CATÓLICA
INSTITUTO DE CIÊNCIAS DA SAÚDE

LISBOA·PORTO

EMOTIONAL FACE PROCESSING IN INSTITUTIONALIZED CHILDREN: ADDRESSING THE ROLE OF EPIGENETIC MECHANISMS AND QUALITY OF RELATIONAL CONTEXT

Dissertação apresentada à Universidade Católica Portuguesa para obtenção do grau de mestre em

Neuropsicologia Clínica

Por

Beatriz Andion Boullosa Perry da Câmara

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Sob a orientação de Prof.ª Dr.ª Ana Mesquita e Prof.ª Dr.ª Filipa Ribeiro

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Agradecimentos

A Ti, sempre.

Às Professoras Ana Mesquita, Isabel Soares e Filipa Ribeiro, por toda a ajuda, orientação, exigência e ânimo que deram ao longo deste trabalho.

À Filipa, cuja amizade e presença constantes me permitem continuar, mesmo nos momentos mais desafiantes. Tenho a certeza que, sem a sua amizade, não seria quem sou hoje nem teria a vontade e certeza de fazer este caminho.

A todos os meus amigos. Entre jantares, ensaios, músicas e gargalhadas fizeram com que tantas vezes eu mantivesse o espírito leve e orientado para o que mais importa – as relações.

A toda a família. À avó Lici e avô Álvaro, exemplos concretos de resiliência, carinho e atenção. Aos pais, por apoiarem estes meus projetos que por vezes parecem incompreensíveis. Aos Manos e suas famílias, que na multiplicidade de opiniões, humores e sensibilidades (características não negociáveis em famílias numerosas) me acolhem e suportam em todos os momentos. Aos meus sobrinhos – João Maria, Álvaro, Maria da Luz, e a Isabel que aí vem. Testam muitas vezes a minha capacidade de trabalho – esta Tese foi feita entre choros, pedidos incansáveis, fraldas para trocar, embalos para adormecer, biberons e papas para preparar e dar. Mas a sua alegria, humor e carinho derretem-me sempre, por isso terei sempre o colo aberto e disponível entre investigações e trabalhos múltiplos.

Título do Projeto: Processamento emocional de faces em crianças institucionalizadas: analisando o papel de mecanismos epigenéticos e da qualidade do contexto relacional

Resumo: Faces com valência emocional podem ser consideradas como um dos estímulos mais importantes para corretamente processar e interpretar interações sociais. O normal desenvolvimento desta capacidade é sensível às experiências precoces socioemocionais e à qualidade dos cuidados recebidos. A Institucionalização tem sido consistentemente associada a consequências desenvolvimentais negativas, nomeadamente nas capacidades socioemocionais e na capacidade de reconhecer e corretamente diferenciar expressões faciais emocionais. Apesar dos mecanismos que medeiam estas consequências serem ainda desconhecidos, a investigação animal tem analisado mudanças epigenéticas como um fator plausível envolvido nesta mediação. Este estudo examina os efeitos da Institucionalização em marcadores cerebrais associados ao processamento de faces em 69 crianças portuguesas institucionalizadas (M_{idade}= 56.58 meses, *DP* = 10.96) e quais os possíveis mecanismos epigenéticos por detrás de tais marcadores. Procuramos compreender a associação entre o perfil de metilação do gene NR3C1 em crianças institucionalizadas com a sua exposição a experiências adversas pré-institucionalização e com a qualidade de cuidado recebido na instituição. Além disso, exploramos se a exposição a tais experiências adversas e diferenças na qualidade de cuidado recebidos modula a resposta neural da criança (medida através de Eventos de Potenciais Evocados - *ERP*) a face feliz e zangada da sua cuidadora. Os resultados mostram que as crianças que sofreram negligência e falta de condições habitacionais antes da institucionalização apresentam uma hipometilação do gene NR3C1 e que a qualidade de cuidado recebido na instituição não influencia o perfil de metilação do gene NR3C1. Quanto ao processamento neural da valência emocional das faces, as crianças que experienciaram negligência antes da instituição apresentaram diferenças na componente N170; as crianças expostas a falta de condições habitacionais apresentaram diferenças na componente P400 e as crianças com melhor qualidade de cuidados recebidos na instituição apresentaram diferenças nas componentes P100, N170, P400 e P250.

Palavras-chave: Institucionalização; Processamento emocional; Psicopatologia do Desenvolvimento; NR3C1; Epigenética

Title of Project: Emotional face processing in institutionalized children: Addressing the role of epigenetic mechanisms and quality of relational context

Abstract: Facial emotional expressions can be considered one of the most important stimuli in order to correctly process and interpret social interactions. The development of this capacity is highly sensitive to early socioemotional experiences and quality of care received. Institutionalization has been consistently associated with negative developmental consequences, namely in socioemotional outcomes and capacity to correctly differentiate and recognize facial emotional expressions. Even though the mechanisms mediating these effects are still unknown, animal research has recently pointed out epigenetic changes as a plausible involved factor in this mediation. The current study examined the effects of institutionalization on brain-based markers of face processing in 69 institutionalized Portuguese children (M_{age}= 56.58 months, SD = 10.96). We looked for associations between the methylation profile variations of the NR3C1 gene in institutionalized children and their exposure to pre-institutionalization adverse experiences and current institutional quality of care. Furthermore, we explore whether exposure to such adverse experiences and differences in the quality of care received in the institution modulated the child's neural response (measured through Event Related Potentials, ERPs) to caregiver's happy and angry faces. Our results show that children who experienced neglect and lack of habitational conditions previously to institutionalization have an hypomethylation of the NR3C1 gene. Results show that neglected children and children who experienced lack of habitational conditions before institution have an hypomethylation of the NR3C1 gene, and that quality of care received at the institutions does not influence NR3C1 gene methylation profile. Regarding the neuronal processing of emotional faces, neglected children showed differences for the N170 component, whereas children exposed to lack of habitational conditions showed differences for the P400 component. Children who received better quality of care in the institution revealed significant differences for the P100, N170, P400 and P250 components

Key-words: Institutionalization; Emotional Processing; Developmental Psychopathology; NR3C1; Epigenetics

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1. Institutionalization

The protection of children at risk has been present throughout humanity's history, with special concern for children deprived of parental care. The first official register of a foundling asylum appears at 787 CE, in Milan, Italy, although non-official registers date to the seventh century in Trier, Cologne, Germany (Ryan, 1909). Even though an orphan is considered by UNICEF as a child who has lost one or both parents because of death (UNICEF, 2017), it is estimated that only 15.1 million of the 140 million orphans have no living parent (UNICEF, 2017). Indeed, studies show that 80-90% of institutionalized children do have a living parent (Csáky, 2009; Bilson & Cox, 2005; Parwon, 2006; UNICEF, 2009), having as main reason for admission poverty and lack of accessibility to healthcare and education (Better Care Network, 2017). Estimates show that 604 847 children are living in institutions in central and eastern Europe and the former USSR (data from 2014), 48 775 in Cambodia (data from 2017), 500 000 in Indonesia and 86 000 in China (data from 2016) (Csáky, 2009). In Portugal, 9 065 children were living in institutions in 2020, with the majority of these children (71%) being institutionalized because of parental neglect, followed by temporary absence of familiar support, deviance behavior and abandonment (Instituto de Segurança Social, 2021). On a curious note, 14% of new entries in the Portuguese institutions in 2020 were influenced by the Pandemic situation (Instituto de Segurança Social, 2021).

Despite the preoccupation with these children's lives, the mortality of institutionalized children was roughly around 50-70% in the early years of the 20th century (Funchs, 1984 in van IJzendoorn et al., 2020), mainly because of poor sanitary and nutritional conditions (Jacobi, 1872, in van IJzendoorn et al., 2020). Although great efforts have been made in ameliorating sanitary conditions and quality of food since then, there are still key quality dimensions that account for a great variability in the quality of institutional facilities. These include staff's specific training, caregivers' rotating schedules, child-to-caregiver ratio and other more subtle but essential qualities for the socioemotional development of the child, such as the consistent provision of responsive, sensitive, and cooperative caregiving (van IJzendoorn et al., 2020; Baptista et al., 2018; Corval et al., 2017; Soares et al., 2014; Baptista et al., 2013; Oliveira et al., 2012). Nowadays, children in institutional care are, therefore, still at risk of poor socioemotional and cognitive development (van IJzendoorn et al., 2020).

1.1 Effects of Institutionalization on Child's Development

The risks associated with this type of care became clear with the Bucharest Early Intervention Project (BEIP), where part of a group of children from Romanian institutions were randomly assigned to high quality foster care (FCG), while some remained in the institutions (CAU) (Zeanah et al., 2003). An extensive baseline assessment was made to a total of 136 institutionalized children in Bucharest, after which 68 of these children were randomly assigned to a high-quality foster care program (Zeanah et al., 2003). Furthermore, a control group of age-matched, typically developing community children was formed (NIG), which allowed for an additional measurement of comparison from a normative care environment (Zeanah et al., 2003). Data from this project showed how institutionalized children have substantial developmental delays and deviations, namely at physical growth, brain, cognitive and socioemotional development (Carr et al., 2018). Indeed, institutionalized children present poorer health outcomes such as low-weight, short-stature, smaller head circumference and abnormal neurobiological development (Bick & Nelson, 2015). In terms of cognitive development, institutionalized children show lower IQ (less 17 to 20 IQ points), learning disorders and consequently school completion difficulties, memory and executive function impairments when compared to biological family-reared children (Carr et al., 2018). Not surprisingly, these children are also at greater risk of developing mental health and behavioral problems. These include anxiety disorders, higher difficulty in emotion regulation, and disturbed social behaviors such as inhibited and indiscriminate social behavior (Sonuga-Barke et al., 2017). For discussion about the etiology and development of the former pathologies, please see Zeanah & Gleason (2015), Lyons-Ruth (2015), and Zeanah et al. (2009).

1.2 Length of Institutionalization, prior and current relational experiences

Age of admission, length of institutionalization and age at adoption are common variables in institutionalization studies that try to explain the individual differences in outcomes observed in these children. Indeed, literature shows less deleterious effects for shorter periods of institutionalization and more severe and harder to reverse outcomes for those exposed to institutionalization for longer periods of time (van IJzendoorn et al., 2020). However relevant and important in the institutionalization literature, this duration and timing effect is not always present

(Oliveira et al., 2012; Soares et al., 2014; Zeanah et al., 2009), and thereby there has been a focus on other proximate caregiving variables that may work as key contributions to the development and maintenance of the negative socio-emotional outcomes of institutionalized children. Indeed, most investigations regarding the socioemotional development of institutionalized children are mostly based on a research design that compares the average functioning of institutionalized children with home-reared children. Consequently, variation in the institutionalization experience is often overlooked.

For example, in the study of Merz and McCall (2010), the authors compared a group of 6 to 18 years old Russian adopted children that had experienced institutional rearing with adequate physical resources but lacking consistent and responsive caregiving, frequent changes in caregivers and low levels of caregiver-child social interaction, with two other groups of post-institutionalized children: 1) a group of post-institutionalized children from around the world with different levels of deprivation and quality of institutional settings and care (Gunnar et al., 2007), and 2) a group of severely deprived post-institutionalized Romanian children. The study concluded that children from severely deprived Romanian institutions were at greater risk of developing various kinds of behavioral problems when compared to the other two groups; furthermore, when compared to never-institutionalized children, even the less deprived institutions (*i.e.* with good physical resources) but high on child to caregiver ratios, had higher rates of attentional and externalizing problems. Relatedly, in a Portuguese sample of institutionalized children aged 12- to 30-months-old, not having a preferred caregiver (adult with whom the child has a preferred affective relationship at the institution) predicted indiscriminate social behavior, over and above 3 pre-institutionally composite experiences created by the study authors (Soares et al., 2014). These composites of pre-institutional risk composites included maternal physical disease, substance use, unsupervised pregnancy and child's premature birth (pre-natal risk composite); government financial aid, domestic violence, prior evaluation of the family by social workers as at risk and prior institutionalization or adoption of target child's sibling (family-relational risk composite), and parental neglect as reason for the child's institutionalization, mother's prostitution, substance abuse, psychopathology or mental retardation (neglect risk composite). Similarly, being the favorite child of a caregiver is a protective factor to the development and severity of indiscriminate social behavior, given that these children exhibit lower levels of this behavior (Smyke et al., 2002). Additionally, in an experimental setting, the same study (Smyke et al., 2002) developed an

intervention that had as main goal improving the consistency of the caregivers during the child's waking hours; the results showed that poorer caregiving quality (higher caregiver-to-child ratio) was related to more negative behavior among 5–31-month-olds residing in institutions, even after taking into account child gender and length of institutionalization. These results are congruent with previous research showing that pathological social behavior in institutionalized children is common even in high-quality institutions where there is a lack of caregiver emotional investment (Tizard & Rees, 1975; Tizard & Hodges, 1978; Lyons-Ruth et al., 2009).

Indeed, research shows that caregiver's interaction quality towards the child - such as sensitivity, cooperation and sensitive responsiveness - is associated with better child's developmental and socioemotional outcomes (Ainsworth, 1969; Leerkes et al., 2009). Caregiver's quality of care can be assessed through Ainsworth's Maternal sensitivity scales (Ainsworth, 1969) that evaluate 2 different but related constructs: 1) **caregiver's sensitivity**, which captures the caregiver's capacity to perceive and accurately interpret, and appropriately and promptly respond to the signals and implicit communication of her child's behavior and 2) **caregiver's cooperation,** that evaluates caregiver's interventions in terms of timing and quality considering the child's state, mood and interests, or if it interrupts or cuts across the child's ongoing activity. These two scales are highly correlated and previous research has derived what is called caregiver's **sensitive responsiveness,** by averaging the results of both scales, forming a new composite that takes into consideration both the caregiver's sensitivity and cooperation aspects (Baptista et al., 2014). Especially relevant for this work, it is known that a caregiver's sensitive responses to the child's negative emotions promote a child's better self-regulation and influences their perception of negative emotional expressions as more tolerable (Leerkes et al., 2009). Furthermore, Taylor-Colls & Fearon (2015) found that a caregiver's sensitivity positively influences the child's attentional bias towards positive facial emotions.

The abovementioned results show how quality of institutional care is associated with variation in the socioemotional outcome among these children. Nevertheless, it is unlikely that institutional care experience alone can explain the variations seen in these children. Indeed, some studies point to the influence of non-institutional factors, especially pre-institutional risk conditions, that compromise the normative socioemotional development of institutionalized children. The adverse family experience that most of these children go through before entering an

institution is relevant in the early emergence of emotional and behavioral problems. For example, Oliveira et al. (2012) found that a prenatal risk composite (*i.e.*, maternal physical disease and maternal substance abuse during pregnancy) predicted higher levels of indiscriminate social behavior in institutionalized children and that a maternal emotional neglect risk composite (which included maternal emotional unavailability and psychopathology) also mediated the association between prenatal risk and indiscriminate social behavior in institutionalized toddlers. Similarly, Baptista et al. (2014) found that the interaction between an history of maltreatment in the family (*i.e.*, physical/sexual abuse, physical neglect, and emotional maltreatment) and unstable caregiving (characterized as higher child-to-caregiver ratio; higher number of caregivers; higher rotating shifts; unstable working schedules and higher turnover of caregivers) in the institution predicted high levels of externalizing problems. Taking all together, it is important to consider how early child experiences (pre-institutionalization) along with the acknowledgement of the quality of care that the child experiences at the institution influences simultaneously the developmental differences seen in these children.

1.3 Face Processing in Institutionalized Children

Cognitive and emotional processes have been particularly studied in this specific population by examining face processing abilities (Parker & Nelson, 2005a,b; Moulson et al. 2009a,b). The ability to recognize face and emotions in oneself and others plays an essential role in adequate social adaptation and development (Cicchetti & Curtis, 2005). Regarding our social interactions, faces are one of the most important visual stimuli to us as humans, and there is strong evidence suggesting a discrete neural circuit subserving this capacity (as opposed to other objects) (Farah et al., 1998). Bowlby (1969) speculating that this capacity was innate and important to the young infant to recognize potential caregivers' and emotional signals prior to the development of language. Nevertheless, the development of the neural response to face recognition is seen by some authors (*e.g.*, Nelson, 2001) as an experience-expectant process, mainly because of studies showing the sensitivity of this system to the social environment. Indeed, children who experience severe early life adversity such as neglect or abuse show disruptions in processing facial emotions (Cicchetti & Curtis, 2005; Nelson & McCleery, 2008). In fact, the study of neural activity regarding social stimulus such as emotional faces, is not only useful in understanding core

neurobiological elements impairments related to complex cognitive and emotional functions, but also in understanding how these impairments may underlie higher social difficulties and deficits seen in institutionalized children (Moulson et al. 2009a).

One way to measure the neurological correlates of information processing such as face processing is through Electroencephalography (EEG), which measures the electrical activity of the brain with a superior temporal resolution compared to other methods. Of special interest are the event related potentials (ERPs) measures extracted from EEG, that capture the brain's electrical activity during time locked events. Regarding the processing of facial expressions, some early and late-occurring ERP are especially relevant, including the P100, N170 and N250 (Curtis & Cicchetti, 2011; Nelson & McCleery, 2008).

The **P100** is an occipital component associated with early processing of visual information and selective attention and occurs between 80 and 120 milliseconds after the stimulus presentation (Mangnun, 1995). Even though it is not specific to faces, this component is linked with attentional influences on early face processing (de Haan, 2007). Among 5-years old children, P100 latency appears to be modulated by affective facial expressions (Batty & Taylor, 2006), in such that it occurs earlier in response to positive and neutral emotions, and later for fear and disgust. Moreover, the same authors also found sex differences in the latency response, in a way where boys had shorter latencies for angry and sad faces (compared to all other emotions) than girls. P100 also differentiates the response to angry faces between maltreated and nonmaltreated children (Curtis & Cicchetti, 2011), showing greater amplitude towards angry faces when compared to their non-maltreated peers. Furthermore, among 3-year-old children, the P100 amplitude was greater for fearful faces compared to neutral ones (Vlamings, Jonkman, & Kemner, 2010).

The **N170** is also a temporal-occipital component occurring between 150-300 milliseconds after stimulus presentation and is associated with structural processing of face (Schyns et al., 2003). Data from N170 response and modulation to facial expression is unclear, with some studies showing that N170 amplitude is modulated by facial expression (Batty & Taylor, 2003; Caharel et al., 2005; Eger et al., 2003; Miyoshi, Katayama, & Morotomi, 2004) and others showing no discrimination from N170 component in response to emotional expressions (Eimer et al., 2003; Herrmann et al., 2002). Nevertheless, Vlamings, Jonkman, & Kemner (2010) show that N170

amplitude is larger for neutral compared to fearful faces in 3 years old, although in one study with 4-15 years old participants this effect was lost (Batty & Taylor, 2006).

The **P400** component occurs between 250-500 milliseconds after stimulus presentation and is known to be responsive to emotional facial expression processing in children (Kobiella et al., 2008; Leppanen, Moulson, Vogel-Farley, & Nelson, 2007) and also sensitive to the child's early adversity experience - for example, in Curtis & Cicchetti (2011) the authors show that 42-month-old maltreated children had greater P400 response to angry facial expression compared to their non-maltreated aged-matched peers.

Other relevant component in the face processing literature is the **Nc**, a large negative ERP component located at the frontal and central EEG leads, and that occurs about 400-800 milliseconds after the stimulus onset (Courchesne *et al.*, 1981). The Nc is thought to be associated with attention allocation to salient/relevant and familiar stimuli (de Haan et al., 2007). In 4 to 6 years old children, Batty & Taylor (2006) found that Nc amplitude is greater in response to fear, disgust and sad faces compared to happy faces. Nc is also more responsive (larger and more rapid) to angry compared to happy faces (Lewis et al., 2007; Nelson & Nugent, 1990). In one study, it was also considered that Nc response to emotional faces serves as regulation of anxiety, given the fact that this component was elicited by angry faces and also because among more anxious children, Nc response was elicited earlier (Lewis et al., 2007).

Finally, the P250 is an early positive frontocentral component and discriminates between neutral and emotional expressions (da Silva et al., 2016; Wronka & Walentowska, 2011).

Table 1. Summary of the ERP components considered in this study

ERP component	Function	Emotional Processing
P1 Occipital negative component around 80 and 120 ms	Reflects early, rapid processing of both simple and complex stimuli; shows sensitivity to configural changes (Batty & Taylor, 2003)	- **Negative** compared to neutral or positive emotions elicited later P1 latencies in 4–6 year olds but not older children or adults (Batty & Taylor, 2006); - No significant amplitude or latency effects for the P1 due to the familiarity or emotional expression of faces (Todd et al., 2008).
N170 posterior negative potential around 170 ms	Detection of faces (particularly the eyes); thought to reflect the structural encoding of faces (Batty & Taylor, 2003)	- Faster or of greater amplitude for emotional faces (Batty & Taylor, 2003); - N170 may not be sensitive to emotional faces in preschool to school-aged children (Batty & Taylor, 2006); - NIG exhibited greater N170 amplitudes to **sad expression**; institutionalized children displayed greater N170 amplitudes to the fear expression (Parker et al. 2005b)
Nc frontocentral negative deflection around 200–700 ms	Sensitive to facial familiarity in infants may also index stimulus salience and memory recognition (de Haan & Nelson, 1997); reflects infants' allocation of attention (Nelson & Collins, 1991)	-Nc amplitudes are larger and latencies faster in response to **negative** facial emotion compared to happy faces in children (Batty & Taylor, 2006; Lewis et al., 2007; Todd et al., 2008). -Nc may be related to both increased attention to emotional faces and the ability to regulate responses elicited by emotional faces. -7- month- old infants display larger Nc to **fearful** than happy faces (de Haan et al., 2004; Grossmann et al., 2011; Leppänen et al., 2007; Taylor-Colls & Pasco Fearon, 2015).
P400 semi-medial occipital sites	Associated with structural processing, encoding, and attention to faces early in development (de Haan & Nelson, 1999; de Haan, et al., 2003).	-Larger amplitude to **fearful** than to happy and neutral faces; -Greater amplitude in response to fearful face compared to both happy and neutral expressions (Leppänen et al., 2007) -Greater in response to fearful faces compared to angry Faces (Kobiella et al., 2008) -Greater P400 response to angry than happy and fearful faces (Xie et al., 2018)
P250 early positive frontocentral component	Matching between a currently perceived stimulus representation to stored, structural representations (Caharel et al., 2002); involved in higher-level semantic processing (Marzi & Viggiano, 2007).	-NIG and IG children manifested greater P250 amplitudes in response to sad and fear expressions, respectively (Parker et al., 2005b); -Relative to the NIG children, the IG exhibited a larger P250 amplitude across all emotions (Parker et al., 2005b)

In general, there is evidence that institutionalized children show a general cortical hypoarousal, compared to their never-institutionalized peers, when exposed to pictures of both familiar and unfamiliar faces using ERP procedure. Specifically, changes in the ERP components N170 and Nc (Parker & Nelson, 2005a) and P1 and P400 components (Moulson et al., 2009a) were observed. Furthermore, behavioral studies with previously institutionalized children show that they are less accurate, compared to controls, at identifying emotions such as happiness, sadness, fear, and anger, as well as matching emotional scenarios to the correct emotional face (Wismer Fries & Pollak, 2004). Indeed, institutionalized children show larger N170 amplitude in response to fearful *versus* sad faces and larger P250 amplitude to happy and sad *versus* angry faces, whereas family-reared children show larger N170 amplitude in response to sad *versus* fearful faces and greater P250 to fear *versus* sad faces (Moulson et al., 2009b; Parker & Nelson, 2005b). Nevertheless, the role of pre-institutional environment *versus* current relational experiences as well as the neurobiological mechanisms that precede these neurophysiological manifestations of institutionalized children to emotional face stimulus are still unknown.

1.4 DNA Methylation as a Neurobiological Response to Adversity

Recent research has focused its attention on how stress-induced neurobiological changes (*e.g.*, genome activity regulation) from adverse caregiving precedes and influences phenotypic manifestations, impacting child's psychological and emotional development (Cole, 2014; Harrison & Baune, 2014; McCrory, De Brito, & Viding, 2010). Epigenetics is a genome activity regulation mechanism and refers to the chemical modification occurring on both DNA and/or on histones, proteins to which DNA is wrapped around. Epigenetic modifications are therefore mechanisms that control the state of the chromatin. A tight chromatin disfavors transcription factor binding, which results in transcriptional silencing (low gene expression), while a loosed chromatin will favor transcription factors binding to DNA and consequently favor gene expression. DNA methylation (DNAm) is one of the most common and widely studied epigenetic mechanisms (Hochberg et al., 2011) and consists of the addiction of a methyl group in the DNA CpG sites (Bird, 1986), associated with transcriptional gene silencing, *i.e.*, low gene expression (Hochberg et al., 2011).

Pioneering animal studies showed a causal link between early life adversity and epigenetic changes, focusing especially on how maternal neglect or deprivation altered epigenetic states, influencing offspring development. Indeed, initial animal studies showed that offspring who received low levels of liking and grooming (LG, *i.e.,* a measure of maternal care) had reduced expression of hippocampal glucocorticoid receptors, impairing the negative feedback on the hypothalamic-pituitary-adrenal axis (HPA axis) and consequently showing higher stress responsivity (Liu et al., 1997). More recently, Weaver et al. (2004a) showed how this variation in the quality of maternal care causes DNAm alterations on the glucocorticoid receptor gene (NR3C1) emerging in infancy and lasting into adulthood. The authors concluded that offspring from the Low-LG had higher levels of DNAm on the NR3C1 gene compared to the High-LG group; moreover, this result proved to be reversible when offspring of the Llow-LG mothers were cross-fostered to High-LG mothers. Other studies have shown this behavioral programming through epigenetic modifications, focusing on maternal separation or deprivation (Francis et al., 2002; Kalinichev et al., 2002) and artificial rearing in case of maternal absence (Gonzalez et al., 2001; Yasuda et al., 2016). In sum, offspring of absent or "poor" quality maternal care reveal long-term detrimental neurobehavioral effects (Weaver et al., 2004a,b; Chen et al., 2012; Kalinichev et al., 2002; Caldji et al., 1998; Yasuda et al., 2016; Moffett et al., 2006; Huot et al., 2001). These effects are *imprinted* by lasting epigenetic modifications that have a functional relevance, given the fact that the regulation of the expression of genes such as the glucocorticoid receptor gene NR3C1 can explain the differences seen on how individuals respond to stress (Weaver et al., 2004a,b). These findings are especially relevant when considering the long-lasting impact adverse childhood experiences can have at the gene regulatory mechanisms level that can perpetuate the effect of these exposures long after the exposure has ended.

Human studies have also corroborated the hypothesis of epigenetic changes as a biological embedding mechanism of adverse childhood experiences in general (Essex et al., 2013; Jawahar et al., 2015; McGowan et al., 2009; Naumova et al., 2016; Turecki & Meaney, 2014; Wright et al., 2017), and childhood institutionalization in particular (Esposito et al., 2016; Naumova et al., 2019; Naumova et al., 2012; Non et al., 2016; Kumsta et al., 2016). Indeed, there is evidence of altered DNAm across genes related to either stress-response or behavioural affiliative processes in institutionalized children (Kumsta et al., 2016; Non et al., 2016). Specifically, the FKBP5 gene (a member of the glucocorticoid receptor complex that is responsible for the cessation of cortisol

stress response by altering this receptor negative feedback (Binder, 2009), and the SLC6A4 gene, responsible for encoding the serotonin transporter (5-HTT), well established associated with early life adversity, maternal care, and long-term child developmental outcomes (Non et al., 2016), and the CYP2E1 gene, which appears to have a role in modulating behavior and cognitive processes (Penas-Lledo et al., 2009). The NR3C1 gene in particular can be considered as a plausible candidate to better understand the stress response to relevant social and emotional stimuli in institutionalized children. In fact, NR3C1 gene DNAm has previous empirical associations with variation in the quality of maternal care received, in both animal (Weaver et al.,2004a) and human studies (for a review, see Provenzi et al., 2019). Furthermore, there is evidence that NR3C1 hypermethylation occurs in both early exposure to maltreatment and/or abuse (Cicchetti and Handley, 2017; Labonté et al., 2012; Radtke et al., 2015), and in exposure to less sensitive or responsive maternal behavior (Murgatroyd et al., 2015; Conradt et al., 2016, 2019), experiences commonly lived by institutionalized children. Little is known, however, about how previous *versus* current institutionalization experiences differentially impact NR3C1 gene methylation.

2. Research Aims and Hypotheses

2.1 Rationale

All things considered, the studies reviewed above show how the quality of early environment impacts development in a wide rather than focused way, showing pervasive effects not only at the genome level (through epigenetic modifications), but also at the neurobiological functioning and behavioral levels. Here we aim to investigate how pre-institutional adverse experiences and current quality of the relational context at the institution are associated with both epigenetic modification (through DNAm level of the NR3C1 gene) and with neuronal processing of facial emotions in institutionalized children.

2.2 Study Hypotheses

This study will explore, in current-institutionalized children, the DNAm pattern of the NR3C1 gene and the ERPs amplitude and latency in response to familiar faces with 2 different emotional expressions (Happy and Angry faces). For this, we will compare institutionalized children exposed to different **pre-institutional risk factors** and experience different **quality of**

institutional relational care. Taking into consideration the above-mentioned studies showing how quality of care influences DNAm patterns in children (Essex et al., 2013; Jawahar et al., 2015; McGowan et al., 2009; Naumova et al., 2016; Turecki & Meaney, 2014; Wright et al., 2017), we hypothesize:

1. Exposure to **pre-institutional risk factors** (*i.e.,* adverse experiences that led to the institutionalization of the child) are related to DNAm of the NR3C1 gene. More specifically, we expect to find a hypermethylation of the NR3C1 gene in exposure to pre-institutional risk factors.

2. Differences in the **quality of institutional relational care** (conceptualized as the caregiver's level of sensitive responsiveness towards the child) is related to DNAm of the NR3C1 gene. Specifically, we will investigate whether quality of care is inversely correlated with DNAm of the NR3C1 gene in institutionalized children.

Giving the lack of literature on the contributions of quality of care in the processing of emotional face stimulus in institutionalized children we will further explore:

3. If exposure to pre-institutional risk factors and quality of institutional relational care influences the child's amplitude and/or latency response on ERP components (Nc, P100, N170, P400, and P250) when processing emotional stimuli (happy and angry faces) of the caregiver; We hypothesize that children exposed to more adverse pre-institutional contexts and to low quality of institutional relational care will differ from the others in the latency and amplitude of the ERP components (Nc, P100, N170, P400, and P250) when processing emotional stimuli (happy and angry faces).

3. Methods

3.1 Sample

3.1.1 Institutionalized children

This study is part of a larger research project that began in January 2010, that focuses on the development of Portuguese institutionalized children. Exclusion criteria included having

severe mental or physical impairments, genetic diseases, autism spectrum disorder and being institutionalized for less than 6 months. A total of 94 participants underwent ERP testing, but 24 were excluded because of insufficient usable data due to excessive noise in the EEG or less than 25 good trials per condition. One participant was excluded for missing data. The final sample for analysis consisted of sixty-nine institutionalized children (47 boys, 68.1%), who were recruited from 23 Portuguese institutions, along with their caregivers. Children were between 36 and 78 months old (M = 56.58, SD = 10.96) by the time of assessment. Age of admission ranged from 3 to 69-months-old (M = 38.03, SD = 14.36), and 7.2% of children were admitted to the institution before 12 months of age. Time in institutions ranged from 6 to 54 months (M = 18.06, SD = 10.98), and most of our sample was institutionalized for 12 or more months (n = 46, 66.7%).

Table 2. Child's Variables

	n	**M (SD)**	**Min-Max**
Age at assessment (months)	69	56.57 (10.96)	36-78
Age at admission (months)	69	38.03 (14.36)	3-69
Time in institutional care (months)	69	18.06 (10.98)	6-54
Developmental Quotient	69	100.18(9.74)	80.18 - 127.12
Gestational Weeks	54	38.67(2.27)	27-42

N= 69. When the whole sample was not available due to missing data on children's case files, the subsample available for analysis is indicated

Neglect was the most common reason for institutional admission in our sample (n = 57, 82.6%), followed by lack of parental competencies (n=34, 49.3%), exposure to family violence (n=24, 34.8%), previously signalized family by social services (n=23, 33.3%), exposure to drug abuse (n=21, 30.4%), lack of socioeconomical conditions (n=18, 26.1%), lack of habitational conditions (n=12, 17.4%), abandonment from primary caregivers (n=11, 15.9%), parental psychopathology (n=8, 11.6%), risk prevention based on previous social services signaling (n=8, 11.6%), psychological abuse (n=7, 10.1%), incest and/or promiscuous relations (n=6, 8.7%), physical abuse (n=6, 8.7%), exposure to criminal practices (n=4, 5.8%), homelessness (n=3, 4.4%), parent's physical inability (n=3, 4.3%), parental cognitive incapacity (n=3, 4.3%), exposure

to maternal prostitution (n=3, 4.3%), effective abandonment (n=2, 2.9%), and abandonment at birth (n=1, 1.4%). In our sample, 48 children (69.57%) had at least 3 pre-institutional risk factors that were considered as reasons for institutional admission. Table 3 shows the cumulative number of pre-institutional risk factors that led for each child's institutionalization.

Table 3. Number of reasons (pre-institutional risk factors) for institutional admission for each child

Number of reasons for institutional admission	n (%)
1	11 (15.9)
2	10 (14.5)
3	18 (26.1)
4	10 (14.5)
5	9 (13.0)
6	3 (4.3)
7	5 (7.2)
8	3 (4.3)

Note. N = 69

3.1.2 Institutional Caregivers

Fifty-six institutional caregivers participated in this study (1 male, 1.8%). Age of caregivers ranged from 21 to 67 years (M = 38.13, SD = 11.20). One (1.9%) had 4 years of education, two (3.7%) had 6 years of education, fifteen (27.8%) had 9 years of education, seventeen (31.5%) had a high-school diploma and 16 (29.6%) had graduated from college. Twenty-six (51%) did not have specific training for their role and thirty-three (61.1%) had rotative schedules. On average, caregivers spent 35.33 minutes (SD = 49.80) individually with each child per day. Regarding the number of children responsible per day, the number ranged from 4 to 28 children responsible per day (M = 11.57, SD = 5.63), with twenty-eight (59.6%) of the caregivers having 10 or more children for whom they were responsible per day.

Table 4. Caregiver's Variables

	n	M (SD)	Min-Max
Age	53	38.13 (11.20)	21-67
Sensitivity	54	5.30 (1.77)	1-9
Cooperation	54	5.44 (1.51)	2-9
Sensitive Responsiveness	54	5.37 (1.52)	1.5-8
Number of children responsible for per day	47	11.57 (5.63)	4-28
Time dedicated to individual child care per day	38	35.33 (49.80)	0-240

Note. N = 56 (1 male)

4. Procedure

This study is part of a larger research project that began in January 2010. It was obtained approval by the Portuguese Social Services and the National Commission for Data Protection. The study was then presented to the staff at each institution. Written informed consent was obtained from biological parents, institution directors, and participating caregivers.

Children were recruited based on their age and exclusion criteria. After deciding which children met the criteria for participating in the study, institutional staff was consulted to identify the assigned caregiver to each child. To characterize a child's prior experiences to institutionalization, information was gathered from his/her file. This information served, as well as with the staff's contribution, to complete the child's sociodemographic questionnaires. The child's mental development and caregiver's quality of care were assessed by trained examiners. Children's files were filled by social workers based on the information that was available to them.

5. Measures

5.1 Sociodemographic Information

This questionnaire assesses both pre and current experiences and life contexts of the institutionalized child. It was completed with the help from a member of the technical team of the institution (*e.g.*, social worker, psychologist) and with access to the child's individual process. Through this questionnaire, information about (1) child's identification (*i.e.*, sociodemographic information, date of institutionalization, reason of admission); (2) filiation (*i.e.*, parents, siblings, household composition); (3) socioeconomic status (*i.e.*, housing, and economic situation); (4) health and developmental history of the child and (5) kindergarten characterization and child's adaptation to it is gathered. Answers are either descriptive and/or categorical (*e.g.*, yes/no). Early family risk factors and pre-institutional experiences were collected through the child's files at the institution.

5.2 Child's Mental Development

The Griffith's Mental Development Scales (1984) was used to assess child's general mental development. Composed of 6 subscales, each pertains to specific areas of development: locomotor, personal-social, language, eye-to-hand coordination, performance, and practical reasoning. All subscales were used and calculated, giving a total score reflecting general development for each abovementioned component. A final overall developmental quotient was calculated by averaging the different sub-quotients (Cronbach's $\alpha = 0.728$). The mean score of our sample was 100.18 (*SD* = 9.74), with a minimum of 80.18 and a maximum of 127.12.

5.3 Quality of Institutional Caregiving

5.3.1 Caregiver's sensitivity towards the child, which was assessed during a fifteen-minutes interactive and video-taped task, that was divided in three episodes: i) play with a challenging toy; ii) monitoring the child during a sham questionnaire, during which the child only possess one uninteresting toy to play, with others more interesting toys to play nearby s/he is instructed not to play with and, iii) free play followed by a clean-up. Scorings of these recorded

sessions were carried out using Ainsworths' Maternal Sensitivity Scales (1969), adapted to the preschool years. Two independent coders rated all cases for the sensitivity vs. insensitivity scale who were blind to the type of relationship of the dyad and to other data collected in this inquiry. The ratings evaluated the ability of the caregiver to perceive and interpret the child's cues and communication, and to correctly respond to them. Four more aspects of caregiver sensitivity are considered: i) awareness of the child signals; ii) correct interpretation of those signals; iii) accurate response to them, and iv) promptness of response. The scores range from 1 (highly insensitive) to 9 (highly sensitive). When disagreements occurred within classification of a case, discussion was carried out until consensus was obtained. Cohen's K was run to determine if there was agreement between two researcher's judgements on caregiver's sensitivity. There was a moderate agreement between the two judgments, $k = .483$, $p < .005$, for the cases classified by the two researchers (n=30). The mean score for caregiver's sensitivity of this study was 5.31 (*SD* = 1.73, range 1-9).

5.3.2 Caregiver's Cooperation based on Ainsworth (1969) Maternal Sensitivity scales. This scale focus on the caregiver's interventions with child's ongoing activity, that is, whether the caregiver breaks into, interrupts or cuts across the child's activity. Two aspects are considered when evaluating the degree of interference: i) the actual extent of physical interference during child's activity, and ii) frequency of interruptions. The scores range from 1 (Highly interfering) to 9 (Conspicuously cooperative). In this study, the mean score for caregiver's cooperation was 5.37 (*SD* = 1.50, range 2-9).

5.3.3 Caregiver's Sensitive responsiveness, which is in line with previous studies (Baptista et al., 2013) and takes into consideration the two highly correlated scales of sensitivity and cooperation ($r = 1.00$, $p < .001$), by averaging the two scores. Our sample's mean score for sensitive responsiveness was 5.34 (*SD* = 1.49, range 1.5-8).

5.4 Event Related Potentials

5.4.1 Procedure

At a distance of 100 cm, the child was seated in front of the computer screen, with the center of the eyes in the midpoint of the screen. Instructions about the task were given to the child, followed by a brief training procedure. Child's caregiver was in the room, standing behind the child, and a researcher monitoring and recording the child's behaviour and quality of the EEG signal. The paradigm had a total duration of 30 minutes, and was divided into three blocks, with an interval of 10 minutes between each block, where the child was free to move around.

5.4.2 Task

The EEG task, procedure and recording were those used in previous research (Mesquita et al., 2015; see Figure 1). To assess the neural emotional face processing of institutionalized children, a face processing Go/NoGo Task based on Todd et al. (2008) was created, using the Presentation (Neurobehavioral Systems Inc., Berkeley, CA) software (Cf. Mesquita et al., 2015). In the present study, only the caregiver's face will be considered for analysis of emotional face processing. In each trial, a fixation cross appears for 400ms. After this, it is presented the face of a familiar caregiver with three possible emotional expressions (happy, neutral, or angry), for a period of 1,000-1,500ms. Then, a frame appears surrounding the face, either filled with circles (Go trials) or stripes (NoGo trials). Go/NoGo trials were counterbalanced across participants. In the correct Go trials (face with the frame with circles) the stimulus automatically disappears when participants press the button. If the button is not pressed within the following 600-1,000ms after the presentation, a red cross appears and remains on the screen for 700ms. In the correct NoGo trials (face with the frame with strips), the stimulus remains for 1,500ms before it disappears. If the button is incorrectly pressed in the NoGo trial, a red cross appears and remains for 700ms. There are a total of 180 Go and 180 NoGo trials (each one preceded by a different facial emotional expression). The intertrial interval is 1,000ms.

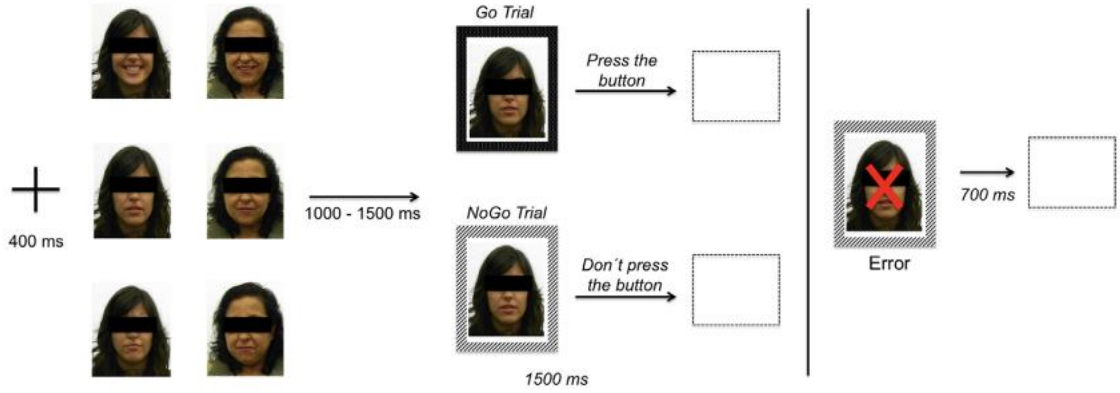


Figure 1. Go/NoGo task design. Retrieved (with copyright permissions) from Mesquita et al. (2015).

5.4.3 EEG Recording

The EEG recording was conducted with Brain Vision Recorder system, using Quickamps amplifier with 32 sintered Ag/AgC1 electrodes set, accordingly to the extended 10-20 International System, at Fp1, Fp2, F7, F3, Fz, F4, F8, FC5, FC1, FC2, FC6, C3, Cz, C4, CP5, CP1, CP2, CP6, T7, T8, TP9, TP10, P7, P3, Pz, P4, P8, PO9, PO10, O1, Oz, O2. Vertical and horizontal electrooculographic activity from above and below the left eye and outer edges of the two eyes were recorded bipolarly, in order to control blinks and eye movements. Impedance of the electrodes was kept below 10 kO. EEG signals were amplified and digitized continuously at a rate of 250 Hz and filtered online with a 0.3-70 Hz band pass filter. The EEG data were corrected for ocular artifacts using the Independent Component Analysis method (ICA; Jung et al., 2000). Subsequently, the EEG was segmented into epochs of 1100 ms, from 100 ms pre- to 1000ms post-stimulus after face stimuli. The data were baseline-corrected to 100 ms before face onset, and segments exceeding +/- 100 μV at any scalp electrode were rejected. The ERPs were averaged and analyzed with a semiautomatic peak detection procedure at the corresponding electrodes for each component. All components were individually checked. For the current thesis, five components, namely **P100, N170, Nc, P250, and P400** were detected in response to caregiver's happy and angry faces. The P100, N170 and P400 components were identified and marked as, respectively, the largest positive peak between 80–220ms after stimuli onset, the largest negative peak between 150–350ms, and the largest positive peak between 250–550ms, at electrode sites O1 and O2

(occipital), and PO9 and PO10 (parieto-occipital). For the **Nc** a temporal window between 330 and 550ms was selected, being its latency the largest negative peak detected in that time frame. Nc electrodes were marked at C3, Cz and C4 electrode sites. The **P250 component** was identified in a temporal window between 175 and 375ms and marked at electrode site C3, Cz and C4.

5.5 Epigenetic Profile Analysis

Saliva samples were collected from institutionalized children of our sample with an ORAGene® (OG-250) device for later DNA isolation, following the manufacturer's instructions. Using the Epityper technology from Agena Bioscience, DNA methylation level were analyzed in enriched CpG sites target regions of the Exon 2 of the gene NR3C1 (*NR3C1_2*, see Figure 2). First, a bisulfite treatment of extracted genomic DNA from each sample was made, in order to convert non-methylated cytosine residues into uracil (methylated cytosine residues remain unaffected), promoting changes in the DNA template. Preserving the bisulfite-induced sequence changes, the target region of NR3C1 gene was amplified through PCR using T7-promoter tagged reverse primers. Second, it was performed a SAP treatment followed by in vitro RNA transcription and base-specific RNA cleavage. After transferring the reaction products onto a chip for data acquisition, the fragments obtained were analyzed using MALDI-TOF mass spectrometry. Using EpiTYPER Software, the methylated and non-methylated cytosine residues in the original genomic DNA were well discriminated and methylation levels were determined. It is possible, by using base-specific cleavage for methylation analysis with MALDI-TOF-mass spectrometry detection, to quantitatively determine methylation level at the CpG sites of our target gene.

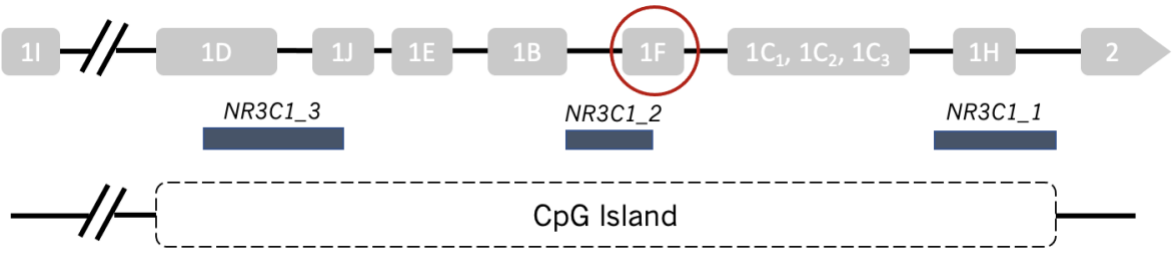


Figure 2. Schematic representation of the glucocorticoid receptor gene (NR3C1). Amplicons (NR3C1_1, NR3C1_2 and NR3C1_3, shown in blue) are shown in relation to the NR3C1 CpG island (dotted box) and untranslated first exons (line boxes) upstream of exon 2. The red circle indicates the 1F exon, analyzed in the current study. Image based on van der Knaap et al. (2014).

6. Data Analysis

Data analysis was run with IBM® SPSS®-26 software. Descriptive statistics were firstly run in order to better characterize our sample and to assess whether parametric tests could be used. The assumptions needed to use parametric tests were met.

To test hypothesis 1 and 2, correlational analyses were run between DNAm NR3C1 gene and quality of relational care with the institutional caregiver (*i.e.,* caregiver's level of sensitive responsiveness), and pre-institutional risk factors (*i.e.,* reasons for child's institutional admission).

For goal 3, and considering the significant results that emerged from hypothesis 1 and 2, a repeated measures ANOVA analysis was run using as a dependent variable the ERP component response (P100, N170, P400, P250, and Nc), in latency (ms) and amplitude (in μV). We conducted three sets of different repeated measures ANOVA, altering the between subject factor: first, we used caregiver's sensitive responsiveness (with two levels: responsive and unresponsive); then we used neglect (with two level: yes and no) and lastly with lack of habitational conditions (with two levels: yes and no). All group analysis used as within group factor emotion (with two groups: happy and angry). An alpha level of .05 was used, and whenever appropriate, degrees of freedom were corrected by the appropriate estimate test. Levene's tests of homogeneity of variances and Box's tests of equality of covariance were checked. When significant ($p < .05$) main or interaction effects emerged, *post hoc* paired comparisons were performed with the Bonferroni adjustment for multiple comparisons, also with an alpha level of .05.

7. Results

7.1 Correlation between DNAm of NR3C1 gene and pre-institutional risk-factors

To test the first hypothesis, Point Biserial correlation analysis was run between NR3C1 DNAm and abandonment experiences (0 = no and 1 = yes), namely abandonment at birth (r_{pb} = .151, *p* = .234), effective abandonment (r_{pb}= -148, *p* = .242), and parental abandonment (r_{pb} = .123, *p* = .331).

Point biserial correlation analysis was run between NR3C1 DNAm and previous family risk signaling from social services (0 = no and 1 = yes), namely as previous family of risk and sibling risk signaling from social services and no significant correlations were found (r_{pb}= .046, *p* = .720 and r_{pb} = -.008, *p* = .950, respectively).

Point biserial correlation analysis was run between NR3C1 DNAm and family risk experiences (0 =no and 1 =yes), such as exposure to substance abuse (r_{pb} = .168, *p* = .185), exposure to criminal practices (r_{pb} = -.066, *p* = .603), exposure to prostitution (r_{pb} = -.108, *p* = .394), and exposure to parental psychopathology (r_{pb} = -.049, *p* = .702).

Point biserial correlation analysis was run between NR3C1 DNAm and experiences of abuse and maltreatment, including experiences of physical abuse (r_{pb} = .015, *p* = .904), psychological abuse (r_{pb}= .006, *p* = .966), testimony of family violence (r_{pb} = .144, *p* = .257), lack of parental competencies (r_{pb} = -.217, *p* = .084), and **parental neglect (r_{pb} = -.439**, *p* <.001).**

Point biserial correlation analysis was run between NR3C1 DNAm and adversity socioeconomic status and parental incapacity, namely with lack of parental competencies (r_{pb} = -.217, *p* = .084), parental physical incapacity (r_{pb} = .060, *p* = .638), **lack of habitational conditions (r_{pb} = -.261*, *p* = .037)**, and homelessness (r_{pb} = .125, *p* = .330).

Table 5. Bivariate associations between NR3C1 DNAm and pre-institutional relational experiences

		NR3C1 DNAm
Abandonment experiences	Abandonment at birth	.151
	Effective abandonment	-.148
	Parental abandonment	.123
Previous signaling from Social Services	Family of risk	.046
	Siblings at risk	-.008
Family risk experiences	Exposure to substance abuse	.168
	Exposure to criminal practices	-.066
	Exposure to maternal prostitution	-.108
	Parental psychopathology	-.049
Abuse and maltreatment	Physical abuse	.015
	Psychological abuse	.006
	Testimony of family violence	.144
	Parental neglect	**-.439****
Socioeconomic and Parental Incapacity	Lack of parental competencies	-.217 (n=63)
	Parental Physical incapacity	.060 (n=63)
	Lack of habitational conditions	**-.261*** (n=63)
	Lack of socioeconomic conditions	-.066 (n=63)
	Homelessness	.125 (n=63)

Note: N = 64.
**p* < .005*;* ***p* < .001

No significant results were found between NR3C1 DNAm and the cumulative number of pre-institutional risk factors for institutional admission for each child. We then divided our sample

in two groups: 1) children who experienced 3 or less pre-institutional risk factors (n=39) and 2) children who experienced 4 or more pre-institutional risk factors (n=30). An independent sample T Test was run between NR3C1 DNAm and these two groups and no significant difference emerged, t(62) = -.286, p =.776.

7.2 Correlation between DNAm of NR3C1 gene and quality of relational care with the institutional caregiver

To test the second hypothesis, Pearson correlation analysis was run between NR3C1 DNAm and caregiver's sensitivity, cooperation, and sensitive responsiveness levels (r = -.038, p = .772, r = .076, p = .555, and r = .016, p = .900, respectively), and no significant results emerged.

Table 6. Correlation analysis between DNAm of NR3C1 gene and caregiver's quality of care

	1.	**2.**	**3.**	**4.**
1.Caregiver's Sensitivity	-			
2.Caregiver's Cooperation	.706** (n=67)	-		
3.Caregiver's Sensitive Responsiveness	.934** (n=67)	.912** (n=67)	-	
4. NR3C1 DNAm	-.038 (n=62)	.076 (n=62)	.016 (n=62)	-

N= 69. When the whole sample was not available due to missing data on children's case files, the subsample available for analysis is indicated
**p < .001

7.3 Proximal and Distal quality of care and ERP response to emotional face stimulus

Considering the significant results found in hypothesis 1, we conducted three sets of a repeated measures ANOVA for all the ERP components (P1, N170, Nc, P250, and P400), having as dependent variable latency (in ms) and/or amplitude (in μV) of each component. For the first set, we used **neglect** as the between-subject factor (with two levels: yes (n=57) and no (n=12) and

the within group factor emotion (with two levels: happy and angry). For the second set, we used **lack of habitational conditions** as the between-subject factor (with two levels: yes (n=12) and no (n=57) and the within group factor was emotion (with two levels: happy and angry). We also run the same statistical model using **quality of relational care of institutional caregiver** as the between subject factor (with two levels: high responsive caregiver (n=41) and low responsive caregiver (n=26)).

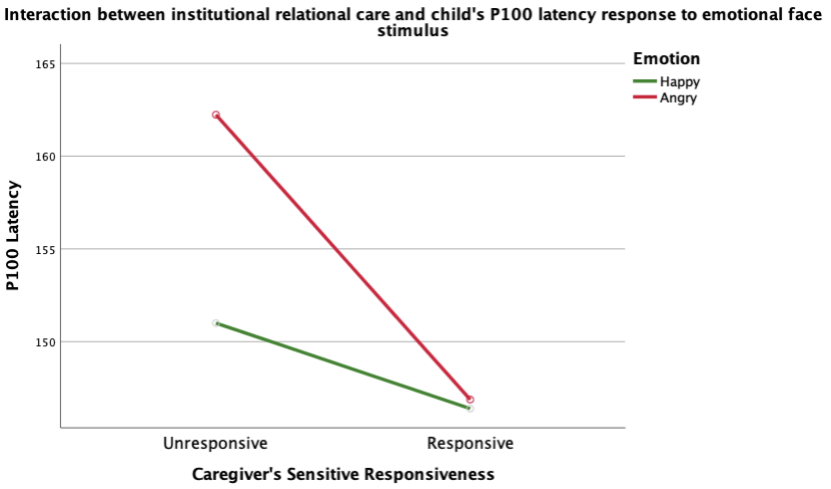
P100 Component

Comparing children with and without experience of Neglect **-** no significant results were found for P100 amplitude and latency response.

Comparing children with and without experience of Lack of habitational conditions **-** no significant results were found for P100 amplitude and latency response.

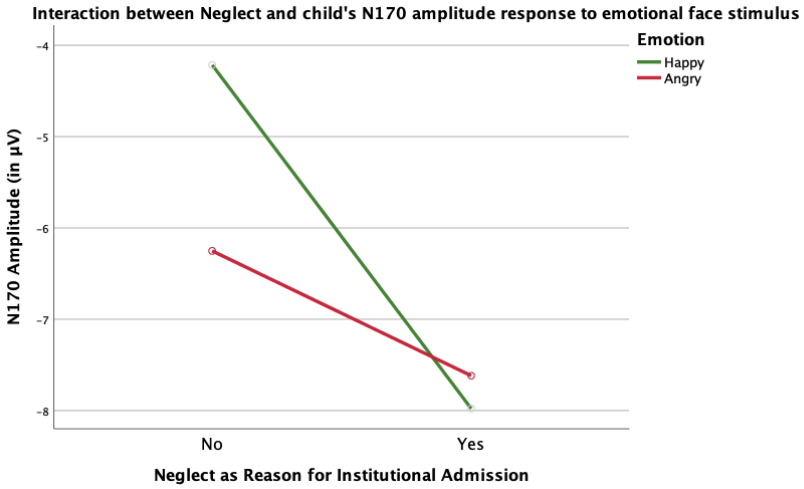
Comparing children experiencing high and low quality of Caregiver's Sensitive Responsiveness - For P100 Latency, there was a main **effect for Emotion, F (1, 65) = 7.787, *p* = .007,** η_p^2= .107, and a significant interaction effect for **Quality of relational care x Emotion**, F (1, 65) = 6.544, *p* = .013, η_p^2= .091. *Post-hoc* analysis revealed that for the low responsive care group, there was a significant difference (*p* <.001) for emotion (happy: *M* = 151, *SD* = 20.89; angry: *M* = 162.23, *SD* = 21.99) and that a significant interaction emerged for **Quality of care x Angry face**, F (1, 65)=7.206, *p* =.009, η_p^2= .100. This effect shows that children cared by more responsive caregivers are quicker (displaying decreased P100 latency) when processing angry faces, than children cared by low responsive caregivers.

No significant results were found for P100 amplitude response.

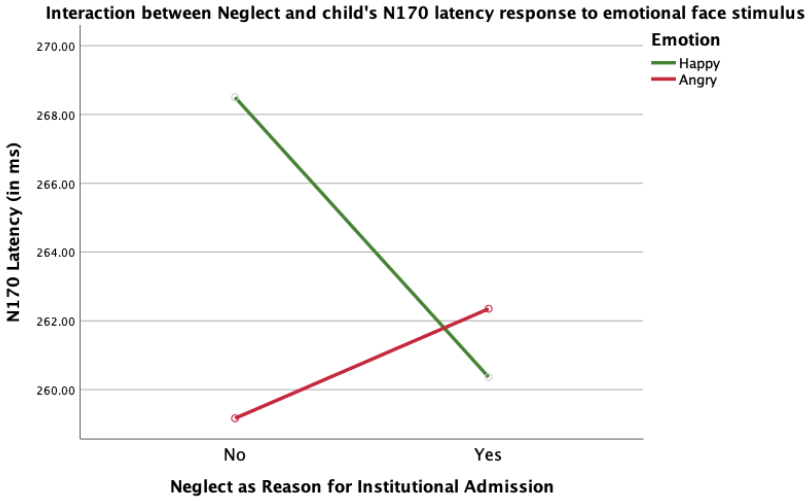


N170 Component

Comparing children with and without experience of Neglect **-** For N170 amplitude**,** a significant effect emerged for **Neglect x Emotion**, **F (1, 67) = 4.762, *p* = .033,** η_p^2= .066. *Post hoc* analysis revealed that there was a significant effect for Emotion in the group of children who did not experienced neglect, F (1, 67) = 4.160, *p* = .045, η_p^2=.058, where these children had greater **amplitude** (*M*=-6.252, *SD* = 2.088) for angry faces than for happy faces (*M*= -4.216, *SD* = 1.987)).

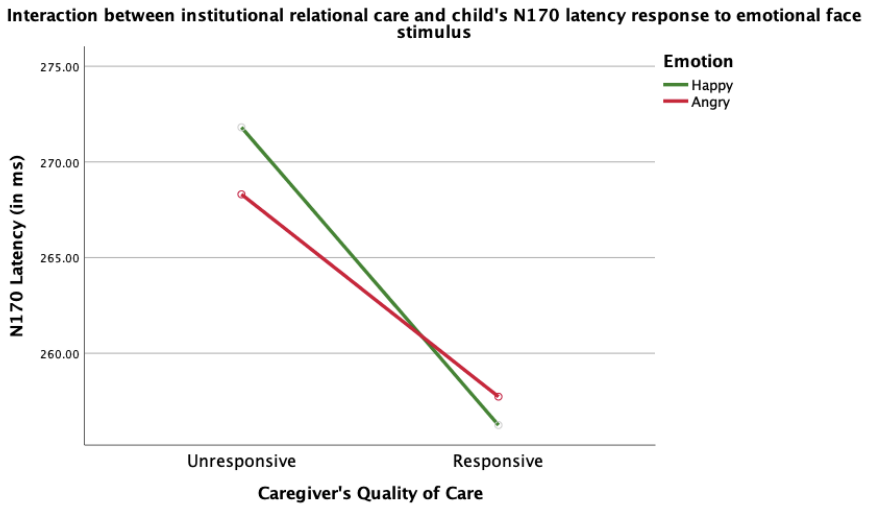


For N170 Latency, a significant effect emerged for **Neglect x Emotion**, F (1, 67) = 5.432, p =.023, η_p^2= .075. *Post hoc* analysis revealed that there was a significant effect for Emotion (happy faces) in the group of children who did not experienced neglect, F (1, 67) = 4.474, p = .038, η_p^2=.063, where these children displayed earlier N170 peaks for angry faces (M=259.17, SD = 6.5) than for happy faces (M = 268.5, SD = 6.29).



Comparing children with and without experience of Lack of habitational conditions - no significant results were found for N170 amplitude and latency response.

Comparing children experiencing high and low quality of Caregiver's Sensitive Responsiveness - For N170 latency, a significant effect emerged for quality of relational caregiving (F (1, 65) = 6.752, p = .012, η_p^2 = .094, independently of the emotional valence of faces. Pairwise comparison test showed that the N170 peaked significantly earlier for happy faces (F (1, 65) = 8.982, p =.004, η_p^2 = .121) in the high responsive (M = 256.244 ms, SD = 20.24) *versus* the low responsive care group (M = 271.81 ms, SD = 21.45). No significant results were found for N170 amplitude response.



Nc Component

No significant results were found relative to neglect, lack of habitational conditions, or caregiver's sensitive responsiveness regarding Nc latency or amplitude response to emotional face stimulus.

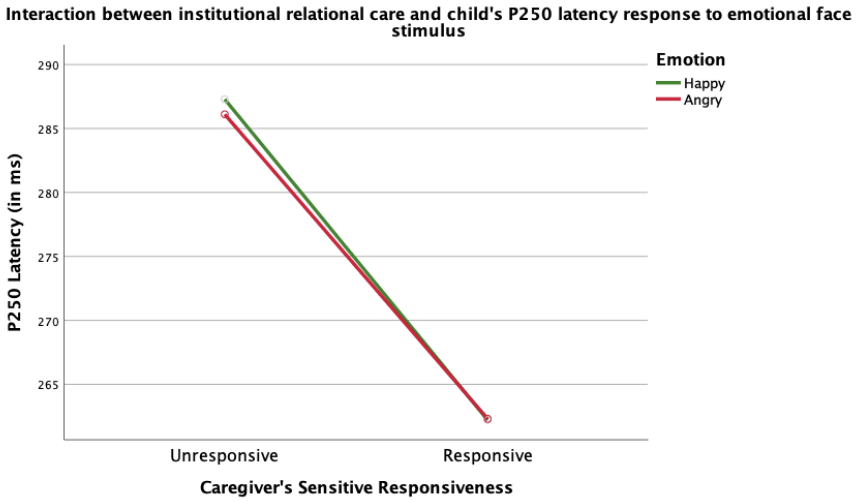
P250 Component

Comparing children with and without experience of Neglect **-** no significant results were found for P250 amplitude and latency response.

Comparing children with and without experience of Lack of habitational conditions **-** no significant results were found for P250 amplitude and latency response.

Comparing children experiencing high and low quality of Caregiver's Sensitive Responsiveness - for P250 latency, a significant effect emerged for quality of relational care (F (1, 65) =15.529, p<.001, η_p^2=.193). The P250 peaked earlier for both happy and angry faces in the high responsive care group *versus* low responsive care group. *Post-hoc* analysis showed that there were significant differences for happy (F (1, 65)=13.004, p<.00,η_p^2=.167) and angry (F(1, 65)=11.746, p<.00,η_p^2=.153) faces between high and low quality of care conditions.

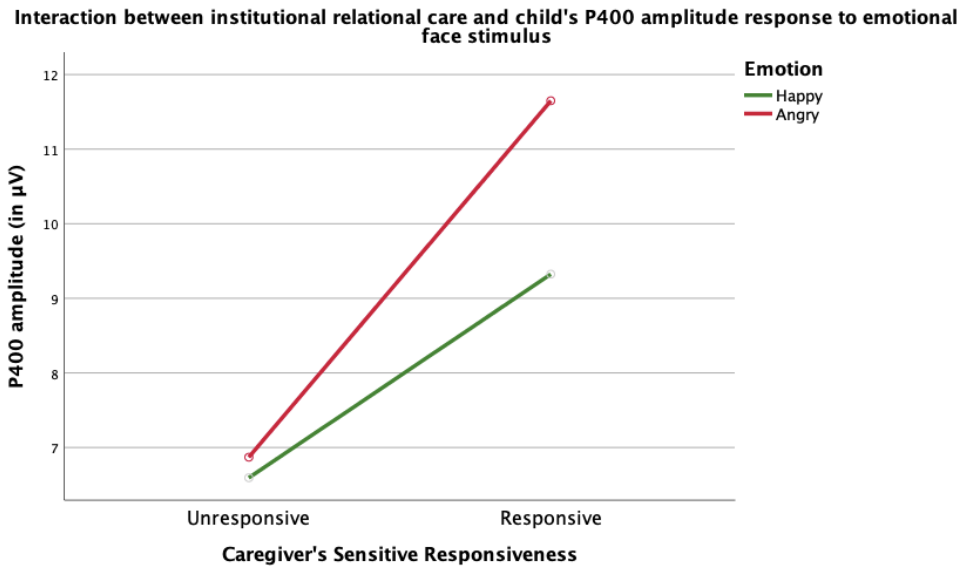
No significant results emerged for P250 amplitude.



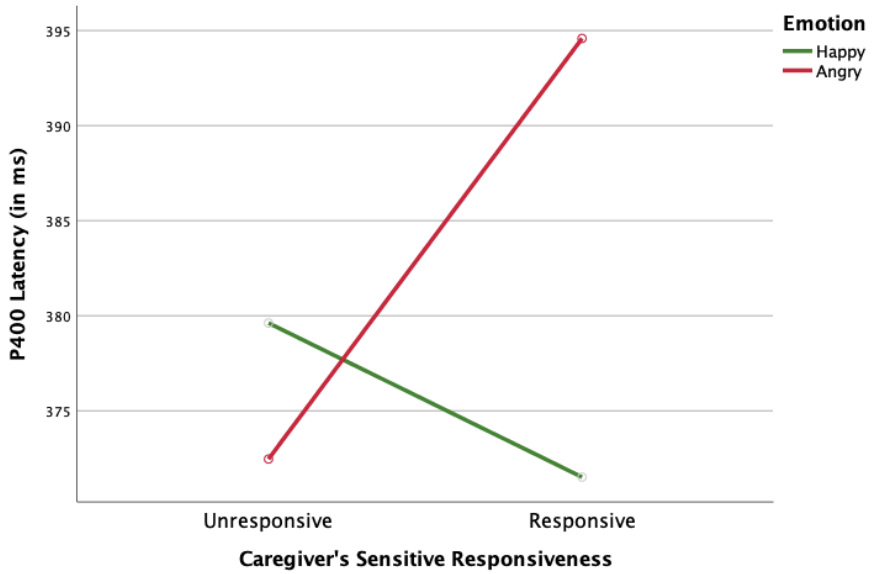
P400 Component

Comparing children with and without experience of Neglect **-** no significant results were found for P400 amplitude and latency response.

Comparing children with and without experience of Lack of habitational conditions **-** For P400 amplitude, a significant result emerged for Emotion, $F(1, 67)=3.972$, $p = .050$, $\eta_p^2 = .056$, showing that children who experiences lack of habitational conditions had greater amplitudes to happy (M=9.32, SD=10.745) and angry (M=11.65, SD=13.606) faces compared to children who did not experienced lack of habitational conditions (happy faces: M=6.59, SD=6.861); angry faces: M=6.87, SD=6.299). *Post hoc* analysis revealed no further significant effects or interactions. No significant results were found for P400 latency.



Comparing children experiencing high and low quality of Caregiver's Sensitive Responsiveness - For P400 latency, a significant interaction effect emerged for Quality of relational caregiving x Emotion (F (1, 65)=6.153, *p*=.016, η_p^2=.086. *Post-hoc* tests showed that for the high responsive care group, a significant effect (F (1, 65)=9.239, *p*=.003, η_p^2=.124) emerged for emotion, where these children displayed earlier and later P400 peaks for happy and angry faces, respectively (*M*=371.51, *SD*=7.91; *M*=394.59, *SD*=7.91).



8. Discussion

Two main goals were set within the scope of this work: 1) to understand if pre-institutional risk-factors and current quality of relational care experiences influenced DNAm levels of the NR3C1 gene and 2) to explore how these previous and current experiences of care influenced the child's neural processing of emotional face stimuli.

For our first goal, we only found significant results concerning NR3C1 DNAm and experiences of neglect and lack of habitational conditions. The direction of this significant result came against our predictions. We found a significant negative correlation between NR3C1 DNAm and having experienced neglect and lack of habitational conditions, that is, children who lived such experiences exhibited an hypomethylation of this gene. Indeed, we were expecting the reverse direction of the NR3C1 gene DNAm, considering the above-mentioned studies such as Weaver et al. (2004a), Cicchetti and Handley (2017), Labonté et al. (2012), and Radtke et al. (2015) showing how adverse social environment alters promoter methylation (more specifically, through hypermethylation of the gene) in the brain of offspring. Nevertheless, and although it has been more widely associated with hypermethylation, early-life adversity is also associated with hypomethylated promoters (for a review, see Watkeys et al., 2018). For example, in a genome-wide study of french-canadian men with a history of severe abuse during childhood, Labonté et al., 2012 found that 31.5% of the analysed probes were hypomethylated among suicide completers with history of childhood abuse. Furthermore, specifically concerning the NR3C1 gene, there are mixed findings for its methylation profile in response to early adverse experiences. For example, exposure to maternal smoking significantly predicted placental NR3C1 CpG-specific hypomethylation at birth (Stroud et al., 2014, 2016). Similarly, a reduced methylation was found at five weeks post-partum in infants of depressed mothers (Murgatroyd et al., 2015), although an hypermethylation of this gene associated with higher levels of maternal depression and/or anxiety is also found in several studies (Braithwaite et al., 2015; Hompes et al., 2013; Murgatroyd et al., 2015; Oberlander et al., 2008; Stonawski et al., 2018). Taken together, and considering previous studies, our results suggest that early life stress and neglect relate to DNA demethylation of the NR3C1 gene at the CpG2 site, which go in line with a dynamic and somehow bidirectional perspective on the alterations of methylation mechanisms in response to early-life adversity (Labonté et al., 2012). Still concerning NR3C1 DNAm, it was a surprise for us not to find

associations with current quality of care. Indeed, sensitive caregiving was found in previous research as a protective factor for DNAm of the NR3C1 gene in children (Murgatroyd et al., 2015; Conradt et al., 2016, 2019). Our results indicate that, for our sample in specific, it seems that NR3C1 DNAm is more sensitive to past, early adverse experiences, than current experiences of relational care.

For our second goal, we did find an influence of the quality of relational care and the child's neural processing of emotional face stimuli. For the **P100** component we found that its **latency response to angry** faces was modulated by caregiving quality. Specifically, we found that children from the low responsive caregiving group were slower at processing happy and angry faces compared to children in the high responsive caregiving group. Furthermore, the P100 response latency peaked earlier for **angry** faces in the high responsive care group *versus* the low responsive care group. The P100 component is responsible for early, rapid processing and detection of visual stimuli (Batty & Taylor, 2003), so it is interesting to note that children with poorer quality of care take more time to process the emotional valence of faces. This result is especially relevant considering that these children present socioemotional difficulties (van IJzendoorn et al., 2020; Oliveira et al., 2014; Oliveira et al., 2012; Soares et al., 2014). Indeed, the laborious processing of the emotional valence of faces that these institutionalized children present may be beneath such socioemotional difficulties, and somehow compromise the adequate response to interpersonal relationships and interactions.

For the **N170** component, we found that the group of children who received high responsive care was faster (*i.e.* had earlier latency) at processing happy faces compared to the low responsive care group of children. A significant result also emerged when considering exposure to neglect, with children **who have not experienced neglect** showing larger N170 amplitude for angry *versus* happy faces and early N170 latency peaks for angry *versus* happy faces.

It seems that experiencing neglect or having variations in caregiver's sensitive responsiveness works differently in the response of the N170 component to emotional face processing in institutionalized children. Indeed, our results show that N170 is especially sensitive to happy faces in both conditions, but children who do not experience neglect [a more positive condition] have larger amplitude for angry *versus* happy faces and earlier N170 peaks for angry

versus happy faces, while children who experience high responsive care [also a positive condition] display earlier N170 latency peaks for happy faces. to happy faces. These results are contradictory to previous research showing that N170 is faster in latency and greater in amplitude to the negative valence of emotions (Batty & Taylor, 2003; Parker et al. 2005b), and that neglected children have greater difficulty discriminating emotional expressions (Pollak et al., 2000). Further research is needed to enlighten the role of more proximal relational experiences in the response of the N170 component to different valences of emotional face stimuli.

For the **P250** we also found that children from the high responsive caregiving group were faster at processing emotions (happy and angry) than children from the low responsive caregiving group. This result goes in line with previous research showing that never institutionalized children exhibited earlier latencies to the P250 compared to institutionalized children (Parker & Nelson, 2005). Furthermore, these group differences in emotion response observed at the P250 and N170 (early latency components) suggest differences at the perceptual, rather than cognitive level of information processing.

For the P400, we found a significant effect for emotion on children who experienced **lack of habitational conditions**, who showed greater amplitudes compared to the group of children who did not experience lack of habitational conditions. Interpretation of this result is limited because there is no literature, to our knowledge, that explores the impact of lack of habitational conditions on the children's neuronal processing of emotional stimulus. Nevertheless, our results show that children who go through such experience - which probably implies a severe degree of deprivation in terms of housing conditions that can probably compromise adequate relational contexts among family members - dedicate a higher amount of cognitive resources for processing emotional faces compared to children who did not have such experience. Animal research has well established that the environmental conditions (enriched *versus* poorer environments) impact structural neuronal changes such as increased number of neurons, synapses and dendrites, which in turn is related to improved learning and memory of these animals (van Praag et al., 2000). Further research is needed in humans to better understand how habitational conditions impact brain structure and relevant neuronal circuits, especially in infants and children whose developmental stage makes them especially sensitive to environmental input. Regarding quality of institutional care, we also found a significant interaction effect between quality of relational caregiving and

facial emotion for P400 latency. Indeed, children from the high responsive group showed later latency to angry faces, whereas children from the low responsive group showed an earlier response to angry faces. The P400 component is associated with structural processing and encoding of faces and, being a later occurring component, is also associated with attentional allocation (de Haan & Nelson, 1999; de Haan, et al., 2003). Having this in mind, one can deduce that children with poorer quality of care are faster at detecting and allocating intentional attention to angry faces. Although not specific to the P400 component, there is vast literature showing that maltreated children are more reactive to negative stimulus (Pollak et al., 2000; Cicchetti & Curtis, 2005; Pollak & Tolley-Schell, 2003). In fact, neuroimaging studies show that important neuronal regions for fear processing, such as the amygdala, have a hyperactive responsivity profile in children exposed to early maltreatment (Dannlowski et al., 2012; McCrory et al., 2011, 2013). Our P400 results go in line with such research, since it seems like an indicator that there is a faster allocation of neuronal resources to angry faces, although interpretation should be cautiously made given the small sample size of our study.

The lack of significant results regarding the Nc component goes in line with Parker & Nelson (2005) who also did not find emotion differentiation for this component in institutionalized children.

This work goes not without limitations. First, our sample size is considerably small for both the epigenetic and neuronal analysis here studied and is limited to children with experience of institutionalization. A community comparison group, matched by age and other relevant variables, would be a necessary improvement to our analysis. Moreover, this community group would be especially interesting to compare in terms of exposure to early adverse experiences, and to see if such group comparison would show differences in the neuronal response to emotional stimulus. This could be one of the reasons why, for example, we did not find any P100 amplitude differences in response to angry faces in our sample, as opposed to Curtis & Cicchetti (2011), who found differences between maltreated and non-maltreated children. Second, and focusing especially on the epigenetics mechanisms, other relevant variables not included in this study, might better explain the lack of significant results we got for the caregiver's quality of care and NR3C1 gene methylation. Although there is accumulating evidence for the molecular impact of environmental experiences, the precise "steps" through which it occurs are not yet clear (see

Champagne, 2018). Indeed, there are probably other important variables to this cascade of neural, physiological, and molecular response to early caregiving experiences that we did not account for. For example, one such variable, proposed by its attractive evolutionarily conserved pathway on neurodevelopment is tactile stimulation (Hellstrom et al., 2012). The importance of tactile stimulation may be especially relevant in the context of institutionalization, given the fact that most of these institutions have a high caregiver-child ratio, making it more difficult to interact physically and emotionally with each child in particular. Furthermore, other genes (*e.g.*, OXTR gene, SRT, and Avp gene) have been signalized in the literature as also good candidates for this "embedding" of adverse experiences.

In conclusion, our work shows (while taking in consideration the above-mentioned limitations) that institutionalization has a negative impact on the neurodevelopment capacity of emotion recognition. Furthermore, we show that DNAm of the NR3C1 gene is especially sensitive to earlier adverse life events, while current experiences of care seem to affect more directly brain functioning. Further research should try to capture the link between these two mechanisms, in order to better understand the complex physiological and neurological adaptation that these children go through in response to such adverse environment.

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