaluated (see **Table 3**).

The variables NVD and type of delivery were also related [$\chi^2(2, N = 124) = 4.92, p < 0.05$]. Out of the 40 (32.3%) children born by cesarean section, 15 (48.4%) did not pass the NVD scale, whereas out of the 84 (67.7%) children who were born vaginally, 16 (19%) did not pass the NVD scale. It appears that birth by cesarean section nearly triples the likelihood of presenting alterations in NVD, *OR* = 2.55, 95% *CI* [1.10, 5.90] (see **Table 4**). Again, we found no statistical significance between NVD and the other control variables considered (see **Table 4**).

A positive relationship was also found between GD and type of delivery [$\chi^2(2, N = 124) = 9.64, p < 0.01$]. Out of the 40 (32.3%) children born by cesarean section, 17 (42.5%) did not pass the GD scale, whereas out of the 84 (67.7%) who were born vaginally, 14 (21%) did not pass it. Cesarean delivery appears to triple the likelihood of presenting alterations in GD, *OR* = 3.69, 95% *CI* [1.58, 5.90] (see **Table 4**). GD and fetal presentation were also related [$\chi^2(2, N = 124) = 9.20, p < 0.01$]. Out of the 44 (35.5%) children with non-cephalic presentation (breech or transverse), 18 (40.9%) did not pass the GD test, and out of the 80 (64.5%) with cephalic presentation, 13 (16.3%) did not pass the GD test. The associated *OR* indicates that fetal presentation multiplies the probability of presenting alterations in GD by 3.57, *OR* = 3.57, 95% *CI* [1.53, 8.30] (see **Table 5**). We found no statistical significance between GD and the other control variables evaluated (see **Table 5**).

The dependent variable GI and the independent variable type of delivery were also related [$\chi^2(2, N = 124) = 5.42, p < 0.05$]. Out of the 40 (32.3%) children born by cesarean section, 16 (40%) did not pass the GI scale, and out of the 84 (67.7%) who were born vaginally, 16 (19%) did not pass this scale. Again, we observed that cesarean delivery almost triples the probability of risk of difficulties in GI, *OR* = 2.62, 95% *CI* [1.15, 6.00] (see **Table 6**). We found no statistical significance between GI and the rest of the control variables (see **Table 6**).

Subsequently, binary logistic regressions were performed for each of the study's dependent variables [verbal development, non-verbal development, global development, and general intelligence (see **Tables 7**, **8**)], which included the clinically plausible control variables that had more than 10% of cases in each cell in the prior bivariate analyses, a probability associated with a Pearson χ^2 statistic lower than 0.05 in the tests of independence and an *OR* with a statistically significant 95% *CI*.

First, the main relationship studied between VD and type of delivery was adjusted by fetal presentation and the interaction

TABLE 2 | Bivariate associations between independent variable (type of delivery) and the control variables (gestational, obstetric, and neonatal variables).

Control variables	Categories	Total	Independent variable Type of delivery		*χ^2	*p*
		N = 124	Vaginal delivery *n* = 84 (67.7%)	Cesarean *n* = 40 (32.3%)		
Maternal age (years)	Under 35	88	64 (72.7%)	24 (27.3%)	3.44[a]	0.063
	Over 35	36	20 (55.6%)	16 (44.4%)		
Gestational age of newborn (weeks)	Over 37	40	29 (72.5%)	11 (27.5%)	0.61[a]	0.434
	Under 37	84	55 (65.5%)	29 (34.5%)		
Gestational age of newborn (weeks)	Over 34	90	60 (66.7%)	30 (33.3%)	0.17[a]	0.677
	Under 34	34	24 (70.6%)	10 (29.4%)		
Fetal presentation	Cephalic	80	71 (88.8%)	9 (11.3%)	45.53[a]	0.000
	Non-cephalic	44	13 (29.5%)	31 (70.5%)		
Weight of newborn (grams)	Over 1500	112	76 (67.9%)	36 (32.1%)	0.07[b]	0.999
	Under 1500	12	8 (66.7%)	4 (33.3%)		
Apgar 1	Over 7	113	76 (57.3%)	37 (32.7%)	0.13[b]	0.755
	Under 7	11	8 (72.7%)	3 (27.3%)		

**Pearson χ^2. [a]0% of cells with an expected frequency less than 5. [b]25% of cells with an expected frequency less than 5.*

TABLE 3 | Bivariate associations between verbal development, type of delivery and the control variables.

Variables	Categories	Total	Dependent variable Verbal development		*χ^2	*p*	*OR*	95% *CI*	
		N = 124	No VD delay *n* = 92 (74.2%)	VD delay *n* = 32 (25.8%)				Lower	Upper
Independent									
Type of delivery	Vaginal	84 (67.7%)	68 (81%)	16 (19%)	6.21	0.013	2.83	1.23	6.52
	Cesarean	40 (32.3%)	24 (60%)	16 (40%)					
Control									
Maternal age (years)	Under 35	88 (71%)	68 (77.3%)	20 (22.7%)	1.50[a]	0.221	1.70	0.72	3.99
	Over 35	36 (29%)	24 (66.7%)	12 (33.3%)					
Gestational age of newborn (weeks)	Over 37	40 (32.3%)	30 (75%)	10 (25%)	0.02[a]	0.887	1.06	0.44	2.53
	Under 37	84 (67.7%)	62 (73.8%)	22 (26.2%)					
Gestational age of newborn (weeks)	Over 34	90 (72.6%)	65 (72.2%)	25 (27.8%)	0.66[a]	0.414	0.67	0.26	1.74
	Under 34	34 (27.4%)	27 (79.4%)	7 (20.6%)					
Fetal presentation	Cephalic	80 (64.5%)	64 (80%)	16 (20%)	3.97[a]	0.046	2.28	1.00	3.97
	Non-cephalic	44 (35.5%)	29 (65.9%)	16 (36.4%)					
Weight of newborn (grams)	Over 1500	112 (90.3%)	83 (71.1%)	29 (25.9%)	0.05[b]	0.999	0.95	0.24	3.76
	Under 1500	12 (9.7%)	9 (75%)	3 (25%)					
Apgar 1	Over 7	113 (91.1%)	84 (74.3%)	29 (25.7%)	0.01[b]	0.999	0.92	0.23	3.70
	Under 7	11 (8.9%)	8 (72.3%)	3 (27.3%)					

*VD, verbal development; OR, odds ratio; CI, confidence interval. *Pearson χ^2. [a]0% of cells with an expected frequency less than 5. [b]25% of cells with an expected frequency less than 5.*

between type of delivery and fetal presentation. Fetal presentation was considered a potential modifier of the main effect under study as well as a potential confounding variable because it was statistically associated with both the independent variable (see **Table 2**) and the dependent variable (see **Table 3**). Following adjustment by VD and type of delivery and eliminating the interaction term [Wald $\chi^2(1, N = 124) = 0.00$, $p = 0.944$] and the variable fetal presentation [Wald $\chi^2(1, N = 124) = 0.37$, $p = 0.541$] in two successive steps, the third estimated model was statistically significant [$\chi^2(1, N = 124) = 5.97$, $p < 0.05$], and the independent variable type of delivery was the only significant variable [Wald $\chi^2(1, N = 124) = 5.98$, $p < 0.05$], with an *OR* = 2.83, 95% CI [1.23, 6.52]. Therefore, cesarean birth is a risk factor for VD delay, such that the *OR* obtained for the variable type of delivery indicates that the risk of VD delay is 2.83 times more likely among infants born by cesarean section than those born vaginally. Regarding the explanatory capacity of this model, 7% of the variability in the response variable is explained by the estimated logistic regression model (Nagelkerke's $R^2 = 0.07$) (see **Table 7**).

TABLE 4 | Bivariate associations between non-verbal development, type of delivery and the control variables.

Variables	Categories	Total *N* = 124	Dependent variable Non-verbal development		*χ^2	*p*	*OR*	95% *CI*	
			No NVD delay *n* = 93 (75%)	NVD delay *n* = 31 (31%)				Lower	Upper
Independent									
Type of delivery	Vaginal	84 (67.7%)	68 (81%)	16 (19%)	4.92	0.027	2.55	1.10	5.90
	Cesarean	40 (32.3%)	25 (62.5%)	15 (48.4%)					
Control									
Maternal age (years)	Under 35	88 (71%)	65 (73.9%)	23 (26.1%)	0.21[a]	0.648	0.81	0.32	2.02
	Over 35	36 (29%)	28 (77.8%)	8 (22.2%)					
Gestational age of newborn (weeks)	Over 37	40 (32.3%)	28 (70%)	12 (30%)	0.78[a]	0.375	0.68	0.29	1.59
	Under 37	84 (67.7%)	65 (77.4%)	19 (22.6%)					
Gestational age of newborn (weeks)	Over 34	90 (72.6%)	65 (72.2%)	25 (27.8%)	1.35[a]	0.245	0.55	0.20	1.50
	Under 34	34 (27.4%)	28 (82.4%)	6 (17.6%)					
Fetal presentation	Cephalic	80 (64.5%)	64 (80%)	16 (20%)	3.00[a]	0.083	2.07	0.90	4.74
	Non-cephalic	44 (35.5%)	28 (63.6%)	15 (34.1%)					
Weight of newborn (grams)	Over 1500	112 (90.3%)	85 (75.9%)	27 (24.1%)	0.49[b]	0.729	1.57	0.43	5.63
	Under 1500	12 (9.7%)	8 (66.7%)	4 (33.3%)					
Apgar 1	Over 7	113 (91.1%)	86 (76.1%)	27 (23.9%)	0.83[b]	0.465	0.55	0.15	2.02
	Under 7	11 (8.9%)	7 (63.3%)	4 (36.4%)					

*NVD, non-verbal development; OR, odds ratio; CI, confidence interval. *Pearson χ^2. [a]0% of cells with an expected frequency less than 5. [b]25% of cells with an expected frequency less than 5.*

TABLE 5 | Bivariate associations between global development, type of delivery and the control variables.

Variables	Categories	Total *N* = 124	Dependent variable Global development		*χ^2	*p*	*OR*	95% *CI*	
			No GD delay *n* = 93 (75%)	GD delay *n* = 31 (25%)				Lower	Upper
Independent									
Type of delivery	Vaginal	84 (67.7%)	70 (83.3%)	14 (21%)	9.64	0.002	3.69	1.58	8.64
	Cesarean	40 (32.3%)	23 (57.5%)	17 (42.5%)					
Control									
Maternal age (years)	Under 35	88 (71%)	65 (73.9%)	23 (26.1%)	0.21[a]	0.648	0.81	0.32	2.02
	Over 35	36 (29%)	28 (77.8%)	8 (22.2%)					
Gestational age of newborn (weeks)	Over 37	40 (32.3%)	30 (75%)	10 (25%)	0.00[a]	0.999	1.00	0.42	2.38
	Under 37	84 (67.7%)	63 (75%)	21 (25%)					
Gestational age of newborn (weeks)	Over 34	90 (72.6%)	67 (74.4%)	23 (25.6%)	0.05[a]	0.816	0.89	0.35	2.25
	Under 34	34 (27.4%)	26 (76.5%)	8 (23.5%)					
Fetal presentation	Cephalic	80 (64.5%)	67 (83.8%)	13(16.3%)	9.20[a]	0.002	3.57	1.53	8.30
	Non-cephalic	44 (35.5%)	26 (59.1%)	18 (40.9%)					
Weight of newborn (grams)	Over 1500	112 (90.3%)	86 (76.8%)	26 (23.2%)	1.96[b]	0.291	2.36	0.69	8.07
	Under 1500	12 (9.7%)	7 (58.3%)	5 (41.7%)					
Apgar 1	Over 7	113 (91.1%)	85 (75.2%)	28 (24.8%)	0.03[b]	0.999	0.88	0.22	3.54
	Under 7	11 (8.9%)	8 (72.3%)	3 (27.3%)					

*GD, global development; OR, odds ratio; CI, confidence interval. *Pearson χ^2. [a]0% of cells with an expected frequency less than 5. [b]25% of cells with an expected frequency less than 5.*

In addition to the main relationship between the dependent variable NVD and the independent variable type of delivery, no significant relationship was found between any of the control variables in the previous analyses (see **Table 4**). Following adjustment by type of delivery, the logistic model was significant [$\chi^2(1, N = 124) = 4.73$, $p < 0.05$], and therefore the independent variable was also significant [Wald $\chi^2(1, N = 124) = 4.76$, $p < 0.05$] with an *OR* = 2.55, 95% *CI* [1.10, 5.90]. According to the estimated model, it was observed that cesarean delivery is also a risk factor for NVD delay. In addition, the *OR*

TABLE 6 | Bivariate associations between general intelligence, type of delivery and the control variables.

Variables	Categories	Total *N* = 124	Dependent variable General intelligence		*χ^2	*p*	*OR*	95% *CI*	
			No GI delay *n* = 91 (73.4%)	GI delay *n* = 33 (26.6%)				Lower	Upper
Independent									
Type of delivery	Vaginal	84 (67.7%)	68 (81%)	16 (19%)	5.42	0.020	2.62	1.15	6.00
	Cesarean	40 (32.3%)	24 (60%)	16 (40%)					
Control									
Maternal age (years)	Under 35	88 (71%)	68 (77.3%)	20 (22.7%)	2.34[a]	0.126	1.92	0.82	4.46
	Over 35	36 (29%)	23 (63.9%)	13 (36.1%)					
Gestational age of newborn (weeks)	Over 37	40 (32.3%)	32 (80%)	8 (20%)	1.32[a]	0.250	1.69	0.68	4.19
	Under 37	84 (67.7%)	59 (70.2%)	25 (29.8%)					
Gestational age of newborn (weeks)	Over 34	90 (72.6%)	64 (71.1%)	26 (28.9%)	0.87[a]	0.351	0.63	0.24	1.64
	Under 34	34 (27.4%)	27 (79.4%)	7 (20.6%)					
Fetal presentation	Cephalic	80 (64.5%)	61 (76.3%)	19 (23.8%)	0.94[a]	0.331	1.49	0.66	3.39
	Non-cephalic	44 (35.5%)	30 (68.2%)	14 (31.8%)					
Weight of newborn (grams)	Over 1500	112 (90.3%)	83 (74.1%)	29 (25.9%)	0.31[b]	0.731	1.43	0.40	5.11
	Under 1500	12 (9.7%)	8 (66.7%)	4 (33.3%)					
Apgar 1	Over 7	113 (91.1%)	81 (71.7%)	32 (28.3%)	1.89[b]	0.285	3.95	0.48	32.13
	Under 7	11 (8.9%)	10 (90.9%)	1 (9.1%)					

*GI, general intelligence; OR, odds ratio; CI, confidence interval. *Pearson χ^2. [a]0% of cells with an expected frequency less than 5. [b]25% of cells with an expected frequency less than 5.*

TABLE 7 | Multivariate logistic regression analysis for verbal and non-verbal development, adjusted by potential interaction and confounding factors.

Variables		*b*	*SE*	Wald χ^2	*df*	*p*	*OR*	95% *CI*	
								Lower	Upper
VD									
Model 1	**Type of delivery**[(a)]	0.80	0.77	1.08	1	0.298	2.23	0.49	10.10
	Fetal presentation[(b)]	0.29	0.72	0.16	1	0.688	1.33	0.32	5.55
	Type of delivery × Fetal presentation	0.07	1.07	0.00	1	0.944	1.07	0.13	8.91
	Constant	−1.49	0.36	23.75	1	0.000	0.22		
	*χ^2(3, *N* = 124) = 6.34, *p* = 0.096								
Model 2	**Type of delivery**[(a)]	0.84	0.53	2.46	1	0.116	2.32	0.81	6.62
	Fetal presentation[(b)]	0.32	0.53	0.37	1	0.541	1.38	0.48	3.94
	Constant	−1.50	0.29	25.99	1	0.000	0.22		
	*χ^2(2, *N* = 124) = 6.34, *p* = 0.042; χ^2(2, *N* = 124) = 0.05, *p* = 0.997; R^2 = 0.07								
Model 3	**Type of delivery**[(a)]	1.04	0.42	5.98	1	0.014	2.83	1.23	6.52
	Constant	−1.44	0.27	27.11	1	0.000	0.23		
	*χ^2(1, *N* = 124) = 5.97, *p* = 0.015; R^2 = 0.07								
NVD									
Model 1	**Type of delivery**[(a)]	0.93	0.43	4.76	1	0.029	2.55	1.10	5.90
	Constant	−1.44	0.27	27.11	1	0.000	0.25		
	*χ^2(1, *N* = 124) = 4.73, *p* = 0.030; R^2 = 0.05								

*VD, verbal development; NVD, non-verbal development; OR, odds ratio; CI, confidence interval. Variables reference categories: (a) = Vaginal delivery; (b) = Cephalic. *Goodness-of-fit tests for logistic regression models: Global test χ^2; Hosmer–Lemeshow χ^2; Nagelkerke R^2.*

obtained indicated that the risk of NVD delay is 2.55 times more likely among infants born by cesarean section than among those born vaginally. Regarding the explanatory capacity of the model, 5% of the variance of the NVD variable is explained by the estimated model (Nagelkerke's R^2 = 0.05) (see **Table 7**).

As with VD, the main relationship studied between GD and type of delivery was adjusted by fetal presentation and interaction between type of delivery and fetal presentation because, in this case, fetal presentation was statistically related both to the independent variable (see **Table 2**) and the dependent variable (see **Table 5**). After eliminating the interaction term [Wald $\chi^2(1, N = 124) = 0.22$, $p = 0.636$] and the variable fetal presentation [Wald $\chi^2(1, N = 124) = 2.14$, $p = 0.143$] in two successive steps, the third estimated model was significant [$\chi^2(1, N = 124) = 9.21$, $p < 0.01$], and only the independent variable type of delivery was significant [Wald $\chi^2(1, N = 124) = 9.08$, $p < 0.01$], with an *OR* = 3.69, 95% *CI* [1.58, 8.64]. Therefore, cesarean birth is a risk factor for GD delay, such that the *OR* obtained for the variable type of delivery indicated that the risk of NVD delay was 3.69 times more likely among infants born through cesarean section than those born vaginally. 10% of the variance for the variable non-verbal development is explained by the estimated model (Nagelkerke's $R^2 = 0.10$) (see **Table 8**).

Regarding the dependent variable GI, no significant relationship was found between type of delivery and the control variables in the previous analyses performed (see **Table 6**), in addition to the main relationship studied. Therefore, GI was adjusted by type of delivery, generating a significant estimated binary logistic model [$\chi^2(1, N = 124) = 5.22$, $p < 0.05$], and therefore, the independent variable type of delivery as well [Wald $\chi^2(1, N = 124) = 5.24$, $p < 0.05$] with an *OR* = 2.62, 95% *CI* [1.14, 6.00]. Therefore, birth by cesarean section was again a risk factor for developing GI difficulties, such that the *OR* obtained for the variable type of delivery indicated that the risk of GI difficulties was 2.62 times more likely among infants born through cesarean section than those born vaginally. In relation to the variance of the response variable explained by the model, 6% of the variance found for the variable GI is explained by the variable included in the estimated model (Nagelkerke's $R^2 = 0.06$) (see **Table 8**).

DISCUSSION

The objective of this study was to obtain an unbiased estimate of the relationship between neuropsychological development and general intelligence on the one hand, and type of delivery of the other.

The results confirm these relationships and indicate that cesarean delivery in twin births is a possible risk factor, independent of the other factors considered, for the presence of disorders in VD, NVD, and GD and for difficulties in the children's GI. None of the control variables considered modified the effect of type of delivery on neuropsychological development and general intelligence. Specifically, the risk of having a neuropsychological developmental disorder and intellectual difficulties at 6 years of age is about three times more likely among newborns in twin births through cesarean section than those born vaginally. However, the discrete explanatory capacity of the estimated models must be considered, which agrees with the final inclusion of a single independent variable.

TABLE 8 | Multivariate logistic regression analysis for global development and general intelligence, adjusted by potential interaction and confounding factors.

Variables		*b*	*SE*	Wald χ^2	*df*	*p*	*OR*	95% *CI*	
								Lower	Upper
GD									
Model 1	**Type of delivery**[(a)]	1.11	0.78	2.01	1	0.156	3.05	0.65	14.21
	Fetal presentation[(b)]	0.99	0.69	2.08	1	0.149	2.71	0.70	10.50
	Type of delivery × Fetal presentation	−0.49	1.05	0.22	1	0.636	0.61	0.07	4.78
	Constant	−1.80	0.34	28.09	1	0.000	0.16		
	*$\chi^2(3, N = 124) = 11.54$, $p = 0.009$; $\chi^2(2, N = 146) = 0.00$, $p = 0.999$; $R^2 = 0.13$								
Model 2	**Type of delivery**[(a)]	0.83	0.53	2.42	1	0.120	2.31	0.80	6.62
	Fetal presentation[(b)]	0.78	0.53	2.14	1	0.143	2.19	0.76	6.25
	Constant	−1.75	0.32	30.43	1	0.000	0.17		
	*$\chi^2(2, N = 124) = 11.32$, $p = 0.003$; $\chi^2(2, N = 124) = 0.22$, $p = 0.894$; $R^2 = 0.13$								
Model 3	**Type of delivery**[(a)]	1.31	0.43	9.08	1	0.003	3.69	1.58	8.64
	Constant	−1.61	0.29	30.22	1	0.000	0.20		
	*$\chi^2(1, N = 124) = 9.21$, $p = 0.002$; $R^2 = 0.10$								
GI									
Model 1	**Type of delivery**[(a)]	0.96	0.42	5.24	1	0.022	2.62	1.14	6.00
	Constant	−1.37	0.27	25.50	1	0.000	0.25		
	*$\chi^2(1, N = 124) = 5.22$, $p = 0.022$; $R^2 = 0.06$								

*GD, global development; GI, general intelligence; OR, odds ratio; CI, confidence interval. Variables reference categories: (a) = Vaginal delivery; (b) = Cephalic. *Goodness-of-fit tests for logistic regression models: Global test χ^2; Hosmer–Lemeshow χ^2; Nagelkerke R^2.*

The results, therefore, are in line with studies that establish that the type of delivery involves risks for babies, and that, in particular, cesarean sections present more risk than vaginal births in the psychological development of children (Hogle et al., 2003; Villar et al., 2007; Pasupathy and Smith, 2008; Wallin et al., 2010; Asztalos et al., 2016). Most of these studies relate birth by cesarean section to poorer neonatal outcomes, even after eliminating confounding factors related to the loss of fetal intrapartum well-being (Smith et al., 2004; Villar et al., 2007; Liston et al., 2008). Only in the case of breech presentations do cesareans provide greater protection compared to vaginal birth, both in single (Villar et al., 2007) and multiple pregnancies (Hogle et al., 2003). These unfavorable results after birth by cesarean section have been linked to onsets of respiratory distress and transient tachypnea secondary to a minor release of catecholamines by the fetus when it avoids the birth canal (Villar et al., 2007; Polidano et al., 2017), or even to defects in the expression of certain genes (UCP2) in the neurons of the fetal hippocampus in response to the absence of the physiological stress associated with vaginal delivery (Simón-Areces et al., 2012).

Several mechanisms could link the mode of delivery and child development. First, a cesarean section reduces the probability of breastfeeding and difficult maternal bonding, and induces changes in infant microbiomes, contributing to changes in the children's metabolic pathways (Ajslev et al., 2011). Biochemical differences have been described in animal studies according to the route of birth. Dopamine concentrations in some areas of the prefrontal cortex, nucleus accumbens and striatum of rats and guinea pigs may be different depending on the mode of delivery, inducing differences in dopamine-mediated behaviors (El-Khodor and Boksa, 1997; Vaillancourt and Boksa, 2000). Additionally, amygdala and thalamus noradrenaline concentrations in adult rats may differ according to cesarean or vaginal birth (El-Khodor and Boksa, 2003). Although it is difficult to know the real implications of these differences in children, some studies show the influence of cesarean deliveries on an offspring's microbiome. These studies indicate an alteration in the central nervous system that would affect short-term memory, motivation, mood and reactivity to stress, and raise questions about its long-term effects (Cryan and Dinan, 2012; Galland, 2014).

On the order hand, the rate of prematurity in the study sample is high in relation to the prevalence of prematurity in our environment. According to the data provided by the Andalusian Institute of Statistics (Institute of Statistics and Cartography of Andalusia, 2012), in our community, 5.9% of single pregnancies and 51.3% of twin pregnancies ended prematurely. The proportion of premature births in the sample is 67.7%, significantly higher than expected, which may be related to local clinical criteria, which reveal the existence of some bias in sample selection because the sample was made up of twin pregnancies that ended at a maximum level of complexity, which is an obstetric and perinatal center in the context of health-care in the province. However, we selected births from pregnancies that were more than 32 weeks, attempting to avoid cases in which extreme prematurity would directly influence the children's cognitive development outcomes. The long-term effect of extreme prematurity is well documented (Wood et al., 2000). There is increased risk of cerebral palsy as well as problems in the development of basic or cognitive executive functions, conditioning low academic performance (Marlow et al., 2005, 2007; Wolke et al., 2008). The long-term effects of moderate (32–34 weeks) or late prematurity (34–37 weeks) are less obvious and, although there are some studies that reveal the existence of certain difficulties at school and in the cognitive development of infants born between 34 and 37 weeks (Lindstrom et al., 2007; Morse et al., 2009; Chyi et al., 2010), they have received less attention and there is less evidence of this (Odd et al., 2012). In the sample studied, 63% of the children born prematurely were born between 34 and 37 weeks, so the impact of prematurity on the outcomes of school performance, cognitive development, and intelligence is reduced. In addition, it is necessary to consider that any association between prematurity and psychological development may be due more to the causes of prematurity than to prematurity itself (Odd et al., 2012). To control the biases, we tried to control obstetric and prenatal variables, although it was not possible to obtain the effect of the type of delivery on the dependent variables analyzed, controlling the possible bias of the cesarean section, due to the number of cesarean sections found in the study sample. The relationship of this variable and other obstetric and perinatal variables (for example: antenatal drug/toxin exposure, congenital infection, and respiratory distress) with the psychological disorders of children would be relevant to analyze in larger samples in future studies. Likewise, factors in the initial design that could be related both to psychological development and prematurity have not been considered, as is the case, for example, with the mental health of parents and social class (Strenze, 2007; von Stumm et al., 2010; Odd et al., 2012; Polidano et al., 2017). These variables, however, could be analyzed together with prenatal and obstetric variables in later studies since, according to some studies, they are related with psychological development and intelligence. The lower the parental level of education and mental health, the lower the quality found in parental education practices, and in the development of the children (Strenze, 2007; von Stumm et al., 2010; Odd et al., 2012; Polidano et al., 2017).

On the other hand, although there is not a complete consensus in the literature (Ravi et al., 2018; Sacchi et al., 2018), we agree on the possible impact of intrauterine growth restriction (IUGR) on the long-term neurocognitive development of the children. The effects could be related to the hemodynamic and metabolic changes due to chronic fetal hypoxemia, fetal tissue hypoxia and acidosis that may occur during pregnancy (frequently due to placental insufficiency). The lack of information about the impact of IUGR on neurodevelopment can be considered one of the limitations of our study. In our initial design we included the assessment of the effects of fetal weight on neurodevelopment but, unfortunately, umbilical or cerebral arteries doppler velocimetry indexes were not available for most of the cases. As a short-term respiratory or metabolic outcome marker, we registered the scores of the Apgar test at the first and the fifth minute after birth, finding that although 8.9% of the children (11 cases) scored under 7 at first minute, the fifth minute score was fortunately normal in all cases, suggesting

a good respiratory and metabolic state. The long-term effects of intrauterine growth restriction and specifically mild chronic hypoxemia could be considered in future studies.

The results confirm the importance of prenatal and perinatal variables, such as type of delivery, in psychological development, as shown by some studies (Calame et al., 2004; David and Dean, 2007; Lollar and Cordero, 2007; Cattani et al., 2010; Dall'oglio et al., 2010; Bidzan and Bieleninik, 2013; González-Valenzuela et al., 2015a; Asztalos et al., 2016). In addition, they are in line with those studies that establish negative effects of cesarean section on children's microbiome and on psychological development (Hogle et al., 2003; Villar et al., 2007; Wallin et al., 2010; Cryan and Dinan, 2012; Galland, 2014; Asztalos et al., 2016; Polidano et al., 2017). However, these results should be considered with caution given the size of the sample, due to the difficulties encountered in assembling it. The creation of a provincial and national census on multiple births carried out by health institutions, as occurs in other countries (Bentley et al., 2016), would facilitate the availability of larger samples for study, as well as to carry out a follow-up and prevention of the possible psychological problems that this population may have. In future studies, it would be necessary to continue applying retrospective cohort designs to larger samples and at younger ages (2–3 years old) in order to generalize the results and ascertain whether the link between type of delivery and psychological development and intelligence is also manifested before compulsory schooling. It would also be interesting to carry out randomized prospective studies along the lines suggested by some authors (Barzilay et al., 2015), in order to find out if the difficulties in children's psychological development diminish with time or if, on the contrary, they cause difficulties in school learning.

Finally, one of the implications of the results obtained could be that, in clinical practice, it might be advisable to carry out programs aimed at health professionals and parents to disseminate the risks of the type of delivery, in order to make known the effects of cesarean births, even in twins, with the idea of avoiding performing cesareans on demand or without medical indication. On the other hand, in light of the results obtained, another implication could be that detection protocols should be activated for possible infantile psychological difficulties in double births by cesarean at a very early age, through the neonatal and pediatric services within the health system.

AUTHOR CONTRIBUTIONS

EG-M, DL-M, and M-JG-V contributed conception and design of the study. DL-M, OC-G, and EG-M organized the database. DL-M performed the statistical analysis. DL-M, EG-M, and M-JG-V wrote sections of the manuscript. All authors wrote the first draft of the manuscript and contributed to manuscript revision, read and approved the submitted version.

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APPENDIX

TABLE A1 | Child Neuropsychological Maturity Questionnaire -CUMANIN- (Portellano et al., 2009).

Tests	Task/objective	Development area
Articulation	Repeating 15 words of increasing articulatory difficulty	VD
Expressive language	Repeating 4 sentences of increasing difficulty	
Comprehensive language	Answering 9 questions about the content of a story the child has heard	
Psychomotricity	Hopping on one leg Touching the nose with the finger Stimulation of the fingers Walking in balance Jumping with feet together Remain squatting with arms crossed Touching all the fingers of the hand	NVD
Spatial structuring	Performing 12 activities of spatial orientation through increasing difficulty Offered a psychomotor Graphomotor response	
Visuoperception	Reproducing 15 geometric drawings of increasing complexity	
Iconic memory	Memorizing 10 drawings of simple objects	
Rhythm	Reproducing 7 rhythmic series of increasing difficulty, through auditory presentation	

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MINI REVIEW
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Early Life Stress, Physiology, and Genetics: A Review

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Early life stress (ELS) is a widely studied concept due to both its prevalent nature and its (presumed) detrimental consequences. In this review, we discuss the relationship between ELS and its underlying physiology spanning the sympathetic nervous system, hypothalamic-pituitary-adrenal axis, and markers of inflammation related to immune function in both human and animal literature. We also consider the potential role of genetic and epigenetic factors on the ELS-health outcome relationship. We conclude with recommendations to overcome identified shortcomings in a field that seeks to address the health consequences of ELS.

Keywords: early life stress, sympathetic nervous system, hypothalamic-pituitary-adrenal axis, inflammation, genetics, epigenetics

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INTRODUCTION

Early life stress (ELS), including physical, sexual, and emotional forms of abuse and neglect experienced by the developing child (Centers for Disease Control and Prevention, 2018), has been linked to a host of physical and psychological sequelae into adulthood (Chapman et al., 2004; Gluckman et al., 2008; Middlebrooks and Audage, 2008; Pechtel and Pizzagalli, 2011). Such adverse experiences are surprisingly common, according to a large-scale epidemiological study on adverse childhood events, which reports that approximately 65% of people in the United States experienced at least one, and 12.5% experienced as many as four, adverse early life events (Middlebrooks and Audage, 2008). Given these statistics, a deeper understanding of the pathophysiology of ELS could produce better long-term prognosticators of adverse sequelae for vulnerable individuals, and promote the development of patient-centered interventions with specific strategies to mitigate the ill effects of ELS.

To elucidate the connection between ELS and negative health outcomes, research examined putative physiological mediators, including the sympathetic nervous system (SNS), the hypothalamic pituitary adrenal (HPA) axis, and cytokines linked to inflammation (Mayer, 2000; Pariante and Lightman, 2008). Dysregulation of these systems has been associated with a host of disorders in and of themselves (Cohen et al., 2000; Cowen, 2010; Lamers et al., 2013). In this review, we will examine the connections between ELS and these physiological pathways in both human adults and mature non-human animals. We will additionally consider the potential role of candidate gene polymorphisms and DNA methylation in genes linked to the stress response and to mood disorders as moderators of the ELS-negative health outcome relationship. Finally, we will offer recommendations for a growing field examining the effects of ELS into adulthood.

AN OVERVIEW OF ACUTE STRESS PHYSIOLOGY AND INFLAMMATORY MARKERS

Stress promotes physiological responses across several different systems, most prominently the SNS, HPA axis, and immune system, which are heavily intertwined (Chrousos, 2009). For instance, brain regions processing sensory and psychological stressors activate preganglionic neurons in brain stem and spinal cord to activate the peripheral SNS. This then prepares the body to "fight or flee" by releasing catecholamines such as epinephrine and norepinephrine to increase heart rate and blood pressure. In parallel, stress activates the HPA axis, leading to the release of corticotropin-releasing factor (CRF) by the hypothalamus, followed by the release of adrenocorticotropin-releasing hormone (ACTH) onto CRH-R1 receptors from the pituitary, and subsequent release of glucocorticoids (including cortisol in humans and corticosterone in animals) from the adrenal glands (Lupien et al., 2009). Both activation of the SNS and HPA axis are correlated with inflammatory markers, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6) (Chrousos and Gold, 1992; Tsigos and Chrousos, 2002; Padgett and Glaser, 2003).

For the purposes of this review, we will focus on these and similar markers to assess the impact of ELS on stress physiology. **Figure 1** presents an overview of these markers, their relationship to one another, and their potential changes in the presence of stress.

EARLY LIFE STRESS, SYMPATHETIC NERVOUS SYSTEM, HYPOTHALAMIC-PITUITARY-ADRENAL AXIS, AND INFLAMMATION IN HUMANS

Several studies have found a relationship between ELS and cardiovascular/HPA axis effects. For instance, Dong et al. (2004) saw a direct relationship between ELS and ischemic heart disease later in life, although this was partially mediated by presence of additional psychological risk variables such as anger levels and depressed affect. Gatt et al. (2009) observed that an increase in the number of ELS events in healthy adult participants was associated with higher resting and activated heart rates. Similarly, Heim et al. (2000) reported elevated heart rate in response to the Trier Social Stress Test (TSST) in a group of depressed women who had experienced sexual and/or physical abuse early in life, compared to non-depressed women without ELS. Within the same group, depressed ELS women displayed heightened cortisol reactivity and dysregulated ACTH (the latter was seen in women with ELS regardless of depression status; Heim et al., 2000). In line with a heightened cortisol response, individuals in a community-based sample with ELS and a psychiatric history displayed poorer recovery and exhibited significantly stronger cortisol reactivity to a cognitive stress task, compared to the non-ELS group (Goldman-Mellor et al., 2012). Yet, others reported *diminished* cortisol associated with ELS. For instance, women who had experienced ELS showed lower baseline cortisol levels preceding a

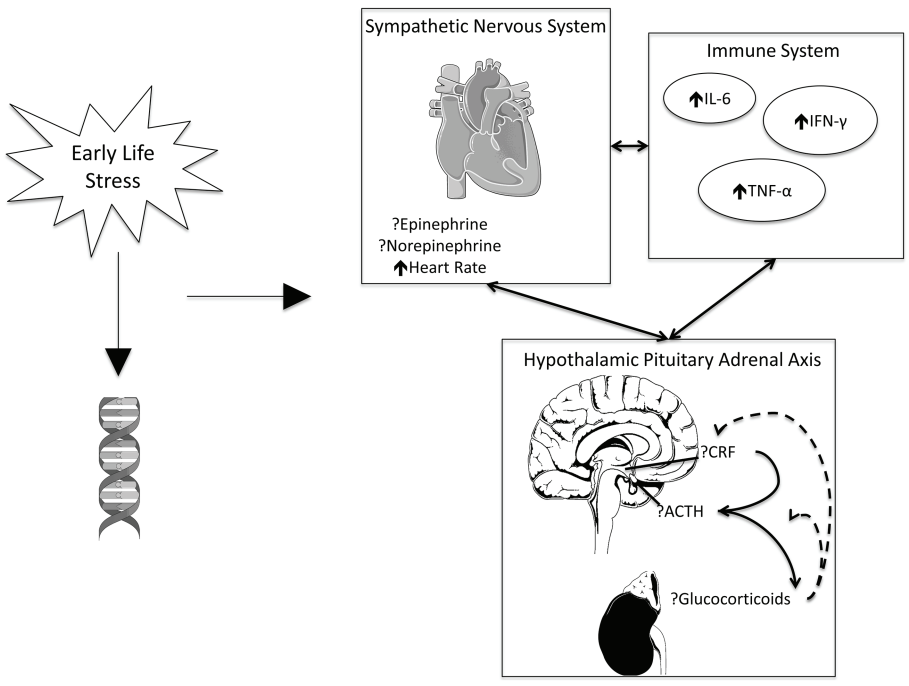

FIGURE 1 | ELS may impact gene methylation. ELS, working in conjunction with genetics, is associated with increased heart rate and inflammation. Evidence is inconsistent on the effects of catecholamines and signaling pathways associated with the HPA axis. The SNS, HPA axis, and immune system exhibit reciprocal effects on one another. Images adapted from open license image site smart.servier.com.

corticotropin-releasing factor (CRF) stimulation test (a bolus of CRF given in the afternoon), but produced a similar post-stressor cortisol response, compared to non-ELS controls (Heim et al., 2001). ELS was also associated with blunted cortisol in another common HPA outcome measure, the cortisol awakening response, in a different study (Li et al., 2015). Given the conflicting literature, we conducted a comprehensive meta-analysis, in which we found no significant association between early life stress and cortisol (Fogelman and Canli, 2018). We did, however, note strong heterogeneity across studies, suggesting that "type of stressor" may be an important moderator of subsequent cortisol physiology. For instance, experiencing abuse was associated with increased cortisol awakening response levels, suggesting that some forms of trauma may be more severe and hence have a more profound and lasting impact on physiology.

ELS is also associated with increased inflammation, both basally and in response to acute stress. One longitudinal study that followed participants since childhood, demonstrated a link between ELS and high-sensitivity C-reactive protein (hsCRP) levels (Danese et al., 2007). ELS was also associated with heightened IL-6 in response to the TSST (Carpenter et al., 2010) and with heightened IL-1β, IL-12, and TNF-α levels (Li et al., 2015) in healthy community samples. In a patient cohort of depressed individuals, there was no significant main effect between ELS and inflammatory markers; however, depressed patients displayed greater ELS and an increase in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and IL-6 in response to the TSST (Pace et al., 2006).

In addition to ELS being linked to the SNS, HPA axis, and immune system, these systems have been shown to influence one another. For instance, Fagundes and Way (2014) proposed that increased sympathetic activity and dysregulated cortisol attributed to ELS may persist to low-grade inflammation over time. The "neuro-immune network" hypothesis by Nusslock and Miller (2016) puts forth the notion that ELS strengthens cortico-amygdala neural circuitry, thus "priming" and responding to heightened SNS, HPA axis, and immune function over time. Taken together, these findings suggest that ELS, at least in certain groups and under certain conditions, has a potent effect on mediating pathways related to the cardiovascular system/HPA axis and markers of inflammation.

EARLY LIFE STRESS, THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS, AND INFLAMMATION IN ANIMALS

In addition to studying the effects of ELS on health in human subjects, results from animal studies may offer helpful insights given the greater ability to manipulate experimental conditions and the simplified nature conditions surrounding ELS relative to human studies. Overall, experimental studies conducted in animal models of ELS tend to concur with human data suggesting heightened reactivity following ELS. For instance, one study found that rats who as pups had experienced maternal separation for either 15 or 180 min per day displayed higher levels of neuropeptide-y (a highly prevalent peptide associated with the CNS) than non-handled controls (Husum and Mathe, 2002), mirroring greater CNS reactivity we often see in human studies of ELS. In the same study, neuropeptide-y/CRF ratios were significantly lower in the maternal separation groups and the effect was dose dependent, such that more maternal separation was associated with lower ratios (Husum and Mathe, 2002); greater neuropeptide-y has been seen as protective in depression-like behavior in rats (Stogner and Holmes, 2000), therefore a lower ratio represents a concern for increased rates of depression-like behavior in the presence of ELS. Other maternal separation studies reported elevated heart rate and inflammatory responses to a physiological stressor (Loria et al., 2010), as well as elevated corticosterone, TNF-α, and IFN-γ levels, and increased production of anxiety-like behaviors (O'Mahony et al., 2009) in maternally deprived rats, compared to controls. In mice, ELS has also been linked with heightened basal corticosterone (Murgatroyd et al., 2009). These findings bolster concerns of up-regulation of physiological response, similar to that seen in human studies.

Yet, as with the human literature, reports on the impact of ELS on physiology are not always consistent. For instance, social isolation in early development was associated with *lower* corticosterone levels in a recovery period following restraint-stress exposure in adult rats (Lukkes et al., 2009). According to one review, rats and primates experiencing maternal separation early in life overall display heightened CRF, but diminished ACTH and glucocorticoid levels; in primates, these levels are initially elevated during development, suggesting that the timing of when measurements are obtained may be an important moderating factor (Pryce et al., 2002). On the whole, the animal literature mostly supports the conclusion that animal models of ELS produce later increases in SNS, HPA axis, and inflammatory markers. The larger degree of consistency across animal studies, compared to the human literature, may have been aided by greater experimental controls, more uniform models of ELS, and homogeneity in the choice of rodent strains.

EARLY LIFE STRESS AND NEGATIVE HEALTH OUTCOMES: THE ROLE OF GENETICS

Thus far, we have reviewed evidence surrounding ELS and its relationship to the SNS, HPA axis, and immune function, as these may be pathways to negative health later in life. However, ELS may also lead to poorer health outcomes through genetic mechanisms. Indeed, numerous studies have considered the role of genetics, both at the structural level of gene variants and at the level of epigenetic regulation of DNA expression (Heim and Binder, 2012). Variants of well-known candidate genes may potentially increase susceptibility to stressful environmental conditions, increasing risk for mood and anxiety disorders (Nugent et al., 2011). In a seminal longitudinal study of approximately 1,000 individuals, Caspi et al. (2003) reported a significant interaction between presence of the serotonin-transporter-linked polymorphic region (5-HTTLPR) S allele

and life stress history predicting depressive symptoms. Later replication studies produced conflicting results and meta-analyses came to opposite conclusions. One the one hand, one meta-analysis concluded that ELS significantly interacted with presence of the S allele in 5-HTTLPR of *SLC6A4* to predict greater stress sensitivity and risk of depression (Karg et al., 2011). On the other hand, a recent large-scale meta-analysis used harmonized analyses across 31 previously published datasets representing more than 38,000 individuals and failed to identify an interaction between the 5-HTTLPR S allele, life stress, and depression (Culverhouse et al., 2018). The authors concluded that "if an interaction exists in which the S allele of 5-HTTLPR increases risk of depression only in stressed individuals, then it is not broadly generalisable, but must be of modest effect size and only observable in limited situations" (p. 134).

Some other candidate genes included *FKBP5* (a co-chaperone to glucocorticoid receptors as part of the HPA axis leading to stress response regulation and anxiety); the gene encoding the brain-derived neurotrophic factor, *BDNF* (hypothesized role in mood disorders; Nugent et al., 2011); and the oxytocin receptor gene, *OXTR* (oxytocin receptors are concentrated in the hypothalamus and dysregulation has been associated with depression and anxiety). For example, Binder et al. (2008) identified four SNPs in the *FKBP5* gene interacting with ELS to predict heightened PTSD symptom severity; similar findings were reported by Klengel et al. (2013), however their results provided robust findings for only the rs1360780 SNP. Presence of the met allele in the val66met *BDNF* polymorphism in conjunction with ELS predicted greater depression and anxiety symptoms, although this was specifically mediated through diminished left prefrontal cortex brain volume and heightened heart rate (Gatt et al., 2009). The rs139832701 SNP in *OXTR* interacted with ELS to predict increased stress and depression scores (Myers et al., 2014).

Candidate gene studies have often been criticized for being statistically underpowered, and large-scale replication attempts have not supported previously reported gene-by-environment interactions. In addition to the Culverhouse et al. (2018) study, another recent large-state analysis imputed data (i.e., did not genotype directly, but inferred genotype from tagging single nucleotide polymorphisms) from large population-based and case-control samples (Ns ranging from 62,138 to 443,264 across subsamples) for 18 candidate genes (including *SLC6A4* and *BDNF,* but not *FKBP5* or *OXTR*) and also failed to find support for any main effect or interaction predicting depression (Border et al., 2019). These authors went further than Culverhouse et al. (2018), concluding "it is time for depression research to abandon historical candidate gene and candidate gene-by-environment interaction hypotheses" (p. 386).

The criticism of candidate genes raises important concerns about sample size and false positive results. Yet, it is also possible that analyses that are limited to DNA sequence variations and one specific disease outcome miss much of the complexity by which genes, life experience, and health outcomes are interrelated. For example, it is possible that additional mechanisms that have not been considered in the prior literature, such as alternative splicing of the human *SLC6A4* (Bradley and Blakely, 1997), contribute to inconsistent findings across study population. The effects of alternative splicing, as well as epigenetic mechanisms (discussed next), would be evident at the level of mRNA or protein expression, for which there are currently only limited datasets available.

The current literature on epigenetics and life stress suggests important mechanisms linking ELS to epigenetic gene regulatory mechanisms, particularly DNA methylation. DNA methylation is the process by which a methyl group binds to, most typically, Cytosin-Guanine (CpG) sites on DNA and regulates its expression (for further information see Moore et al., 2013). Such methylation may be caused by life stress. As early as *in utero*, experiences related to maternal nutrition and health can lead to an increased risk for metabolic dysregulation (e.g., in insulin and leptin) later in life with ELS-induced adaptations in DNA methylation as a proposed pathway to such a relationship (Gluckman et al., 2008). Therefore, examining how ELS is associated with methylation may provide insight into one pathway toward dysregulation.

Several studies have reported increased DNA methylation in the presence of ELS. First reported in rats in a seminal study by Weaver et al. (2004), poor maternal care was linked to increased methylation of the glucocorticoid receptor (GR) gene promoter, reduced GR expression in the hippocampus, and increased adult stress reactivity, which could be reversed with administration of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). A study by McGowan et al. (2009) extended these findings to humans, showing increased GR methylation and reduced GR mRNA expression in the hippocampus of suicide victims with a history of childhood abuse, compared to suicide victims without childhood abuse and non-suicide controls. In other work, adult participants who had experienced physical abuse as children had higher *SCL6A4* DNA methylation in blood relative to those who had not (Beach et al., 2010). Those with major depressive disorder (MDD) who had experienced ELS also exhibited a greater percentage of average methylation of *SLC6A4* in blood cells compared to those with MDD who had not experienced ELS (Kang et al., 2013). In those with PTSD, the ELS group had a greater proportion of transcripts with methylated CpG sites in blood cells compared to the no ELS group (Mehta et al., 2013). Evidence for ELS and methylation exists across species as well. Rats that experienced abusive behavior during development by stressed mothers showed increased DNA methylation of *BDNF* in the prefrontal cortex at exons IV and IX (Roth et al., 2009). Finally, the presence of methylation may be a function of both genotype and the presence of ELS. For instance, Duman and Canli (2015) found that presence of the S allele in the serotonin-transporter-linked polymorphic region (5-HTTLPR) interacted with ELS to predict greater average methylation of *SCL6A4* in blood samples; however, this did not correspond to downstream mRNA expression, suggesting more than just DNA methylation effects need to be considered. ELS interacted with rs1360780 in *FKBP5* such that presence of the T allele in conjunction with ELS was associated with decreased methylation percentage of intron 7 in blood cells (Klengel et al., 2013). This evidence

collectively suggests that methylation may represent one pathway to poorer health.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

It is now widely accepted that ELS is linked to significant adverse sequelae in adulthood (Enoch, 2011; Shonkoff et al., 2012). However, this association needs to be better understood in its nuances. For instance, different types of ELS may be differentially associated with health risk; although most forms of ELS are associated with increased risk of psychopathology, surprisingly, this association was weaker for forms of physical neglect (Carr et al., 2013). This may stem from discrepancies in the severity of ELS types or from differences in the response (or lack thereof) to different ELS types. Negative consequences of ELS may be most pronounced when the stress takes on its most severe forms (Caspi et al., 2003; Kendler et al., 2004), when there are numerous instances of ELS (Heim et al., 2002; Dube et al., 2003; Myers et al., 2014), or in the context of other moderating factors. This includes, but is certainly not limited to, genetic disposition (Nugent et al., 2011) and ELS-related epigenetic modifications of genes involved in the stress systems (Heim and Binder, 2012).

Another consideration is that ELS perhaps does not present a unique threat to underlying physiology relative to other forms of stress. In one area of thought, ELS is unique because it takes place during developmentally sensitive periods, where underlying physiology later in life is particularly vulnerable to stressful experiences (Lupien et al., 2009). However, other evidence calls into doubt the singular role of ELS in that some forms of stress have a profound impact regardless of their timing. For example, sexual abuse, whether it occurs early in life or later on in adulthood, has been linked to a host of somatic disorders, including gastrointestinal problems and chronic pain (Paras et al., 2009). Similarly, an increase in stressful life events (regardless of age that the stressors occurred) was associated with increased depression (Risch et al., 2009) and evidence exists that such lifelong stress interacts with the presence of the S allele on 5-HTTLPR to predict increased rates of depression (Caspi et al., 2003), although this finding continues to generate debate (Karg et al., 2011; Culverhouse et al., 2018). Further complicating this narrative is the notion that different types of ELS co-occur (Merrick et al., 2018). Therefore, the detrimental effects of stress may not be restricted to the early developmental period, but rather might be determined by severity and number of different stressors.

Based on these considerations, we make three recommendations for the field to advance: (1) begin with rigorous operational definitions of ELS, thus making it easier to measure and compare findings across studies. At present, there are numerous methodologies to measure ELS (e.g., questionnaires (Bernstein et al., 1994), interview methods (Bremner et al., 2000), or by asking a social worker or caregiver if previous abuse has occurred), all potentially introducing unique variance that may help explain some of the inconsistent results. Showing correlations between instrument types within the same study (e.g., validating a social worker's report with a high score on a standardized questionnaire by the trauma survivor) may set a gold standard to interpret any findings. (2) Consider ELS in a larger genetic and environmental context. This would span genetic profiles for multiple genes of interest or polygenic risk scores (for further information see Dudbridge, 2013), as well as current stressors in the person's life and presence of social support (Heim et al., 2008; Daskalakis et al., 2013). (3) Conduct research in ways that mitigate, reframe, or reverse the consequences of ELS. Teaching children appropriate coping mechanisms and building social networks (Werner, 1995) may act as deterrents to these negative outcomes. Likewise, in applying lessons from the animal literature, environmental enrichment (i.e., cognitive stimulation and physical activity) may act as another mechanism to help mitigate negative effects (Fox et al., 2006).

AUTHOR CONTRIBUTIONS

NF wrote the initial draft. TC revised the initial draft. Both TC and NF conceptualized the material for review.

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Emotional Stress During Pregnancy – Associations With Maternal Anxiety Disorders, Infant Cortisol Reactivity, and Mother–Child Interaction at Pre-school Age

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There is growing evidence that even milder forms of maternal stress or anxiety during pregnancy affect the fetus causing possible long-term consequences for infant and child development. The mechanisms through which prenatal maternal stress may affect the unborn are not yet entirely clarified. Due to limited self-regulatory skills after birth, infants depend on sensitive behavior of their parents to regulate affective states and physiological arousal. Dyadic affect regulation has been linked to various developmental patterns up to adolescence and thereby represents a key element of early social relationships. Aim of the study was to evaluate possible long-term consequences of emotional stress during pregnancy and postpartum anxiety disorders, as well as infant postpartum cortisol reactivity on mother–child-interaction at pre-school age. The sample comprised of N = 63 mother–infant dyads at study entry, n = 28 diagnosed with postpartum anxiety disorders according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), n = 35 were healthy controls. Mothers were interviewed with the Structured Clinical Interview for DSM-IV Disorders at an average infant age of M = 4.1 months and filled out a questionnaire regarding emotional stress during pregnancy. Further, they were videotaped during the Face-to-Face-Still-Face paradigm (FFSF), a widely used mild socio-emotional stressor for infants. To determine infant stress-reactivity, infant salivary cortisol was collected before, immediately after and 20 min after the FFSF. Missing values were estimated by multiple imputations. At the age of M = 5.3 years, mother-child-interaction was re-assessed in a follow-up sample of n = 30 dyads via a free-play situation. Moreover, dimensional measures for anxiety were assessed. Mothers in the clinical group reported significantly higher stress scores than the control group. Infant stress reactivity in the early postpartum period and maternal anxiety symptoms at the 5-year follow-up assessment were significantly associated with dyadic interaction quality at pre-school age. Even though maternal stress during

pregnancy did not directly predict mother–child interaction quality at pre-school age, it was significantly correlated with infant cortisol reactivity during postpartum period. Nevertheless, caution should be taken when interpreting the results considering the small sample size.

Keywords: stress, cortisol reactivity, child development, pregnancy, anxiety disorders

INTRODUCTION

The Influence of Maternal Stress During Pregnancy on Infant and Child Development

A growing body of research indicates that maternal stress during pregnancy exerts strong influence on the development of the unborn (Van den Bergh et al., 2017). Recent studies underline the long-term influence on a variety of developmental domains in the offspring, such as metabolic functioning, cognitive and emotional development (for review see Beijers et al., 2014). To date, however, the mechanisms through which prenatal maternal stress may affect the unborn are not yet entirely clarified (Hocher, 2014). Among others, prenatal environmental influences, known as fetal programming (Seckl, 2004), genetic factors (Hannigan et al., 2018) as well as postpartum environmental factors (Graignic-Philippea et al., 2014; Mughal et al., 2018) are discussed.

To date, prenatal maternal stress is defined very broadly, including psychological distress such as anxiety or depressive symptoms and life events, e.g., trauma, loss, or natural disasters. In this study we focused on emotional stress during pregnancy. This was assessed retrospectively with a questionnaire in the early postpartum period, including items regarding maternal experience of anxiety, sadness, joy, stress, and general tension (Mohler et al., 2006).

Maternal anxiety disorders in the perinatal period are the most common psychiatric disorders with prevalence rates of 11 to17% (Reck et al., 2008; Fairbrother et al., 2016) and are closely linked with alterations in the human stress systems (Bartlett et al., 2017). The hypothalamic-pituitary- adrenocortical axis (HPA axis) is one major regulating system to cope with stress on hormonal level. Its end product cortisol is intensively discussed as an underlying mechanism accounting for the association between maternal stress/anxiety during pregnancy and infant and child development. Research indicates that elevated maternal cortisol levels in response to stress may affect the offspring's HPA axis functioning. Consequences might be increased cortisol levels, increased cortisol reactivity (Luecken et al., 2013; Zijlmans et al., 2015) and, on the long run, an increased risk for developmental problems in the offspring. Evidence, however, is still inconsistent as there are recent studies pointing in the opposite direction (O'Connor et al., 2013; Nazzari et al., 2019). The clinical sample in our study consisted of mothers with anxiety disorders, the vast majority (92,8%) with a prepartum onset and ongoing diagnoses in the postpartum period. Therefore, we assume substantial continuity in maternal stress from pregnancy to the first months postpartum suggesting that prenatal stress is strongly associated with maternal postnatal experiences.

Even though it is difficult to disentangle the influence of prenatal and postnatal maternal stress on infant and child development, current studies try to differentiate between timing effects. Prenatally, maternal emotional stress seems to affect cognitive development. In a study of Lin et al. (2017) prenatal emotional stress induced cognitive deficits independent of postnatal stress, even though, maternal pre- and postnatal emotional stress levels were moderately correlated. Regarding maternal prenatal anxiety, recent studies reported that maternal anxiety during pregnancy is associated with socio-emotional problems (Madigan et al., 2018) and a more difficult temperament in the offspring (e.g., high negative affectivity and poor attentional regulation) (Baibazarova et al., 2013; McMahon et al., 2013). A study of Chong et al. (2016) showed that this prenatal effect was independent of maternal anxiety postpartum. Both, maternal prenatal anxiety and emotional stress are associated with higher reaction intensity in children (Lin et al., 2017), higher negative emotionality (Pluess et al., 2010), significant higher rates of behavioral problems and lower prosocial behavior in 5 year-old children independent of concurrent maternal mood (Loomans et al., 2011) and predicted poorer working memory at 8 years of age (Pearson et al., 2016).

A growing body of studies find significant links between prenatal stress and/or anxiety and child development up to adolescence (for review see, Loomans et al., 2011; Graignic-Philippea et al., 2014) Furthermore, maternal anxiety disorders are discussed to increase the offspring's risk for the development of anxiety disorders (Hettema et al., 2001; Low et al., 2012; Lebowitz et al., 2016). However, caution is required in the assumption of causality as all studies are of observational character.

To the best of the author's knowledge, there is only one study investigating prenatal maternal stress, as reflected in higher depressive and anxious symptoms, on mother–child interaction at pre-school age. In this study by Endendijk et al. (2017) prenatal emotional symptoms were not related to the quality of the mother–child interactive behavior at the age of 23–60 months. As far as known, there are no studies taking postnatal anxiety disorders according to DSM-IV and infants stress reactivity into account.

The Role of Maternal Sensitive Interaction Behavior for Infant Stress Regulation

To pacify stressful experiences and to regulate their own affects, infants only have a limited repertoire of self-regulatory

behaviors during the first months of life, such as hand-to-mouth-movements and non-nutritive sucking. Therefore, infants depend on the co-regulation of their caregivers, playing an important role in the development of stress regulation in infants (Provenzi et al., 2018). Empirical findings highlight the importance of specific patterns of mother–child interaction for infants' affect regulation. For dyadic co-regulation, sensitive reactions of the caregivers are of special importance for the development of affect regulation in the infant as well as for behavioral and physiological reactions (Haley and Stansbury, 2003; Conradt and Ablow, 2010). Sensitive and responsive parents are described as paying attention toward the infant's signals and reacting promptly and appropriate to them. If the caregiver cannot respond adequately to the child's emotions and interpersonal regulation fails, infants engage in self-directed stress regulation and develop lower tolerance to negative affect and lower stress regulation competencies (Gianino and Tronick, 1988; Tronick, 1989; Müller et al., 2016).

For a better understanding about underlying mechanisms through which maternal stress or anxiety affects child development (Leclere et al., 2014; Pratt et al., 2017), it might be helpful to observe dyadic interactional codes, which address the mother–child dyad as a single unit. On the one hand, we therefore focused on dyadic reciprocity which is described as a mutual exchange in which each interaction partner contributes and the interaction is characterized by collaboration and joint activity (Feldman et al., 2013). On the other hand, special attention was paid to dyadic negative states. The interaction behavior of dyads displaying high dyadic negative states is constricted and poor of emotional expressiveness or enthusiasm. Further, the atmosphere is tense and it seems that mother and child feel uncomfortable with each other (Feldman, 1998).

Overall, maternal sensitivity does not seem to be generally limited in anxiety disorders as studies display a heterogeneous picture. Some studies reported less sensitive maternal interaction behavior in mothers with anxiety disorders compared to healthy controls (Warren et al., 2003; Feldman et al., 2009) while others did not find differences regarding maternal sensitive or intrusive behaviors (Murray et al., 2007, 2012; Weinberg et al., 2008; Kaitz et al., 2010). However, some studies suggested that anxious mothers spoke in a less positive emotional tone (Nicol-Harper et al., 2007), smile less, played or imitated their infants less frequently (Field et al., 2005) and showed more anxious facial expressions (Murray et al., 2007) compared to healthy control dyads. Infants of highly anxious mothers showed less positive affect, more withdrawal, more frequent crying, and increased signs of stress during interaction (Stein et al., 2012). As the quality of interaction is of such great importance for infant stress regulation (Bosquet Enlow et al., 2014), infants of anxious mothers might frequently lack sufficient regulatory scaffolding with possible long term consequences for child socio-emotional development (Martinez-Torteya et al., 2014). This interactive dysregulation might be partly responsible for the increased risk for the development of mental disorders in infants of anxious caregivers (e.g., Mäntymaa et al., 2009).

So far, no study has investigated the links between prenatal stress and the quality of mother–child interaction at pre-school age, taking postpartum anxiety disorders into account. As one of the first studies we wanted to focus on maternal emotional stress during pregnancy in a sample of postpartum anxious mothers and its association's with mother-child-interaction at pre-school age taking infant stress reactivity during infancy into account. We assumed that emotional stress during pregnancy would differ significantly in both groups, with mothers in the clinical group reporting higher stress scores. We also expected that prenatal stress would be significantly associated with infant stress reactivity in early infancy. Furthermore, we hypothesized that emotional stress during pregnancy as well as infant cortisol reactivity and maternal anxiety would predict mother–child interaction quality at the 5-year follow-up assessment.

MATERIALS AND METHODS

Participants

This sample ($N = 63$ at study entry) is part of a larger longitudinal study (Reck et al., 2013, 2018a,b; Tietz et al., 2014). Participants were recruited between June 2006 and October 2010. They were reached by distribution of flyers (e.g., to gynecologists) as well as newspaper advertisements. Furthermore, the study team responded to public birth announcements and cooperated with the Heidelberg University Women's Hospital in order to recruit women with anxiety disorders for the clinical group. The clinical group consisted of women diagnosed with at least one of the following anxiety disorders according to DSM-IV: panic disorder with agoraphobia, agoraphobia without history of panic disorder, generalized anxiety disorder, social phobia, obsessive compulsive disorder, post-traumatic stress disorder, and anxiety disorder not otherwise specified (NOS). They were excluded if an acute or former psychosis, a current or former bipolar disorder, current substance abuse, acute suicidal tendency or an acute major depression or dysthymia were diagnosed as primary disorder. Provided the anxiety disorder posed as primary diagnosis, women were not excluded. This was the case for $n = 4$ women ($n = 1$ major depression, $n = 2$ dysthymia, $n = 1$ depressive disorder NOS). Women were only included in the control group if they had no current or antecedent axis I diagnoses according to DSM-IV.

At first assessment $N = 122$ mothers with their infants were reached. For the present analyses, we excluded $n = 14$ dyads who met diagnostic exclusion criteria. In the remaining subsample ($N = 108$), $N = 64$ mothers agreed upon selection of their infant's saliva. $n = 1$ infant was excluded due to cortisone medication. None of the infants met the exclusion criteria of prematurity (born before the completion of the 36th week of gestation), small-for-gestational-age or congenital abnormalities. Thus, the final sample consisted of $N = 63$ mother–infant dyads at study entry. $n = 28$ of the mothers were diagnosed with at least one anxiety disorder (clinical group) and $n = 35$ mothers had no clinical disorder (control group). A detailed report of total recruitment and case exclusions is demonstrated in **Figure 1**.

The distribution of diagnoses in the clinical group was as follows: $n = 20$ mothers had more than one anxiety disorder: $n = 14$ mothers had a panic disorder with or without agoraphobia

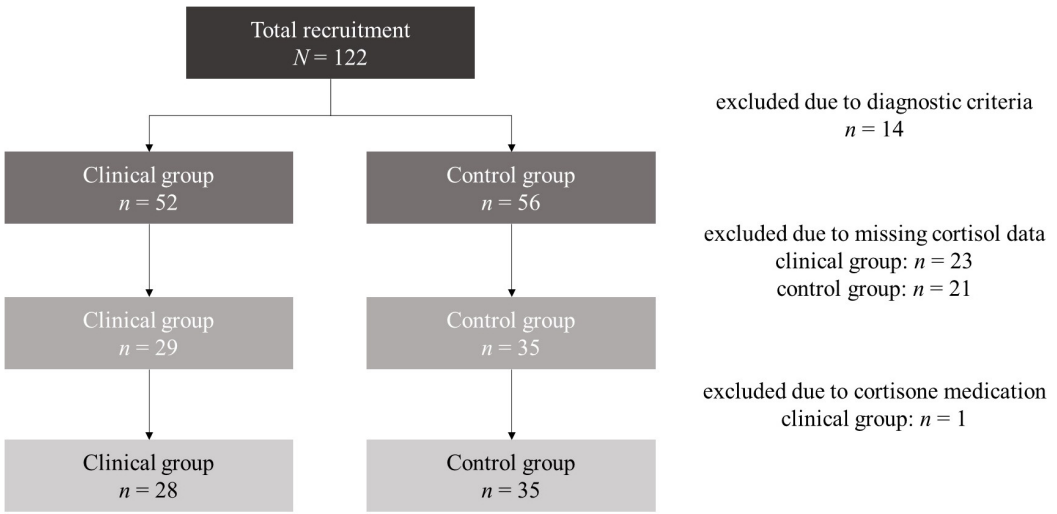

FIGURE 1 | Flow chart of recruitment process and case exclusions.

or an agoraphobia without history of panic disorder. n = 14 mothers were diagnosed with a generalized anxiety disorder. n = 13 mothers were diagnosed with an obsessive-compulsive disorder. n = 9 mothers had a social phobia. n = 9 women were diagnosed with a specific phobia. n = 1 woman had a post-traumatic stress disorder. n = 2 mothers had an anxiety disorder NOS. The vast majority of the clinical sample (n = 26, 92,8%) was diagnosed with a prepartum onset of their anxiety disorder(s).

At the 5-year follow-up, only a sub-sample of N = 32 mother–infant dyads could be reached (n = 19 control group; n = 13 clinical group). This high dropout rate was primary due to life changes in the recruited families (change of address and contact data) and changes of interests (rejection of or non-response to study invitation) within the assessment gap of 5 years. n = 6 still suffered from at least one current anxiety disorder and further n = 3 from at least one partially remitted anxiety disorder. Of these, n = 1 mother had four and n = 1 mother had three anxiety disorders. Overall, there was n = 1 mother with a current agoraphobia without history of panic disorder and n = 1 mother with a fully remitted panic disorder. n = 2 women were diagnosed with a current generalized anxiety disorder (n = 1 partially remitted); n = 6 women suffered from an obsessive-compulsive disorder (n = 4 partially remitted); n = 1 woman had a social phobia; n = 2 mothers were diagnosed with a specific phobia; and n = 1 woman was diagnosed with a partially remitted anxiety disorder NOS. Maternal and infant characteristics at both assessment points and stratified for both groups are presented in **Table 1**. Of n = 2 mothers the interactive situation was missing at the 5-year follow-up. Consequently, only N = 30 dyads were analyzed for dyadic interactive abilities.

Procedures

Assessments took place at the Heidelberg University Hospital, Germany. The study was approved by the independent ethics committee of the Heidelberg University Medical Faculty. According to the Declaration of Helsinki, written informed consent was obtained from all mothers both for their own participation as well as for the participation of their infant prior to the first assessment and after a full explanation of all study procedures. At first assessment, the Face-to-Face Still-Face paradigm (FFSF; Tronick et al., 1978) was videotaped. The FFSF paradigm consists of three episodes, 2 min each (for a detailed description see Tronick et al., 1978). Afterward, the Structured Clinical Interview-I for DSM-IV Disorders (SCID-I, Wittchen et al., 1997) was carried out. Questionnaires were filled out at home and sent back via mail. Five years postpartum, mother–child dyads were re-invited to the lab. At this follow-up assessment, mothers and their children engaged in a 20-min free-play situation which was video-recorded. Following, mothers again were interviewed via the SCID-I and filled out questionnaires at home.

Infant Cortisol Reactivity

According to recent literature at the time of study planning (for review see Gunnar et al., 2009) infant salivary cortisol was collected immediately before (C_1), immediately after (C_2) and 20 min after the FFSF paradigm (C_3). Even though the circadian rhythm of the HPA-axis is not fully developed during the first months postpartum (de Weerth et al., 2003; Bright et al., 2012) salivary cortisol can be seen as a valid marker for infant stress reactivity in early infancy (Jansen et al., 2010). Saliva samples were stored at −20°C until required for analysis according to standard procedures (Schwartz et al., 1998) with a detection limit of the used assay between 0.1 and 15.0 ng/ml and intra-assay variances of 5.95% Vol. for 2.6 μg/100 ml, 1.59% Vol. for 17 μg/100 ml and 4.62% for 26.6 μg/100 ml). In order to control for effects of circadian rhythm on cortisol reactivity, the assessments were scheduled between 10.00 and 11.00 AM (M = 11.0 AM, SD = 1.8 h).

TABLE 1 | Demographics and tests on comparability of subgroups.

	General	Control	Anxiety	*t (p)*
Infant age at study entry (months)[a] *M* (*SD*)	4.1 (1.4)	4.0 (1.4)	4.3 (1.5)	−0.92 (0.36)
Gestation age (weeks)[b] *M (SD)*	39.5 (1.5)	39.7 (1.3)	39.2 (1.6)	1.31 (0.18)
APGAR (average)[c] *M (SD)*	9.5 (0.6)	9.5 (0.6)	9.5 (0.6)	0.07 (0.94)
Child age at 5-year-follow-up (years)[d] *M* (*SD*)	5.3 (0.4)	5.3 (0.4)	5.5 (0.5)	−1.25 (0.22)
Infant gender (frequencies)	**General**	**Control**	**Anxiety**	χ^2 *(p)*
Female	42	24	18	0.13[e] (0.79)
Male	21	11	10	
	General	**Control**	**Anxiety**	*t (p)*
Maternal age at study entry (years) [f] *M* (*SD*)	32.4 (5.6)	33.0 (5.5)	31.8 (5.6)	0.89 (0.38)
Maternal age at 5-year-follow-up (years) [g] *M* (*SD*)	39.8 (5.6)	40.2 (5.3)	39.3 (6.2)	0.46 (0.65)
Maternal education (frequencies)	**General**	**Control**	**Anxiety**	*U (p)*
University degree	34	19	15	463.0 (0.70)
University entrance qualification	12	8	4	
High secondary qualification	14	7	7	
Low secondary qualification	3	1	2	
Marital status (frequencies)	**General**	**Control**	**Anxiety**	χ^2 *(p)*
Married	42	26	16	2.15[h] (0.23)
Not married	15	6	9	

[a]*Min = 2.5; max = 7.8;* [b]*min = 36.3; max = 41.9;* [c]*min = 7.0; max = 10.0;* [d]*min = 5.01; max = 6.06;* [e]*0 cells has expected count less than 5, minimum expected count is 9.33;* [f]*min = 22.0; max = 45.0;* [g]*min = 27; max = 50;* [h]*0 cells have expected count less than 5, minimum expected count is 6.58.*

However, n = 14 infants (22.2% of study sample) were assessed after 11 AM (M = 13.8 AM, SD = 1.2 h) due to postponed study appointments. Thus, we considered sampling daytime as a confounder. Furthermore, cortisol reactivity is correlated with prior napping and feeding. Consequently, additionally to the instruction of keeping the infants rested and fed as usually, time to and length of prior feeding and sleeping periods were considered as additional confounders. Salivary cortisol levels were interpreted on the basis of the area under the curve with respect to increase (AUC_I) which allows a sensitive measure of physiological changes over time (Pruessner et al., 2003).

For two infants the C_1 and the C_2 as well as for n = 18 infants the C_3 value was missing due to low amounts of saliva or interruption of assessment by feeding or napping. Mean values in all measurements (C_1: M = 1.34 ng/ml, SD = 1.35 ng/ml, min = 0.10 ng/ml, max = 7.10 ng/ml; C_2: M = 1.31 ng/ml, SD = 1.28 ng/ml, min = 0.10 ng/ml, max = 6.50 ng/ml; C_3: M = 1.07 ng/ml, SD = 0.98 ng/ml, min = 0.10 ng/ml, max = 3.90 ng/ml) were in range of normative parameters (Tollenaar et al., 2010). Mean of the AUC_I was negative (M = −4.65 ng/ml × min, SD = 17.08 ng/ml × min, min = −46.00 ng/ml × min, max = 39.20 ng/ml × min), indicating that cortisol levels declined from the first (C_1) to the last (C_3) assessment. In the group of infants with three valid cortisol measurements (n = 42) there were n = 13 responders (31%); responders were defined as infants whose AUC_I was one SE (2.63 ng/ml × min) above zero. Low to medium responder rates and consequently declining cortisol means are often found in infant and child samples (Gunnar et al., 2009; Jansen et al., 2010). However, all valid infant AUC_I values were considered for the analyses. No outlying values (defined as values deviating more than three interquartile ranges from the median) were identified for the AUC_I measure. Furthermore, there were no significant correlations (all $p > 0.15$) with potential confounder variables (infant and maternal age, infant gender, marital status, financial concerns, gestational age, PDA, breastfeeding, number of infants, APGAR values, daytime of assessment, time passed since and duration of prior meal or sleeping periods, frequency and duration of daytime naps and nighttime awakes, sleeping arrangement and childcare). Consequently, we disregarded these factors as confounders for the main and additional analyses.

Maternal Anxiety Disorders

The German version of the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I, Wittchen et al., 1997) is semi-structured and a widely used interview for the diagnosis of selected Axis I disorders. It was used by trained and experienced clinical psychologists for the assessment of maternal postpartum anxiety disorders. According to DSM-IV, we included participants with the following diagnoses: generalized anxiety disorder, panic disorder with and without agoraphobia, agoraphobia without history of panic disorder, specific phobias, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder, and the anxiety disorder NOS.

Maternal Anxiety Symptoms: Agoraphobic Cognitions Questionnaire (ACQ), Body Sensations Questionnaire (BSQ) and the Mobility Inventory (MI: MIA/MIB)

Core symptoms of anxiety disorders at the 1st assessment and the 5-year follow-up were assessed with the German version of the Agoraphobic Cognitions Questionnaire (ACQ), the Body Sensations Questionnaire (BSQ) and the Mobility Inventory (MI) (Ehlers et al., 2001). The three questionnaires combine cognitions, physical symptoms and avoidance behavior, all core symptoms of anxiety disorders. The ACQ consists of 14 questions about the frequency of typical anxiety cognitions with scales ranging from 1 ("thought never occurs") to 5 ("thought always occurs when I am nervous"). The BSQ consists of 17 items and measures the extent of fear of anxiety related physical symptoms from 1 ("not frightened or worried by this sensation") to 5 ("extremely frightened by this sensation"). The MI consists of 27 items describing predominant agoraphobic situations and the avoidance of these situations. Moreover, it also indicates the severity of the anxious symptomatology. The MI is subdivided into the Mobility Inventory Alone (MIA) and the Mobility Inventory Backened (MIB; i.e., accompanied by a person trusted). In our sample, internal consistency ranged from Cronbach's $\alpha = 0.88$ (ACQ at both assessments) to 0.95 (MIB at both assessments); MIA = 0.95 at 1st assessment and 0.94 at 5-year follow-up; BSQ = 0.92 at both assessments. Thus, the internal consistency was comparable to the ones reported by the authors and can be evaluated as good to excellent.

The mean scores for the anxiety questionnaires at the 1st assessment were as follows: ACQ: $M = 1.33$ ($SD = 0.38$, min = 1.00, max = 2.50), MIA: $M = 1.31$ ($SD = 0.53$, min = 1.00, max = 3.56), MIB: $M = 1.11$ ($SD = 0.22$, min = 1.00, max = 2.12), BSQ: $M = 1.69$ ($SD = 0.59$, min = 1.00, max = 3.53). Anxiety questionnaire data was missing for $n = 3$ women in the 5-year follow-up sample, thus the sample size for analyses controlling for current anxiety symptoms was reduced. The mean scores for the anxiety questionnaires at the 5-year follow-up were as follows: ACQ: $M = 1.31$ ($SD = 0.44$, min = 1.00, max = 3.07), MIA: $M = 1.30$ ($SD = 0.46$, min = 1.00, max = 2.96), MIB: $M = 1.13$ ($SD = 0.30$, min = 1.00, max = 2.42), BSQ: $M = 1.68$ ($SD = 0.63$, min = 1.00, max = 3.41). In this study, we used a composite score averaged over the four scales for each assessment to encounter symptom heterogeneity between the different anxiety disorders. This composite score reached an excellent internal consistency ($\alpha = 0.99$ at 1st assessment; $\alpha = 0.93$ at 5-year follow-up) and ranged from min = 1.02 to max = 2.51 ($M = 1.33$, $SD = 0.38$) at the 5-year follow-up respectively from min = 1.00 to max = 2.36 ($M = 1.33$, $SD = 0.33$) at the 1st assessment.

Prenatal Emotional Stress Index

The Prenatal Emotional Stress Index (PESI) is a self-report questionnaire assessing emotional stress during pregnancy (Mohler et al., 2006). It was applied at the first measurement time to assess maternal emotional stress during pregnancy. retrospectively. The questionnaire consists of 11 items per pregnancy trimester (overall 33 items) measuring anxiety, sadness, joy, stress, and tension via visual analogous scales ranging from 0 to 100%. The scale values are the mean item responses per trimester. Cronbach's α was $\alpha = 0.91$ for the first, $\alpha = 0.92$ for the second and $\alpha = 0.93$ for the third trimester, indexing excellent internal consistency. Average scores were $M = 31.92$ ($SD = 24.60$, min = 0,00, max = 92.27) for the first, $M = 30.41$ ($SD = 22.59$, min = 0,00, max = 89.09) for the second and $M = 31.63$ ($SD = 22.93$, min = 0,00, max = 91.27) for the third trimester. The intercorrelations between pregnancy trimesters were as follows: the first trimester significantly correlated with the second ($r = 0.76$, $p < 0.01$) and third trimester ($r = 0.70$, $p < 0.01$) as well as the second trimester correlated significantly with the third trimester ($r = 0.82$, $p < 0.01$). A composite score averaged over the three trimesters was used for the analyses This composite score reached an excellent internal consistency ($\alpha = 0.96$) and ranged from min = 2.42 to max = 86.97 ($M = 31.34$, $SD = 21.41$).

Mother–Child Interaction

At the 5-year follow-up mother–child interaction during a free-play situation was coded by a trained and reliable coder (inter-rater-consistency = 87.6%) using the 4th revision of the macro-analytical tool "Coding Interactive Behavior" (CIB) (Feldman, 1998). The coder was blind to the study hypotheses and maternal psychiatric status.

The CIB is a valid, reliable and widely used (Feldman, 1998) global rating system for interaction sequences between two or more partners. The basic coding scheme consists of 48 scales (22 for adults, 16 for children and 5 dyadic scales), which are scored from 1 to 5 in steps of 0.5 (1 = minimal level of specific behavior/attitude; 5 = maximal level of specific behavior/attitude). The scales assess the macro-analytical nature and flow of the interaction (e.g., reciprocity and adaption) as well as the involvement and interactive style of each partner (e.g., specific behaviors and affective/attentive states). The different scales can be analyzed separately or averaged to 8 composites (3 for both caregiver and child and 2 dyadic composites).

In this study, we focused on both dyadic composites, i.e., "reciprocity" (averaged from the scales "dyadic reciprocity," "adaption-regulation," and "fluency") and "dyadic negative states" (averaged from the scales "constriction" and "tension"). The means were $M = 3.67$ ($SD = 0.71$, min = 2.17, max = 4.83) for reciprocity and $M = 1.70$ ($SD = 0.82$, min = 1.00, max = 3.50) for dyadic negative states.

Statistical Analyses

For all analyses we used the *Statistical Package for Social Sciences* (IBM™ SPSS® v. 24.0.0.0). G-Power v. 3.1.9.2 (Faul et al., 2007, 2009) was used for power-estimations for the confirmative analysis.

Before carrying out the main analyses, we tested if the list-wise case-exclusions (see section "Participants") were valid for our sample and analyses by the use of Little's MCAR-test (Little, 1988). If the MCAR-test is non-significant it is unlikely that excluded cases and the final sample differ regarding considered variables. We considered socio-demographic data (e.g., age),

birth data (e.g., gestational age), self-report data (e.g. PESI), interactive variables (ICEP-R, Reck et al., 2009), cortisol data (and potential confounders) and interaction data (CIB) at the 5-year follow-up for this procedure. Moreover, child and gestational age, APGAR values, child gender, maternal age and education as well as marital status were checked for differences between the included and excluded cases as well as between the control and the clinical group via *t*-tests, *U*-tests and χ^2-tests to ensure comparability.

For the main analysis, Mann–Whitney *U*-tests were carried out to compare the study groups regarding emotional stress, infant cortisol reactivity as well as CIB scores as the distributions of questionnaire data, cortisol values and reactivity as well as dyadic negative states significantly deviated from normal distribution ($p < 0.01$ in Kolmogorov–Smirnov and Shapiro–Wilk test). Especially for small samples and unequally sized groups, general linear modeling may not be sufficiently robust against violations to mathematical assumptions (e.g., normal distribution). Thus it may lead to progressive statistical testing (Bortz and Schuster, 2010). The intercorrelation between variables indexing for symptom severity, i.e., emotional stress (PESI total score) and anxiety symptoms (at both assessments) were explored via Pearson correlations. Furthermore, the association between emotional stress and infant cortisol reactivity was analyzed via generalized linear modeling (with maximum likelihood estimation and stepwise backward procedure) to control for and to evaluate the independent contribution of study variables (emotional stress and anxiety symptoms). Variables were used uncentered. Consequently, the *B*-weights of the regression models were not standardized. To estimate effect sizes, we computed Cohen's d for group comparisons and w^2 ($= \frac{\chi^2}{N}$) for regression coefficients. $d = 0.2$ respectively $w^2 = 0.01$ are interpreted as small, $w^2 = 0.09$ respectively $d = 0.5$ as medium-sized and $w^2 = 0.25$ respectively $d = 0.8$ as large effects (Cohen, 1977).

Effects of group, measurement time and their interaction term on infant cortisol measures were tested by a two-way ANOVA for repeated measures with the between-subject factor "group" and the within-subject factor "measurement time" to account for differences in the infants' cortisol trajectories between the groups and for intraindividual variance. Since mathematically premises are violated (see above) the results of this ANOVA are of purely descriptive quality. Mauchly's procedure was used to test for violation of the assumption of sphericity. This assumption was violated for infant cortisol values (pooled data: $p < 0.01$; $\varepsilon = 0.71$; original data: $p < 0.01$; $\varepsilon = 0.77$). Consequently, repeated measures dfs were Huynh–Feldt corrected.

For the further additional analyses, generalized linear modeling (with maximum likelihood estimation and stepwise backward procedure) was used to control for and to evaluate the independent contribution of study variables (emotional stress, cortisol reactivity, and anxiety symptoms) in explaining the CIB scores.

We used a two-tailed critical α-error of $\alpha = 0.05$. The valid sample size varies as a function of missing values in the original data and the 5-year-follow-up.

RESULTS

Preliminary Data Analyses

The MCAR-test was non-significant ($\chi^2 = 1{,}071.80$, $df = 1{,}168$, $p = 0.98$), indicating that the list-wise case-exclusions were valid for our sample and that this sub-population was representative for the total sample. Additionally, tests on comparability between the excluded and included cases revealed no systematic differences (all $p > 0.13$). Moreover, no differences were found between the groups regarding sociodemographic or birth-related data (see **Table 1**).

We had complete cortisol data (i.e., to all three measurement points C_1, C_2, and C_3) for $n = 42$ infants (66.7% of study sample). However, the infants of the remaining sample ($n = 21$) had at least one valid cortisol value. Furthermore, $n = 8$ PESI-scores (12.7% of study sample) for each trimester as well as anxiety questionnaire scores at the first assessment (ACQ: $n = 8$, MIA: $n = 15$, MIB: $n = 13$, BSQ: $n = 8$; 12.7–23.8% of study sample) were missing. We estimated the missing values for these dyads using multiple imputations (Rubin, 1987). For these estimations, all variables analyzed in this study were used as predictors according to standard procedures (Enders, 2010). We estimated missing values for $N = 20$ data sets (automatic imputation method, linear regression model, maximum 10 iterations). Estimated cortisol values were restricted to the limit of detection of the cortisol assay (0.1–15.0 ng/ml), PESI and anxiety questionnaire scores were restricted to the maximum score range (PESI: 0–100%, anxiety questionnaires: 1–4). **Table 2** contains the means and standard deviations of the pooled imputed values and the pooled sample after multiple imputations. There were no systematic variations of estimated values in the iteration process. Variation occurred within the scope of random variations. The AUC_I, the PESI and the anxiety symptoms composite were then additionally computed for every case including cases with imputed values in every imputed data set.

Since the missing interaction data of the 5-year follow-up in $n = 33$ dyads (52.4% of study sample) was not due to just missing values but real study dropouts over an extended period and since the percentage of missing data exceeded 50%, we refrained from estimating missing values for the 5-year follow-up. All analyses were carried out for (1) the original data set and (2) in each of the 20 imputed data sets. The results of (2) were pooled. Consequently, two results are reported: the pooled results over the imputed data set and the results of the original data set.

Main Analyses

Table 3 summarizes the Mann–Whitney *U*-tests on comparisons between the clinical and the control group. Only emotional stress during pregnancy (PESI total score) differed between the groups in the pooled ($p < 0.01$) and the original data set ($p < 0.01$). The clinical group reported higher scores of emotional stress compared to controls (Cohen's *d* pooled data: $d = 1.75$; original data: $d = 2.19$). There were no group differences between the clinical and the control group regarding infant cortisol reactivity (AUC_I; pooled data: $p = 0.19$; original data: $p = 0.66$) or CIB scores (dyadic negative states: $p = 0.21$; dyadic reciprocity: $p = 0.62$).

TABLE 2 | Pooled imputation result (averaged over 20 data sets) of missing data.

	Imputed values				Data after imputation (N = 63)			
Assessment	***M***	***SD***	**Min**	**Max**	***M***	***SD***	**Min**	**Max**
C_1 (in ng/ml) (n = 2 imputed values)	1.49	0.95	0.81	2.16	1.34	1.34	0.10	7.10
C_2 (in ng/ml) (n = 2 imputed values)	1.15	0.60	0.73	1.58	1.31	1.27	0.10	6.50
C_3 (in ng/ml) (n = 18 imputed values)	1.19	0.84	0.10	2.75	1.10	0.94	0.10	3.90
PESI 1st trimester (in%) (n = 8 imputed values)	31.53	4.84	25.05	39.41	31.87	23.03	0.00	92.27
PESI 2nd trimester (in%) (n = 8 imputed values)	30.36	4.84	23.06	37.36	30.40	21.16	0.00	89.09
PESI 3rd trimester (in%) (n = 8 imputed values)	32.17	5.08	24.21	39.04	31.70	21.49	0.00	91.27
PESI total score	30.92	4.64	24.23	37.59	31.29	20.05	2.42	86.97
ACQ (n = 8 imputed values)	1.42	0.43	1.00	2.14	1.34	0.37	1.00	2.50
MIA (n = 15 imputed values)	1.48	0.53	1.00	2.65	1.36	0.54	1.00	3.56
MIB (n = 13 imputed values)	1.29	0.32	1.00	1.93	1.15	0.26	1.00	2.12
BSQ (n = 8 imputed values)	1.79	0.66	1.05	2.85	1.71	0.60	1.00	3.53
Anxiety symptoms (composite)	1.43	0.25	1.07	1.98	1.35	0.31	1.00	2.36

TABLE 3 | Mann–Whitney U-tests on differences between the study groups regarding emotional stress during pregnancy (PESI total score), infant cortisol reactivity (AUC_I), and dyadic interaction (CIB) at 5 years follow-up.

		Control Group		Clinical Group		Test statistic	
Outcome		***M***	***SE***	***M***	***SE***	***U***	***p***
Emotional stress (PESI total score)	Original data (n = 55)	19.37	1.77	52.29	4.37	40.00	<0.01
	Pooled data (n = 63)[a]	19.37	1.77	46.18	3.66	75.86	<0.01
Infant cortisol reactivity (AUC_I)	Original data (n = 42)	−5.56	2.89	−3.18	5.19	191.00	0.66
	Pooled data (n = 63)[a]	−5.38	3.22	0.18	3.88	374.52	0.19
Dyadic negative states (CIB)	Original data (n = 30)	1.54	0.18	1.94	0.26	80.50	0.21
Dyadic reciprocity (CIB)	Original data (n = 30)	3.75	0.14	3.54	0.26	96.50	0.62

[a] *For pooled analyses: averaged over original and imputed data sets.*

The intercorrelation between emotional stress and anxiety symptoms at the 1st assessment (pooled data: $r = 0.631$, $p < 0.01$; original data: $r = 0.709$, $p < 0.01$) and the 5-year follow up (pooled data: $r = 0.621$, $p < 0.01$; original data: $r = 0.625$, $p < 0.01$) was positive and significant. The intercorrelation between anxiety symptoms at the 1st assessment and the 5-year follow-up was positive and significant in the pooled ($r = 0.636$, $p < 0.01$) and the original data set ($r = 0.709$, $p < 0.01$).

To evaluate the unique and independent association between emotional stress during pregnancy (PESI total score) and infant cortisol reactivity (AUC_I), we used a generalized linear backward regression controlling for anxiety symptoms at the 1st assessment. As maternal disorder was not revealed as meaningful for infant cortisol reactivity, it was neglected in this analysis. In the first step, anxiety symptoms at the 1st assessment were excluded as non-significant for infant cortisol reactivity (pooled data: $B = -0.920$; $p = 0.928$; original data: $B = -6.479$; $p = 0.384$). The second and final step (**Table 4**, Model 1) contained only emotional stress as significant predictor for infant cortisol reactivity: Emotional stress was significantly and positively associated with infant cortisol reactivity (pooled data: $p = 0.036$, $w^2 = 0.125$; original data: $p = 0.034$, $w^2 = 0.084$).

Multicollinearity for this model was approximated by linear regression and the estimation of the variance inflation factor (VIF). Multicollinearity seems unproblematic, as the VIF was low for both variables and both data (pooled data: average VIF = 1.670; original data: VIF = 2.011) (O'Brien, 2007).

For the Mann–Whitney U-tests, large effects ($d = 0.8$) in our pooled sample could be detected by a chance of 1-β = 0.69 (1-β = 0.30 for minimal data set of $n = 30$). Consequently, especially for medium-sized and small effects the power of these comparisons is not sufficient. The power for the final regression model was approximated for the linear multiple regressions with one coefficient. Large effects ($f^2 = 0.35$) for beta weights in our pooled sample could be detected by a chance of 1-β = 0.99 (1-β = 0.93 for original data). The chance to find medium-sized effects ($f^2 = 0.15$) in our pooled sample was 1-β = 0.86 (1-β = 0.62 for original data). Small effects ($f^2 = 0.02$) had a chance of 1-β = 0.20 (1-β = 0.13 for original data) to be detected. Thus, especially small effects could not be sufficiently detected.

Additional Analyses

Repeated Measures Analysis on Infant Cortisol Reactivity

There was neither a significant main effect of measurement time [pooled data: $F(1.42,85.32) = 1.83$, $p = 0.24$; original data: $F(1.54,61.67) = 3.24$, $p = 0.06$] nor of group [pooled data: $F(1,61) = 2.40$, $p = 0.14$; original data: $F(1,40) = 0.83$, $p = 0.37$]. Moreover, the interaction effect between measurement time and

TABLE 4 | Generalized linear regression models on infant cortisol reactivity (AUC_I) and dyadic negative states and reciprocity (CIB).

Model		Parameter	*B*	*SE*	Lower *CI* bound (95%)	Upper *CI* bound (95%)	Wald χ^2 [a]	*p*
Model 1: infant cortisol reactivity (AUC_I) predicted by emotional stress (PESI total score)	original data (n = 36)[b]	PESI total score	0.372	0.175	0.029	0.716	4.512	0.034
		Intercept	−12.350	5.373	−22.881	−1.818	5.282	0.022
		Scale	190.893[c]	44.994	120.270	302.984	/	/
	pooled data (n = 63)[d]	PESI total score	0.235	0.112	0.015	0.456	5.303	0.036
		Intercept	−10.272	4.459	−19.019	−1.525	6.323	0.021
		Scale	329.473	74.212	182.782	476.163	/	/
Model 2: dyadic negative states (CIB) predicted by anxiety symptoms at the 5-year follow-up and infant cortisol reactivity (AUC_I)	original data (n = 17)[e]	Anxiety symptoms (5-year follow-up)	1.202	0.431	0.356	2.047	7.764	0.005
		AUC_I	0.016	0.004	0.009	0.023	20.499	<0.001
		Intercept	0.193	0.591	−0.966	1.352	0.106	0.745
		Scale	0.474[c]	0.162	0.242	0.928	/	/
	Pooled data (n = 25)[f]	Anxiety symptoms (5-year follow-up)	1.280	0.404	0.488	2.072	10.072	0.002
		AUC_I	0.012	0.004	0.005	0.020	13.186	0.002
		Intercept	0.077	0.529	−0.959	1.114	0.042	0.884
		Scale	0.421	0.120	0.186	0.656	/	/
Model 3: dyadic reciprocity (CIB) predicted by anxiety symptoms at the 5-year follow-up and infant cortisol reactivity (AUC_I)	original data (n = 17)[g]	Anxiety symptoms (5-year follow-up)	−1.196	0.336	−1.854	−0.537	12.659	<0.001
		AUC_I	−0.009	0.004	−0.018	−0.001	4.928	0.026
		Intercept	5.197	0.482	4.253	6.142	116.330	<0.001
		Scale	0.293[c]	0.100	0.150	0.574	/	/
	Pooled data (n = 25)[h]	Anxiety symptoms (5-year follow-up)	−1.261	0.325	−1.897	−0.625	15.168	<0.001
		AUC_I	−0.010	0.004	−0.018	−0.002	7.577	0.010
		Intercept	5.269	0.440	4.407	6.131	144.644	<0.001
		Scale	0.288	0.082	0.128	0.448	/	/

[a]*For pooled analyses: averaged over original and imputed data sets;* [b]*Likelihood-ratio χ^2 = 6.258, p = 0.012;* [c]*Maximum likelihood estimate;* [d]*Average likelihood-ratio χ^2 = 4.348, average p = 0.056;* [e]*Likelihood-ratio χ^2 = 8.980, p = 0.011;* [f]*Average likelihood-ratio χ^2 = 12.599, average p = 0.002;* [g]*Likelihood-ratio χ^2 = 10.371, p = 0.006;* [h]*Average likelihood-ratio χ^2 = 15.037, average p = 0.001.*

group was non-significant [pooled data: $F(1.42,85.32) = 1.22$, $p = 0.36$; original data: $F(1.54,61.67) = 0.09$, $p = 0.86$]. Means and standard deviations are demonstrated in **Table 5**.

Although there is a widely accepted practice of log-transforming raw data to achieve normally distributed values (Miller and Plessow, 2013), this technique is not uncritical regarding its aims, success and its effects on data interpretation, and thus have to be used and interpreted with caution (Feng et al., 2014). However, aiming to assure, that the results of the ANOVA were not referable to skewed cortisol raw values, we applied ln-transformation to our original data. The transformed data were checked for outlying values (as defined by more than 1.5 respectively 3 interquartile ranges below the first respectively above the third quartile). There were neither mild nor extreme outliers. However, after transformation data still were significantly skewed [Komogorov–Smirnov test: $p = 0.02$ for $\ln(C_1)$, $p = 0.02$ for $\ln(C_2)$, $p = 0.06$ for $\ln(C_3)$; Shapiro–Wilk: $p < 0.01$ for all three measures]. The results of the ANOVA (Mauchly's-test: $p < 0.01$; Huynh–Feldt-ε = 0.86) with the transformed values as dependent variable remained unchanged: There is neither an effect of time [$F(1.73,69.15) = 3.04$, $p = 0.06$], of group [$F(1,40) = 0.04$, $p = 0.84$] nor an interaction effect [$F(1.73,69.15) = 0.07$, $p = 0.91$].

The power to detect large ($f = 0.40$) between-subject effects in this ANOVA was high (pooled data: 1-β = 0.97; original data: 1-β = 0.87. Additionally, large ($f = 0.40$) and medium-sized ($f = 0.25$) within-subject and interaction effects were sufficiently detectable (pooled data: 1-β > 0.97, original data: 1-β > 0.90). Only medium-sized ($f = 0.25$) and small ($f = 0.10$) between-subject effects as well as small ($f = 0.10$) within-subject and interaction effects could not be excluded in these analyses (pooled data: 1-β < 0.67, original data: 1-β < 0.49). It can be concluded that for between-subject effects only large effects can be ruled out. For within-subject effects large and medium-sized effects can be ruled out.

Dyadic Interaction Quality

As maternal disorder was not revealed as meaningful for dyadic interaction quality, it was neglected in these additional analyses. To evaluate unique and independent relations of emotional stress during pregnancy (PESI total score), infant cortisol reactivity (AUC_I) and maternal anxiety symptoms at both assessments with dyadic negative states and dyadic reciprocity (CIB), we used stepwise backward regressions (with generalized linear modeling) with these predictors as main effects.

TABLE 5 | Descriptive statistics on infant cortisol values by group and measurement time.

			M	*SD*	*n*
Original data	C_1 (in ng/ml)	Control group	1.52	1.50	26
		Clinical group	1.15	0.93	16
		Total	1.38	1.31	42
	C_2 (in ng/ml)	Control group	1.40	1.45	26
		Clinical group	1.12	0.85	16
		Total	1.29	1.25	42
	C_3 (in ng/ml)	Control group	1.13	0.98	26
		Clinical group	0.86	0.76	16
		Total	1.03	0.90	42
Pooled data[a]	C_1 (in ng/ml)	Control group	1.59	1.49	35
		Clinical group	1.03	1.06	28
		Total	1.34	1.34	63
	C_2 (in ng/ml)	Control group	1.50	1.44	35
		Clinical group	1.06	0.97	28
		Total	1.30	1.27	63
	C_3 (in ng/ml)	Control group	1.18	1.03	35
		Clinical group	0.99	0.81	28
		Total	1.10	0.94	63

[a] *For pooled analyses: averaged over original and imputed data sets.*

The following variables were stepwise excluded as non-significant for dyadic negative states: Emotional stress (PESI total score; pooled data: $B = -0.004$; $p = 0.669$; original data: $B = 0.019$; $p = 0.167$) and anxiety symptoms at the 1st assessment (pooled data: $B = 0.632$; $p = 0.304$; original data: $B = 1.046$; $p = 0.112$). For dyadic reciprocity the same variables were excluded, however, in reversed order: Anxiety symptoms at the 1st assessment (pooled data: $B = -0.260$; $p = 0.681$; original data: $B = -0.121$ $p = 0.864$) and emotional stress (PESI total score; pooled data: $B = 0.009$; $p = 0.104$; original data: $B = -0.001$; $p = 0.918$). The third and final step (**Table 4**, Model 2 for dyadic negative states respectively Model 3 for dyadic reciprocity) contained only significant predictors, i.e., infant cortisol reactivity (AUC_I; negative states: pooled data: $p = 0.002$, $w^2 = 0.527$; original data: $p < 0.001$, $w^2 = 1.206$; reciprocity: pooled data: $p = 0.010$, $w^2 = 0.303$; original data: $p = 0.026$, $w^2 = 0.290$) and maternal anxiety symptoms at the 5-year follow-up (negative states: pooled data: $p = 0.002$, $w^2 = 0.403$; original data: $p = 0.005$, $w^2 = 0.457$; reciprocity: pooled data: $p < 0.001$, $w^2 = 0.607$; original data: $p < 0.001$, $w^2 = 0.745$) were both associated to dyadic interaction quality.

As in these analyses generalized linear modeling was used to minimize effects of violations of mathematical assumptions (Feng et al., 2014) and as non-linear transformations (as, e.g., ln-transformation) change the information regarding equality of numerical differences contained in the data (e.g., relevant for computing AUC_I-indices), we refrained from repeating these analyses with ln-transformed data despite skewed infant cortisol raw and reactivity values.

Multicollinearity for these models was approximated by linear regression and the estimation of the VIF. For emotional stress (PESI total score: pooled data: average VIF = 1.961; original data: VIF = 2.379), anxiety symptoms at the first assessment (pooled data: average VIF = 2.050; original data: VIF = 2.681), at the 5-year follow-up (pooled data: average VIF = 2.100; original data: VIF = 2.183) as well as for infant cortisol reactivity (pooled data: average VIF = 1.158; original data: VIF = 1.225) multicollinearity seems unproblematic, as the VIF was low for all variables and both data (O'Brien, 2007).

The power for the final models was approximated for the linear multiple regressions with two coefficients. Large effects ($f^2 = 0.35$) for beta weights in our pooled sample could be detected by a chance of 1-β = 0.87 (1-β = 0.65 for original data). The chance to find medium-sized effects ($f^2 = 0.15$) in our pooled sample was 1-β = 0.53 (1-β = 0.33 for original data). Small effects ($f^2 = 0.02$) had a chance of 1-β = 0.12 (1-β = 0.09 for original data) to be detected. Thus, medium-sized and small effects could not be sufficiently detected in our 5-year-follow-up sample.

DISCUSSION

The present study aimed at evaluating possible long-term consequences of emotional stress during pregnancy and postpartum anxiety disorders on mother–child interaction at pre-school age taking infant stress reactivity during infancy into account. First, our results show that mothers with postpartum anxiety disorders report higher levels of emotional stress during pregnancy. This is in line with previous studies indicating higher levels of perinatal emotional stress in women with postpartum anxiety disorder (Britton, 2008).

Secondly, our study revealed no differences in postpartum cortisol reactivity in infants of mothers with postpartum anxiety disorders and infants of the control group. This is a rather surprising result, as studies describe an association between maternal psychopathology in the postpartum period and the cortisol reactivity of their infants (e.g., Brennan et al., 2008). One explanation for this unexpected result could be that our clinical sample consisted of women with various anxiety disorders. So, it remains unclear whether mothers with different anxiety disorders display the same difficulties regarding mother–infant/child interaction. If the impact of anxiety disorders on mother–infant/child interaction vary this could explain the unexpected result.

Moreover, there might be some methodological reasons for this non-expected finding of our study, for example a missing mean increase in cortisol following the FFSF in our sample. This might be due to the fact, that infant salivary cortisol samples were only taken prior to, immediately after and 20 min after the FFSF. Consequently, we may not have had a full coverage of the possible cortisol peak times, though many studies have used similar intervals as demonstrated in the review of Gunnar et al. (2009). Further research studies should lengthen the observation interval to 30 min as suggested by a review of Provenzi et al. (2016). Second, the finding of a decline in the mean value of infant cortisol-reactivity (AUCI) may be surprising given the established stressful nature of the FFSF. Nevertheless, a low to medium rate of infant cortisol responders (31% for our original data) and thus a decrease in cortisol

means often is found in infant and child stress research (Gunnar et al., 2009; Jansen et al., 2010). It must be noted that the lack of reactivity does not imply that the measurement of cortisol reactivity in response to psychological stressors is not meaningful. Rather, it has been argued that the individual differences might bring to light factors that account for the individual differences as well as potential risk factors that may adversely affect infant development. Moreover, a dampening of cortisol-responses to stressors in rodents and humans during early development (Gunnar and Donzella, 2002) might play a role for our results. Although the reasons and duration of this dampening period is still unknown, there are many factors affecting stress reactivity. These include genetic influences (Montirosso et al., 2015), temperament differences (van Bakel and Riksen-Walraven, 2004), age related changes (Jansen et al., 2010), individual differences in sensitivity to the nature of the stimuli and contexts (Gunnar et al., 2009), and a sculpting of stress reactivity by interactive history (Gunnar and Quevedo, 2007). Furthermore, a recent meta-analysis (Provenzi et al., 2016) showed that a robust effect on salivary cortisol reactivity was only found for the 5-episode FFSF studies.

Even though we did not find group differences regarding infant stress reactivity, emotional stress during pregnancy was significantly correlated with infant stress reactivity. This finding adds to the heterogeneous picture about the association between maternal prenatal stress and infant cortisol reactivity. While some studies report an associations between prenatal stress and modified infant stress reactivity (e.g., Luecken et al., 2013), a current systematic review only finds limited evidence for this association (Bleker et al., 2018). As a majority of women in our clinical sample already had perinatal anxiety disorders, it is likely that our results arise from a longer exposure to stress of the fetus due to maternal stress during pregnancy and in the postpartum period. In contrast, time-limited exposure to stress is usually characterized by high chronicity and are often highly inter-correlated across gestation (Davis and Sandman, 2010). Further research is needed to disentangle timing and duration influences of maternal stress on infant stress reactivity.

The results of our study suggest that maternal stress during pregnancy influences infant development (Davis and Sandman, 2010; Buss et al., 2012). This result of our study could suggest programming influences of maternal psychobiological stress response on the developing fetus, but as the results yield from correlative analyses, we cannot draw causal conclusion. A variety of alternative factors, such as genetics, autonomous nervous and immune system functioning (Van den Bergh et al., 2017) as well as methylation processes (Weaver et al., 2004; Oberlander et al., 2008) have to be considered in prospective studies.

In our study, we did not find differences between the clinical and the control group with regard to the quality of interaction at pre-school age. This result was against our hypotheses and could be due to the heterogeneity regarding the anxiety disorders in our sample. Nevertheless, this finding is in line with a number of studies also reporting no difference in mother–infant interaction in mothers with anxiety disorders (Murray et al., 2007, 2012; Weinberg et al., 2008; Kaitz et al., 2010). Although, data about maternal interaction behavior in course of postpartum anxiety disorders is heterogeneous, as some studies did report reduced sensitivity in mothers with anxiety disorders (Feldman et al., 2009).

Notwithstanding, our results show an association between the quality of dyadic interaction at pre-school age and infant cortisol reactivity in the postpartum period as well as current maternal anxiety symptoms. Therefore, our results revealed the crucial role of maternal current anxiety symptoms for dyadic reciprocity as well as for dyadic negative states. Dyads with mothers suffering from current anxiety symptoms show lower mutual exchange, collaboration, and joint activity in interaction. Furthermore, these dyads display poor emotional expressiveness, more feelings of discomfort and the atmosphere is tense. In addition, our results support the assumption, that a dysfunction of the infant's HPA-axis exerts long-lasting influence on further child development (Lin et al., 2017; Madigan et al., 2018) in dyads with ongoing anxious symptomatology. Our results point toward this direction, as infant cortisol reactivity in the postpartum period significantly predicted child socio-emotional development at pre-school age operationalized as dyadic negative states as well as dyadic reciprocity during mother–child interaction. Both interactive patterns, dyadic negative affect and less dyadic reciprocity, can be seen as markers for a lack of emotion regulation skills (Feldman, 2015). Thus, the present study demonstrates the importance of infant's HPA functioning for long-term healthy development. This is in line with a current study by Neuenschwander et al. (2018), showing that heightened cortisol reactivity is associated with poorer executive functioning in 6-year old children. Furthermore, children's cortisol levels functioned as a mediator between maternal prenatal depressed and/or anxious symptoms and executive functioning. Apart from this, our results are in line with recent studies reporting that negative developmental pathways in children with a dysregulation of their HPA-axis function even increase, if familial risks factors are evident, such as parental social anxiety (Poole et al., 2018) or psychosocial risk factors, such as low parental education or unemployment (Karlén et al., 2015).

Contrary to our hypothesis emotional stress during pregnancy did not predict mother–child interaction quality at pre-school age. This is in line with the findings of Endendijk et al. (2017) and leads to the assumption that there must be other factors, for example child temperament, that could account for dysfunctional interactive behavior at pre-school age. In our study, maternal emotional stress during pregnancy was significantly correlated with infant cortisol reactivity during postpartum period, indicating that especially stress during pregnancy might influence stress reactivity during infancy. Therefore, this research issue should be addressed in further research, at best with a mediation analysis, regarding the influence of infant stress reactivity as a potential mediator between prenatal stress and interaction quality at pre-school age. Moreover, the bidirectional nature of the association between child cortisol and interaction

quality should be addressed, as some studies highlight the importance of maternal interaction behavior for the regulation of child's HPA-axis (Muller et al., 2015). Furthermore, future research should address the impact of further confounders, such as child temperament and parenting behavior postpartum. Unfortunately, our study sample was too small to run these analyses, wherefore further research with larger study samples are urgently needed.

The study has some limitations. Firstly, besides a rather small sample size and a low power, especially at the 5-year follow-up, mothers with different anxiety disorders are included in our clinical sample. Furthermore, a majority of women suffered from more than one anxiety disorder. The sample size does not allow subgroup analyses for the different anxiety disorders, and therefore it is impossible to draw conclusions about the specific effect of different anxiety disorders. Secondly, our sample is characterized by an overproportion of academic degrees, whereby our data is not representative for the overall population. Thirdly, the maternal stress questionnaire (PESI) retrospectively assesses emotional stress during pregnancy via self-report which may have affected postpartum answering tendencies. Moreover, it would be of great interest to assess saliva cortisol samples during pregnancy, enabling future studies to analyze the impact of maternal stress during pregnancy for child development prospectively. Fourthly, infant salivary cortisol was assessed prior to, immediately after and 20 min after the Still-Face paradigm. Due to few samples or the small time frame it is possible that we missed peak cortisol reactivity times, which may in part account for the negative mean cortisol reactivity. Lastly, the study design was observational, causality assumptions are not appropriate.

CONCLUSION

Taken together, our empirical results as well as theoretical assumptions emphasize the importance to further investigate the influence of stress during pregnancy for infant and child development. Our results underline that emotional stress during pregnancy is linked to infant stress reactivity and this in turn influences mother–child interaction up to pre-school age. Regarding maternal stress and its influences on infant and child development, it would be of major importance to disentangle different time-effects as well as different kinds of stressors, such as psychological stress, anxiety, or depressive symptoms and life events (trauma, loss, or natural disasters). Furthermore, potential moderators should be addressed, such as early life experiences, stress coping strategies, social support or maternal face processing, since current studies show the detrimental effect of anxiety (De Carli et al., 2019). With regard to possible long-term consequences for infant and child development early intervention and prevention programs are of vital importance. Recent studies point toward the direction that especially early interventions focusing dyadic reciprocity could improve children's regulatory capacities (Feldman, 2015). In sum, the foundation of socio-emotional competencies and especially affect and stress regulation capacities are laid early in life. They are primarily learned in the context of parent-infant/child-interaction with possible long-lasting effects regarding stress regulation for future relationships and mental health over the lifespan.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the independent ethics committee of the Heidelberg University Medical Faculty with written informed consent from all subjects in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

A-LZ and MM contributed to the analysis and interpretation of the data, drafting of the manuscript, and final approval of the final version of the manuscript. NN and BD contributed to the study and manuscript conception, and approval of the final version of the manuscript. CR contributed to the study conception and design, drafting of the manuscript, and final approval of the final version of the manuscript.

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