# Chronic neuro-behavioural effects of the early-life environment

## rodent and primate studies

**Habilitation Thesis**

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# Chronic Neuro-Behavioural Effects of the Early-Life Environment:

# Rodent and Primate Studies

**Habilitationsschrift**

**of**

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**Overview**

The sequencing of the human genome is a monumental advance and will lead to marked facilitation of the identification of candidate genes involved in the regulation of complex neurobiological, physiological and behavioural traits. A logical consequence of this is that great interest will now be stimulated in identifying the external and internal environmental factors that regulate the individual- and tissue-specific expression of these genes (Gottlieb, 1998). In behavioural neurobiology (Levine, 1960) and psychiatry (Newport et al., 2002) it has long been recognized that postnatal parental care is one of, if not the, most important environmental regulatory factors in terms of individual, i.e. offspring, development. Observable behavioural interactions of parent and offspring mediate nonobservable sensorimotor, thermal and nutrient-based events that have important and widespread regulatory effects (Hofer, 1994). Most importantly, the impact of the quality and quantity of parental care received by the offspring is not restricted to acute effects; rather, via these acute effects the trajectory of development is also altered, leading to chronic effects. Accordingly, there is now a growing body of human epidemiological and clinical evidence and animal experimental evidence that early social experiences influence the functioning of some vital neurobiological, physiological and behavioural processes in adulthood. This situation is presented in Figure 1, which illustrates how (1) infancy constitutes a sensitive period for responsiveness to external environmental factors, (2) parental care constitutes the major such factor at this stage of development, and (3) the impact of this responsiveness is both short and long term.

Ultimately, this life span chain of events will need to be understood at the many epigenic, epigenetic, molecular, and proteomic levels, at which it is occurring (Meaney, 2001). At present, however, considerable research effort is being made to identify (1) the neurobiological, physiological and behavioural traits that are distinctive in human adults who experienced extreme environments, notably abuse or neglect, in early life, and (2) in vivo animal models in which specific manipulations of the parent-infant relationship yield robust acute and/or chronic effects on these same traits (Heim & Nemeroff, 2001; Newport et al., 2002). Recent clinical studies indicate that early exposure to adverse experience increases the risk for the development of, among others, posttraumatic stress disorder, depression, and generalized anxiety disorder. In addition to the abnormalities at the behavioural level that constitute the

diagnostic systems of these diseases, there is physiological evidence for altered functioning of homeostatic/stress systems including the hypothalamic-pituitary-adrenal axis and the autonomic nervous system, and for disrupted brain activity as evidenced by functional neuroimaging studies (Bremner et al., 1997; De Bellis et al., 1999; Heim et al., 2000, 2001). The neurodevelopmental hypothesis for psychiatric disorders proposes that (1) environmental insults during early stages of development cause early brain pathology, and (2) this pathology interacts with normal brain maturation occurring subsequently, to (3) lead to the diagnostic behavioural symptoms of the disorder (e.g. Weinberger, 1986).

With regard to animal models of the chronic neurobehavioural effects of early environmental insult in humans, the rat has been the subject of considerable investigation in terms of the inter-relationships between maturation of neuroendocrine and neurotransmitter systems, the infant-mother relationship, and long-term neurobehavioural function (Meaney et al., 1996; Levine, 2002). Much of this research has focused on the intermittent deprivation of maternal care (Pryce et al., 2002), and this work is reviewed in the section of this report entitled "Long-term neurobehavioural impact of the postnatal environment in rats: manipulations, effects and mediating mechanisms". In nonhuman primates the parent-infant relationship involves, in contrast to the rat, continuous infant-mother body contact over a considerable period of infant development (Pryce, 1996). The major primate research in this area to-date has been based on the absolute separation of the infant from the mother (maternal privation) and human hand-rearing (Kraemer, 1992), so that to-date there has been no attempt to develop primate models based on the intermittent deprivation of parental care in young infants, as used in rat studies. My colleagues and I have conducted a long-term study aimed at developing a primate model of chronic neurobehavioural effects of intermittent deprivation of parenting in the common marmoset monkey. The procedure used and its acute effects are described in the section of this report entitled "Repeated parental deprivation in the infant common marmoset (Callitrhix jacchus, Primates) and analysis of its effects on early development", and some of the chronic effects we have demonstrated are described in the final section of this report "Intermittent deprivation of parenting disrupts development of homeostatic and reward systems in primate offspring".

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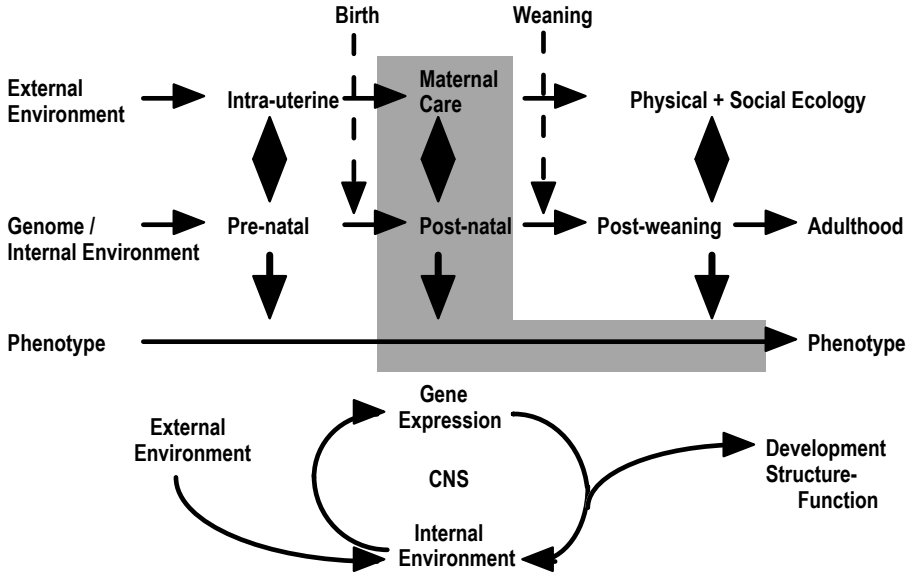
Figure 1. A schematic model of the interrelationships between environment, genome and phenotype that emphasizes the importance of parental care received in the postnatal period between birth and weaning for long-term development of the structure and function of physiological and behavioural traits. The external environment impacts on the internal (cellular) environment, thereby affecting epigenetic processes, gene expression and, ultimately, phenotype. Disruption of infant homeostasis by atpical parental care represents a critical period of sensitivity to these interrelationships. CNS, central nervous system.



**Long-term Neurobehavioural Impact of the Postnatal Environment in Rats: Manipulations, Effects and Mediating Mechanisms**


## Abstract

The major characteristics of the postnatal environment of the rat pup are its mother and littermates. The pup, which is poorly developed at birth, matures rapidly in this environment, and regulates the behaviour and physiology of the dam and littermates, as well as vice versa. The study of the impact of the rat's postnatal environment on its long-term neurobehavioural development is of fundamental importance. In fact, it is one of the major examples -- at the interface of the biological, social and medical sciences -- of animal models for the study of the interaction between the environment and the genome in both the acute and chronic regulation of the phenotype. Specific experimental manipulations of the rat postnatal environment have been demonstrated to exert robust and marked effects on neurobiological, physiological and behavioural phenotypes in adulthood. In the present review we present some of the major findings, including some original data, and discuss what these existing data can tell us about the long-term neurobehavioural effects of the postnatal environment in rats, the external and internal mechanisms that mediate these effects, and the most appropriate directions for future basic and applied research in this area.



**Key words/phrases:** Rat, Postnatal environment, Early handling, Non-handling, Maternal separation, Early deprivation, Early life stress, Maternal behaviour, Emotionality, Cognition


### Introduction

The importance of the environment in the regulation of brain, physiology and behaviour has long been recognised in the biological [1], social [2] and medical [3] sciences. Given that environmental regulation refers ultimately to modulation of the central nervous system's gene expression and proteomic activity [4], and given the marked recent advances at the genomic level resulting from the human and other species' genome projects [5], then we are now clearly at an important junction in the study of the causal relationships between physical and social environmental factors and neurobiological, physiological and behavioural phenotypes.

In mammals, including rodents, nonhuman primates and humans, the mother is the source of infant nutrition and a complex infant-mother relationship has evolved out of this nutritional need, such that the mother provides essential thermal, somatosensory, kinaesthetic, olfactory, visual and auditory stimulation, as well as maternal milk, for an extended period of

postnatal development [6]. Hofer has been pioneering in describing how observable behavioural interactions of parent and offspring mediate non-observable events that have important and widespread homeostatic effects [7]. As such, the maternal environment constitutes one of the most significant environments that any mammal will encounter throughout its entire life span. In humans, there is a growing body of clinical and epidemiological evidence indicating that adverse postnatal environments associated with high levels of early life stress, for example, loss of a parent, parental neglect and/or abuse, or being cared for by a depressed parent, can impact on the long-term neurobiological and psychosocial development of offspring and markedly increase vulnerability to the development of psychiatric disorders [8-13]. Therefore, as well as being of marked biological significance, the mammalian infant-mother relationship -- in particular experimental manipulation thereof -- is potentially also of high biomedical importance [3, 14-17]. Animal models based on the impact of disrupted maternal care and leading to reliable and marked chronic effects on neurobiology, physiology and behaviour in adulthood, if identified, will be characterized by impressive levels of face and construct validity [3, 18].

In laboratory rats, the chronic effects of postnatal manipulation of the infant-mother relationship have been studied experimentally for nearly 50 years, a research field pioneered by the work of Seymour Levine (e.g. [1, 19-23]) and Victor Denenberg (e.g. [24, 25]). Rat dams typically give birth to around 12 pups per litter, and these pups are poorly developed at birth ("altricial") and grow rapidly, with weaning taking place at 3-4 weeks. Maternal care occurs in bouts of retrieving, licking, and nursing, interspersed with periods when the dam is absent from the nest and litter [26]. Mother and littermates both constitute important components of the individual pup's environment and both can provide important sources of thermal, somatosensory, kinaesthetic, olfactory and auditory stimulation. As well as being stimulated by, each individual pup also stimulates, mother and littermates; an elegant example of the complexity of these three-way dyadic postnatal relationships is provided by the observation that arched back nursing (or kyphosis) and milk ejection by the dam are dependent on the combined suckling stimulus of several neonatal pups [26].

The first postnatal manipulation model to be investigated in detail was the comparison between early handling and early nonhandling [1, 19]. In these studies, early handling, or EH, was defined as the experimenter picking up the pup, removing it from the breeding cage and isolating it in a small compartment for several minutes, repeated across days between birth and weaning. Early nonhandling (NH) was defined as the complete absence of handling (both experimental and husbandry related) in the breeding cage between birth and weaning [1]. As adults, EH rats were: more active, explored more, and defecated and urinated less in the open field; exhibited a lower plasma corticosterone (CORT) response to stressors including injection or placement in water; and demonstrated a more rapid

maximal CORT response to electro-foot shock followed by a more rapid return to baseline titres [1, 19-21]. As well as providing landmark data on the importance of the postnatal environment for the long-term development of the emotional phenotype in the rat, Levine's studies also revealed a caveat that has continued to be a major theme, indeed a major problem, in the design and interpretation of rat postnatal manipulation studies conducted to this day. The issue concerns the nature of the control group in the EH-NH model. Given that EH constitutes a physical manipulation and NH a non-manipulation, then it is a logical step to regard the former condition as the experimental group and the latter condition as the control group. However, another interpretation is that rat pups require a minimal amount of stress and/or stimulation in order to develop into adult rats that exhibit a behavioural profile typical for laboratory rats, and that NH is below this minimum and therefore constitutes an experimental group. Levine referred to this caveat in an early review of his own EH-NH emotionality work ([1], p. 81). A clear problem with measures of emotionality is that it is very difficult to define what is normal or typical for a laboratory rat, of any strain, maturing in the constant laboratory environment. Studies based on the actual absence of an otherwise ubiquitous cognitive phenomenon, namely latent inhibition, have subsequently confirmed that NH does indeed yield adults exhibiting abnormal behaviour, and is therefore in itself an experimental treatment. Latent inhibition (LI) consists of retardation in the classical conditioning of a neutral stimulus (e.g. tone, light) to an unconditioned stimulus (e.g. electro-foot shock) as a consequence of its prior nonreinforced preexposure [27]. It is a ubiquitous behavioural phenomenon, interpreted as being highly adaptive as it allows an organism to ignore irrelevant information in its environment. In humans disrupted LI is a common and important feature of the behaviour of schizophrenic patients [27]. In male rats, NH leads to the absence of LI, supporting both the importance of the EH-NH model to biomedicine and the validity of the interpretation that NH constitutes the manipulation in this model ([28-30]; see also [31]). In the last 15 or so years, as reviewed below, a large number of laboratories have applied the EH-NH model to ask questions concerning the importance of the postnatal environment for long-term neurobehavioural development, in the contexts of both emotionality and cognition. The model has also been used in an attempt to explain the mediating mechanisms via which postnatal manipulation can lead to alterations in the adulthood phenotype at the neurobiological, physiological and behavioural levels.

In parallel to this recent increased interest in the EH-NH model, other manipulations (e.g. maternal separation, early deprivation) of the infant-mother-litter relationship have been developed and their effects studied. The findings, again at the neurobiological, physiological and behavioural levels, are reviewed below. Here, it is important to point out that this research area has become very complex, both conceptually and methodologically. Firstly, there are many forms of manipulation that are currently in use and it would seem to be

essential to recognise that a large number of potentially very important variables are being manipulated differently in the many laboratories active in this area. A recent review from our laboratory has addressed this very issue [16]. Second, there is clearly a need for a framework that facilitates recognition of these variables and that provides clear and unambiguous nomenclature for the different forms of postnatal manipulation. Recently [32] we proposed the following: *Maternal separation* (MS) should be used to describe separation of the intact litter from the dam for one or more hours per day across several postnatal days (e.g. [33-36]), and *single maternal separation* to describe separation of the intact litter from the dam for a single 24-h period (e.g. [37-42]). *"Infant"* or *"early"* combined with either *"isolation"* or *"deprivation"* should be used to describe separation of the pup from the dam and the litter for one or more hours per day across several postnatal days (e.g. [43-45]). Our preference is for *early deprivation* (ED), because this indicates similarity to EH and thereby emphasises the important and reciprocal relationship between these two manipulations: the "patent form" of EH constitutes separation of the pup from the litter and the dam (e.g. [1]), as does ED; EH does not constitute deprivation in that the isolation period is shorter than species-typical periods between successive bouts of maternal care, whereas ED clearly does. A third important point with regard to the current complexity of postnatal manipulation research in the rat concerns the nature of the control group. The fragility of NH as a control treatment has already been highlighted above, and recently rats that as pups have experienced the human interventions inherent to cage cleaning have been used as controls. *Animal facility rearing* (AFR) is the title given to this group, and AFR, as reviewed below, has already been applied to study specific long-term effects of EH, MS, ED and, indeed, NH [15, 44-47]. In a recent review, Levine has highlighted that AFR is also not without its problems, given that, "Variations in animal husbandry are as numerous as there are animal facilities." ([31], p. 540).

Against the above background, the overall aim here is to review the evidence for the long-term impact of specific postnatal manipulations of the pup-mother-litter relationships on emotional and cognitive responses to specific environmental challenges in adult rats. The following specific themes are addressed: Long-term neurobehavioural effects of early handling; Long-term neurobehavioural effects of maternal separation; Long-term neurobehavioural effects of early deprivation; Identifying the appropriate control group and, Mediating mechanisms. The long-term effects that are reviewed are neurobiological, physiological and behavioural.

**Long-term neurobehavioural effects of early handling**

As stated in the Introduction, the initial studies with the EH-NH model were aimed at comparing effects on anxiety-like behaviour and hormonal stress responses, and demonstrated

that EH was associated with increased open-field exploration, reduced open-field defecation and urination, suggesting reduced activation of the sympathetic branch of the autonomic nervous system (ANS), and a more rapid and less prolonged plasma CORT response to a range of stressors, suggesting altered activity of the limbic-hypothalamic-pituitary-adrenal (LHPA) system [1]. The long-term changes associated with EH have since been replicated by many laboratories, with the most extensive series of studies being performed by Michael Meaney, Paul Plotsky and colleagues (hereafter refereed to as the Meaney-Plotsky group). These studies have led to marked advances in our understanding of the EH-NH model at the neuroendocrine and neurochemical levels, as summarized below. It is also important to highlight that the interpretation of the Meaney-Plotsky group is that EH is a treatment that exerts anxiolytic and stress-coping effects relative to NH, rather than EH constituting the control and NH the atypical treatment, as demonstrated for LI [28-30]

Adult male rats that had been exposed to EH on postnatal days (PNDs) 2-21 were found, relative to NH, to exhibit a lower peak plasma adrenocorticotrophic hormone (ACTH) stress response as well as a more rapid return to basal titres, following a 5-min period of restraint [48]. The same was the case for their CORT peak response and recovery titres in response to a 20-min period of restraint, whilst basal CORT titres did not differ between EH and NH adult males [48]. Following adrenalectomy, and a sufficient time period to allow clearance of endogenous CORT, radioligand binding was used to demonstrate higher binding capacity of glucocorticoid receptors (GRs) in the hippocampi of EH adult male rats relative to their NH counterparts [48]. Adult male rats exposed to EH on PNDs 2-14 were found, relative to NH, to exhibit lower basal corticotrophin releasing factor (CRF) mRNA levels in the hypothalamus, and lower titres of CRF in the median eminence [36]. The proportion of the median eminence CRF remaining after exposure to 20 min of restraint stress was lower in NH than in EH [36], suggesting that the absolute amount of CRF released from the median eminence into the hypophyseal portal system was higher in NH compared with EH adult male rats. The reduced hypothalamic CRF activity of the EH rats correlates neatly with their reduced pituitary-adrenal endocrine stress reactivity, and it is parsimonious to assume that the low basal hypothalamic CRF causally underlies the latter. This of course leads to the question of what is causally responsible for the down-regulation of CRF. Here the favoured explanation of the Meaney-Plotsky group is that the elevated hippocampal GR expression of EH mediates relative suppression of CRF synthesis in the hypothalamus via enhanced reactive negative feedback from the hippocampus [35]. Certainly the evidence for increased hippocampal GR binding capacity in EH adults, as obtained in adrenalectomized rats, has been supported by evidence for increased hippocampal GR mRNA expression (specifically) in intact EH versus intact NH adults [49]. Arguing against this is the report that hippocampal GR activation actually impairs LHPA reactive negative feedback, via inhibition of

hippocampal MR negative feedback [50]. Also, given that the GR is (as is the MR) a transcription factor that acts via its nuclear effects on gene transcription [51, 52], extreme caution needs to be exercised in attributing an effect that is already occurring within 1 hour, i.e. the interval between the cessation of stress and the return of CORT to basal levels in EH rats, to differences in GR levels. On the other hand, increased hippocampal GR levels are associated with decreased CORT stress reactivity not only in EH versus NH rats, but also in Lewis versus Fischer rats [53] and in transgenic mice that are over-expressing GRs in the hippocampus [54]. Recently, attention has turned to EH inducing down-regulation of amygdala-mediated excitation of the HPA neuroendocrine response [52] in addition to up-regulation of its hippocampus-mediated inhibition (see below). Specifically, the noradrenergic input from the brain stem nuclei to the hypothalamus, which stimulates CRF release and is under amygdala control, has been investigated as another potential route via which EH can impact on adulthood LHPA reactivity [17, 33, 55, 56]. EH has been reported to markedly decrease norepinephrine release in the paraventricular nucleus of the hypothalamus (PVNh) in response to restraint stress relative to NH [56].

Therefore the Meaney-Plotsky group has provided an impressive set of neuroendocrine and neurochemical findings with the EH-NH model that can be integrated to account for the more prolonged CORT stress response that both they, Levine, our selves [44] and other groups, have demonstrated in NH adult rats. However, it is worth noting here that these neuroendocrine findings do not immediately seem to fit together with the demonstration by Levine that EH rats exhibit a more rapid, efficient CORT stress response than NH [21], and in this respect it would therefore be interesting to examine whether the reduced hypothalamic CRF levels of EH rats [36] are associated with increased CRF receptor expression in pituitary corticotrophs.

Of course the LHPA constitutes an important stress system, both in terms of its permissive effects (e.g. stimulation of gluconeogenesis; CRF receptor up-regulation) and suppressive effects (e.g. inhibition of hippocampal neuron excitability; inhibition of CRF and ACTH synthesis and release) [57]. However, the LHPA system is not directly involved in the regulation of stress-related behaviour, which also differs markedly between EH and NH adult rats. One important link between the HPA system and behaviour is the amygdala. The central nucleus of the amygdala not only stimulates activity in the HPA axis as noted above, but it is also critically involved in the excitation of a number of circuits that regulate emotional behaviour. Certain of the neurocircuits that underlie emotional behaviour have been studied recently by the Meaney-Plotsky group and, as with the HPA system, a remarkable constellation of effects has emerged in the EH-NH model. The rationale underlying this research is that the central nucleus of the amygdala is intimately involved in the regulation of behavioural, autonomic and endocrine output in response to environmental challenge [58], so

that neurocircuits involving this nucleus and its projection sites, such as the brain stem nuclei (e.g. locus coeruleus, nucleus of the solitary tract, vis. autonomic and cortical arousal), and bed nucleus of the stria terminalis (vis. HPA activation), might be affected in the EH-NH model [55]. Relative to NH, EH adult rats exhibited increased central benzodiazepine (BZ) receptor binding in the amygdala (central and lateral nuclei) and the locus coeruleus; increased expression of γ2 subunit mRNA of the $GABA_A$ receptor complex (which confers high-affinity BZ binding) in amygdaloid and brain stem nuclei, and increased $GABA_A$ receptor levels in the brain stem nuclei [33].

Although the model does remain to be validated in pharmacological terms, the above constellation of EH-NH differences in the amygdala-brain stem circuit is consistent with the reduced behavioural expression of anxiety and fear to innate negative reinforcers demonstrated by EH adult rats. For example, in the same study as that which identified the relative effects of the EH-NH postnatal environments on the development of the amygdala-brain stem GABA and BZ receptor systems, it was described how EH adult rats exhibit relatively high open-field exploration, low novelty-induced suppression of feeding, and low acoustic startle responsiveness relative to NH [33]. In our laboratory, EH and NH have been compared in these sorts of innate tests of anxiety as well as in additional behavioural tests that are also sensitive to treatment differences in anxiety and fear but that involve learning and memory, in that behavioural responses are elicited by an innately neutral stimulus that has acquired fear-inducing properties (conditioned stimulus, CS) via its association with an innate negative reinforcer (or unconditioned stimulus, UCS), such as mild foot shock. For example, NH adult males exhibit impaired two-way active avoidance relative to EH adult males [29, 45] (Figure 1), a difference that might be mediated by increased fearfulness or decreased stimulus-reinforcer (CS-UCS) association learning, or indeed both processes simultaneously. When fear-conditioned freezing behaviour [59] is used as the dependent measure, EH and NH do not differ, neither in the development of freezing across successive pairings of a tone with foot shock, nor in the subsequent expression of freezing when returned to the same context (context test) or presented with the same tone in a new context (CS test) at 24-48 hours post-conditioning [45] (Figure 2). When, rather than freezing behaviour, pituitary-adrenal endocrine responsiveness to the CS test was used as the dependent measure of fear conditioning, the EH adult males were less reactive than NH, both in terms of ACTH and CORT (Figure 2). This discrepancy between the relative behavioural and endocrine fear responses of the EH and NH subjects strongly suggests that the latter reflect the EH-NH neuroendocrine differences downstream of the amygdala rather than differences in CS fear conditioning [45].

Spatial learning and memory under the negative reinforcement conditions of the water maze have been reported to be unaffected in the EH-NH model when testing is

conducted with young adults [60-62]. This is in contrast to the situation in aged rats where both the Meaney laboratory and our laboratory have reported that aged EH rats exhibit enhanced learning and memory compared with aged NH rats [60, 61, 63]. This presumed age-dependent effect has been attributed to protection from aging-related cognitive decline by EH [60, 61, 64], a theme that is beyond the scope of the present review. Recently, however, we have conducted a study in which, specifically in females, the acquisition of the location of the submerged platform was enhanced in EH relative to NH young (6-month-old) females [45] (Figure 3). As stated in the Introduction, one cognitive process that is clearly responsive in the EH-NH model when conducted with young adults is LI [27, 29, 30, 65, 66]. The effect is specific to males and can be demonstrated in a range of tests of stimulus-reinforcer association learning including two-way active avoidance, fear-conditioned freezing and the conditioned emotional response (Figure 4).

As noted in the Introduction, the demonstration of disrupted LI, a ubiquitous cognitive process, in NH adult male rats provides strong support for the interpretation, that in the EH-NH model it is the latter that constitutes the manipulation ([27]; see also [1, 31]). Recently, considerable further evidence has accumulated, and this from studies that have compared either EH, or both EH and NH, with AFR. If specific forms of stress and/or non-stressful stimulation, and in a sufficient amount, are essential to support normal postnatal neurobehavioural development in all mammalian species (see Introduction), then it is possible that NH constitutes an environment of deficient stress and/or stimulation. Direct comparison of the EH-NH model with AFR is very insightful here. AFR very probably provides more stimulation for pups and dam than does NH; it involves handling 1-2 times per week during cage cleaning, as well as movement of personnel in and out of the colony room which, in our laboratory at least, is not permitted under the NH procedure. Just as would be predicted if NH does constitute the manipulation in the EH-NH model, the differences between EH and AFR young adults are markedly reduced relative to the robust differences between EH and NH young adults [15, 36, 44-46]. Indeed, in the majority of studies performed to-date, it has been demonstrated that EH is without effect relative to AFR. This is the case when EH and AFR adult males are compared in terms of their pituitary-adrenal stress responses to psychological stressors [15, 44] (Figure 5), and males and females in terms of their anxiety behaviour in the elevated plus maze [15], the open field (Figure 6) and the acoustic reflex startle test [44] (Figure 7). Thus, just as EH is associated with reduced innate endocrine and behavioural responses to affective challenge relative to NH, so is AFR [44]. In tests where the measurement of affect involves stimulus-reinforcer association learning, the evidence is less clear cut but certainly quite intriguing. AFR adults are as similarly efficient as EH adults in two-way active avoidance and therefore AFR males are superior to NH males [45] (Figure 1). In fear-conditioned freezing, however, AFR adult males actually exhibit increased freezing

and pituitary-adrenal endocrine responsiveness when confronted with the tone that was associated 48 hours previously with foot shock relative to EH and even NH males (as described above, this measure is unaffected in the EH-NH model) [45] (Figure 2). Spatial learning/memory in the water maze is similar in EH and AFR young adults in males, and in females EH tend to exhibit enhanced performance [45] (Figure 3). EH and AFR adults demonstrate very similar levels of LI (Figure 4).

In summary, the original behavioural findings of differences in emotionality in the EH-NH model as obtained by the laboratories of Levine and Denenberg have since been replicated by a large number of laboratories and, it is important to note, in a number of different strains. The majority of behavioural tests used have assayed responses to innate negative reinforcers, but there are also demonstrations, most notably in two-way active avoidance, that emotional differences persist in the EH-NH model when behaviour is dependent on stimulus-reinforcer association learning. On the basis of this behavioural evidence, as well as the early evidence for EH-NH endocrine differences in responses to environmental challenge, the Meaney-Plotsky group undertook an extensive investigation into EH-NH differences in adult neurobiological status associated with these behavioral and physiological differences. A constellation of inter-related changes has been described: in the LHPA system, and in particular in terms of hypothalamic CRF levels and hippocampal GR levels; and in the amygdala and its projections to the noradrenergic brain stem nuclei, that in turn project to limbic and cortical regions as well as to the sympathetic autonomic nervous system. Underlying the Meaney-Plotsky research is the theory that EH constitutes the experimental manipulations and NH the control group. This is in direct contrast to the evidence provided by the study of LI, and to recent evidence, much of which has been provided by Meaney-Plotsky, that EH is largely without effect on behavioural and neuroendocrine phenotypes relative to AFR.

**Long-term neurobehavioural effects of maternal separation**

There are many instances in the original research reports and reviews of the Meaney-Plotsky group where it is stated that maternal separation (MS) leads to effects opposite to those of EH. For example, “In the rat and mouse, postnatal handling decreases the magnitude of behavioral and endocrine responses to stress in adulthood. In contrast, longer periods (i.e. 3-6 h) of daily separation from the mother increase behavioral and endocrine responses to stress.” ([67], p. 129); “As adults, animals exposed to repeated maternal separation of 180-360 min per day for the first 2 weeks of life showed significantly increased plasma ACTH and corticosterone responses to either restraint or novelty stress compared to 0 min controls (animals reared in the same manner as NH animals).” ([68], p. 253). The research on which these statements are based took the form of simultaneous comparisons of EH, NH and MS

adult male rats and, with NH being used as the control group, the statements are proposing that EH is resulting in adult rats that are less emotionally reactive than NH, while MS is resulting in adult rats that are more emotionally reactive than NH. What the majority of the findings indicate, however, is that, relative to NH, MS is without effect on endocrine or behavioural responses in tests based on innate emotional challenge. In [36] it was reported that adults exposed to 180-min MS on PNDs 2-14 exhibited higher basal CRF mRNA levels in the hypothalamus and higher titres of CRF in the median eminence. Therefore the effects of MS on hypothalamic-median eminence CRF, as measured relative to NH, were indeed the opposite of those obtained with EH ([36], see above). However this did not translate into increased pituitary-adrenal stress reactivity, as evidenced by the absence of a difference in the CORT response to restraint in MS and NH subjects in the same study, whilst a more recent study has since demonstrated that MS adults do tend to exhibit a more prolonged ACTH response, although clearly not a higher peak stress ACTH response, than NH adults [56]. Even in the absence of robust pituitary-adrenal endocrine correlates the reported CRF effects of MS are very interesting and require explanation. MS is reported to increase the restraint-induced release of norepinephrine into the PVNh [56], and this constitutes a neurochemical parameter on which EH and MS exert opposite effects relative to NH, in accordance with the claims of the Meaney-Plotsky group. What of the effect of MS on hippocampal GRs? Here the situation is puzzling because whereas it has regularly been claimed that MS leads to reduced expression of hippocampal GRs and therefore reduced glucocorticoid negative feedback efficiency (e.g. [35], p. 56) we are not aware of any research report where this claim is substantiated with empirical data. This unfortunate gap in the evidence clearly needs to be filled, given the marked importance and implications of the theory that has been developed. Turning to the behavioural effects of MS, there is to-date no published evidence that 180 min MS leads to the opposite effects to those of EH relative to NH. In fact, the vast majority of the evidence is that, in young adult rats, MS is without effect relative to NH. Thus, the Meaney-Plotsky group has demonstrated that MS does not affect exploration in an open field, novelty-induced suppression of feeding, or the acoustic startle response, relative to NH [33].

Recently, the Meaney-Plotsky group has conducted further studies in which 3 groups have again been compared simultaneously; the identity of the groups has shifted, however, from EH-NH-MS to EH-AFR-MS [15, 46]. As would be predicted from the above summary of the lack of differences between EH and AFR, the outlier group in these studies is MS. Thus, the EH-AFR-MS model also fails to yield a situation in which EH and MS yield opposite effects relative to the control group. However, this model does consistently yield MS effects relative to the AFR group and in the direction that MS rats are more emotionally reactive than the control group. Thus, it has been reported that MS rats demonstrate greater ACTH and CORT peak responses and more prolonged responses, using air puff as the

stressor [15]. Behaviourally, MS adults exhibit increased anxiety-like behaviour, as measured in the elevated plus maze, relative to AFR [46].

One possible explanation for the lack of effect of MS relative to NH on a large number of measures of emotional reactivity is that MS does not constitute a sufficiently severe form of deprivation to alter the trajectory of neurobehavioural development (indeed, the same interpretation has recently been proposed by Levine [31]). As we describe above, NH probably constitutes a form of deprivation and therefore an experimental treatment, in the form of under-stimulation. According to this line of reasoning, MS may well constitute a similar treatment: pups are maintained as a litter and removed from the dam for 180 min, a time period equivalent to that for which dams have been observed to be spontaneously absent from the pups [69]. As such, the 180 min MS might not constitute a state in which the absence of specific maternal factors leads to loss of homeostasis; rather its effects, relative to AFR and EH, may have their bases in under-stimulation. At least partial support for this interpretation is provided by another line of research from the Levine laboratory, in which the acute pituitary-endocrine responses of pups to a single MS of varying duration have been investigated [23]. A single MS of 3 hours is not sufficient to elicit a stress response and is also not sufficient to increase sensitivity to stressors additional to the MS, such as saline injection. MS models of longer duration, such as the 6-h model used by Lehmann et al. [34], are approaching the duration that is known to elicit a stress response [23]. Furthermore, such acute stress responses might directly underlie the demonstrated learning-enhancing effects of the 6-h MS model [34].

**Long-term neurobehavioural effects of early deprivation**

Early deprivation, involving deprivation of not only the mother but also the littermates for an extended period of time, and this repeated across days, would appear to constitute a more severe postnatal manipulation than MS, and might be expected to constitute a postnatal stressor. In fact clear evidence that this latter assumption is indeed the case is provided by the study of McCormick and colleagues, which demonstrates that ED for 60 min per day on PNDs 2-8, potentiates the CORT response to ED on PND 9 relative to pups that experienced either EH or NH on PNDs 2-8 and then ED on PND 9 [43]. Therefore, ED might well constitute a model that does lead to opposite effects of EH in the model, EH-NH-ED. Over the last 3-4 years we have performed a quite extensive series of studies with ED, using 240 min of ED on PNDs 1-21 and with pups provided with some exogenous warmth to maintain an ambient temperature of 30°C during ED. In these studies we have, in fact, included two groups with which to contrast the effects of EH and ED, namely both NH and AFR. One hypothesis of how these treatments might affect rat development, and certainly one in line with the reasoning of the Meaney-Plotsky group, is that emotionality will increase

according to the following pattern: ED>NH>AFR>EH. The rationale for this hypothesis is that ED subjects will develop into the most emotionally responsive because they were stressed as pups, then NH will be the next most emotional because they were under-stimulated as pups, and EH will be the least emotional because EH stimulates the dam-litter relationship in a positive direction, and more so than does AFR (see Mediating mechanisms). Our results were clear-cut. They were also far from being in line with the above hypothesis.

In terms of the stress endocrine system, ED adult males did not differ from any of the three other treatments in terms of basal CORT titres (Figure 5). They demonstrated a significantly reduced CORT response to 2-min restraint compared with NH males, and in this respect they were similar to both the EH and AFR males [44] (Figure 5). Turning to behaviour, in terms of their innate acoustic startle response, ED adults (male and female) were less responsive than their NH counterparts and were therefore again similar to AFR and EH [44] (Figure 7). ED adults (male and female) were more active in a novel open field than were NH, with EH and AFR not differing significantly from any other group [44] (Figure 6). In terms of tests of emotional behaviour based on stimulus-reinforcer association learning, firstly, ED males exhibited enhanced two-way active avoidance compared with NH males and did not differ from either EH or AFR males [45] (Figure 1). Relative to AFR males, ED males tended to exhibit reduced freezing behaviour to a tone paired 48 h previously with foot shock, and therefore again behaved similarly to EH males [45] (Figure 2). The ED male pituitary-adrenal response to the same conditioned negative reinforcer was intermediate between that of AFR and EH; that is the response of ED males to the tone CS was increases in both plasma ACTH and CORT titres that were significantly lower than those of AFR males and significantly greater than those of EH males [45] (Figure 2). ED did not affect spatial learning and memory in the water maze relative to AFR, NH or EH in adult males, but it did impact on this behaviour in adult females. Very interestingly, the effect was one of enhanced acquisition performance in ED females relative to NH and AFR with EH females being intermediate in performance and also demonstrating enhanced performance relative to NH [45] (Figure 3). And finally here, ED also exhibited effects in the same direction as EH on LI [66]: when the EH-AFR-NH-ED model is studied in the active avoidance test in subjects either with or without pre-exposure to the conditioned stimulus associated with the foot shock reinforcer, then it is the CS pre-exposed ED adult rats that demonstrate the most marked relative deficit in avoidance behaviour, i.e. the most pronounced LI, followed by AFR>EH>NH (Figure 4).

**Identifying the appropriate control group**

Clearly, throughout the study of the long-term neurobehavioural impact of postnatal manipulations in rats, NH has predominated as the control group. Equally clearly, this approach has shortcomings, as described above, and yet, some 40 or so years after the

pioneering EH-NH stuides there are many experiments where NH is still being used. Our laboratory has also been "guilty" of this practice, and in our self-defence we would argue (as of course others are equally entitled to do) that this was at least partly motivated by the need to ensure that our findings could be compared as directly as possible with those of other laboratories. This includes the findings of the Meaney-Plotsky group; firstly because we were concerned by their statements that EH and MS exert opposite effects on neurobehavioural development (see examples of citations above) whereas the data did not support this, and secondly because our own emerging data also did not support this statement. In fact, as reviewed above, we were finding the very opposite: if maternal deprivation was impacting on behaviour then it was doing so in the same direction as EH, relative to NH, AFR, or both.

We are of the opinion that the approach of including the NH group has been vindicated, because it has provided considerable evidence that: (1) NH is an abnormal postnatal environment as far as laboratory rat neurobehavioural development is concerned; (2) 3-h MS on PNDs 1-21 yields very few neurobiological and no behavioural effects that are *opposite* to EH; and (3) 4-h ED on PNDs 1-21, to the extent that it affects neurobehavioural development, does so in the same direction as EH. As stated above, the Meaney-Plotsky group has recently published studies in which they have moved away from studying NH to AFR, and the reason for this may well be related to the evidence listed directly above. Of course, the abandonment of the NH group does not necessarily render the theories it was confounding more plausible: the more recent approach of using an EH-AFR-MS model does not result in EH and MS yielding opposite effects, rather it shifts the "significant group" from being EH (versus NH) to being MS (versus AFR) (e.g. [15, 46]).

Clearly, research into the long-term neurodevelopmental effects of postnatal manipulations is now at a stage where it is no longer appropriate to continue, in either theory or practice, with the use of NH as a control group. Levine has referred to the unusual circumstances of NH, both in the past [1] and recently [31], and the Meaney-Plotsky group has moved away from NH in EH-MS studies. When three such distinguished scientists in this field are of the same opinion, it would appear to be very pertinent to take note.

Taking time to tease apart the conditions of NH relative to the postnatal manipulations for which it is meant to serve as a control or comparison group brings home the limitations of the NH procedure. In the case of EH, the daily manipulation comprises brief human handling of the dam and pups and brief separation of the pup, either alone or with the littermates, in a different environment. In the case of MS, the manipulation comprises brief human handling of the dam and pups and prolonged separation of the litter in a different environment. ED comprises brief human handling of the dam and pups and prolonged isolation of the pup in a different environment. Furthermore, with EH at least, there is considerable evidence that the maternal care of the dam is chronically altered by the

manipulation (see Mediating mechanisms). As such, NH is of limited value as a control for: EH, because it is unclear whether the marked EH effects are mediated by human handling, brief exposure to a different environment in the absence of the dam (and possibly littermates), altered maternal behaviour in the home cage, or some combination thereof; and ED, because it is unclear whether the marked ED effects are mediated by human handling, prolonged isolation in a different environment, altered maternal behaviour in the home cage, or some combination thereof. As reviewed above, MS is largely without effect relative to NH.

It is possible to gain some insight into (i) which specific factors are mediating the effects of EH and ED and (ii) what will constitute an appropriate control for these treatments, via multiple comparisons of the different treatment and "control" groups. Given that both NH and MS increase emotionality relative to AFR [15, 44], it would appear that a general chronic state of under-stimulation, either of the dam and the litter (NH), or of the litter per se due to a 3 hour absence of the dam (MS), that can be reversed by typical animal facility husbandry, is the major mediating mechanism for both NH and MS. From this, we can conclude that the level of dam and pup stimulation that is provided by AFR is an essential characteristic for the control group in postnatal manipulation studies. For NH and MS pups, chronic under-stimulation could well take the form of insufficient levels of specific patterns of maternal care, and this is discussed in detail below. Then, given that MS is largely without effect relative to NH, it is unlikely that either human handling per se or exposure to a new environment per se (both components of MS relative to NH) is an important mediating factor in any of the other postnatal manipulations. Turning now to EH and ED, if we accept the above conclusions that human handling and exposure to a new environment are not important factors that need to be specifically controlled, this leaves brief isolation and increased maternal care (EH) and prolonged isolation (ED) as the most likely mediating mechanisms and therefore the factors that need to be controlled for. As such, AFR constitutes an appropriate control group for EH and ED. Even so, in the case of effects of EH relative to AFR (e.g. reduced fear-conditioned freezing) it remains unclear whether these are due to brief isolation or to increased maternal care, or a combination thereof. In the case of effects of ED relative to AFR (e.g. enhanced spatial cognition and LI) we would conclude that these are most likely due to effects of prolonged and complete social deprivation, and that AFR will constitute an appropriate control for this. Of course, AFR is currently quite a vague concept for a control group and as such there is a real danger that it will vary widely between laboratories [31]. A more rigorously defined control, probably comprising daily exposure to the disturbance of specific husbandry-like procedures, should be aimed for. Contrary to the recent proposal that in developmental research there are no control groups only comparison groups [31], in the above discussion we have described how fundamentally important

consideration of the control group is in developmental research to the conclusions deduced from the data obtained.

**Mediating mechanisms**

In our above consideration of what constitutes the most appropriate control group for specific postnatal manipulations, we of course also began referring to the mechanisms that mediate the effects of these manipulations. Just as the Meaney-Plotsky group has held a high profile in the study of the effects of postnatal manipulations, so has the Meaney group held a similar profile in theories of the mechanisms mediating these effects. Specifically, this group has proposed that, at least as far as the EH-NH model is concerned, it is altered maternal behaviour that is the mediating mechanism [55, 67, 71-78]. It was first reported quite some time ago that, in the EH-NH model, EH stimulates an increase in maternal behaviour, specifically in the form of pup licking, and not just following dam-pup reunion but throughout the entire light and dark phases of the daily cycle [79]. Based on this finding it was proposed that altered maternal behaviour could contribute to mediation of the observed effects of EH on long-term behavioural and physiological development [80]. Since these early studies, the Meaney group has also observed the effects of EH on maternal behaviour [71], and has gone on to claim: "Variations in maternal care affect the development of individual differences in neuroendocrine responses to stress in rats." ([71]; p. 1659); "The mothers of infant rats show individual differences in the frequency of licking/grooming and arched-back nursing of pups that contribute to the development of individual differences in behavioural responses to stress." ([55]; p. 5335).

The rationale for the jump from the finding that EH increases maternal care to the statements cited above is based on the extensive evidence, from the Meaney group, that the offspring of dams that exhibit spontaneously high levels of specific maternal behaviour patterns exhibit EH-like phenotypes in adulthood. The EH-like phenotype comprises neurochemical (e.g. increased central BZ receptor density in the amygdala and locus coeruleus; decreased CRF receptor density in the locus coeruleus), neuroendocrine (e.g. increased hippocampal GR mRNA; decreased hypothalamic CRF mRNA; reduced ACTH and CORT stress reactivity), and behavioural (e.g. increased exploration; reduced novelty-induced suppression of feeding) traits [55, 71]. The spontaneous differences in maternal care that were observed took the form of licking/grooming (LG) and arched-back nursing (ABN) (see [47] for a clarification of these terms). The dams in which high-LG-ABN versus low-LG-ABN were observed were all NH dams [55, 71]. Therefore, the complete constellation of the EH-phenotype that had been identified in the EH-NH model had now also been identified within the NH group, by comparing the 25% highest LG-ABN versus the 25% lowest LG-ABN NH dams. Even more than this, the young adult offspring of high-LG-ABN NH mothers

exhibited enhanced spatial learning and memory in the water maze relative to low-LG-ABN NH mothers [76], an effect not observed until senescence in the EH-NH model [60].

These maternal care studies have used a correlational design in which genetic and experiential effects on the mother-infant relationship and offspring development can be confounded, and at several different levels [81]. In further studies from the Meaney group, partial fostering of pups between low LG-ABN and high LG-ABN dams has provided evidence that differences in the LG-ABN received may make a direct (i.e. causal) contribution to open field exploration (offspring born to low LG-ABN dams and reared by high-LG-ABN dams (Low to High) demonstrated increased exploration relative to Low to Low, and High to Low offspring demonstrated decreased exploration relative to High to High [75]), and partial evidence that differences in LG-ABN received make a direct contribution to water maze spatial learning and memory (offspring born to low LG-ABN dams and reared by high-LG-ABN dams (Low to High) demonstrated enhanced spatial memory relative to Low to Low, but High to Low offspring demonstrated spatial memory that was equivalent to that of High to High [76]). It will therefore be interesting to examine how the many additional behavioural, physiological and neurobiological correlates of LG-ABN behave in fostering studies, where genetic and experiential effects can to some extent be teased apart.

Even in cases where fostering studies provide evidence that offspring phenotype is related to the maternal care received, extreme caution needs to be exercised in deducing that the relationship is causal. There are several grounds for such caution. Firstly, all of the fostering studies performed by the Meaney group to date have been based on a background of NH rearing conditions (e.g. [73, 75, 76]) and, as described in detail above, NH constitutes an environment of under-stimulation for pups [31] that leads to an abnormal phenotype [27]. Accordingly, many of the offspring that are the subjects of these studies will be outside of the normal range of phenotypes present in most laboratory (AFR) colonies. Second, maternal care does not take place in a vacuum but constitutes a complex set of dyadic interactions between dam and litter as well as between littermates. Thus, inter-dam differences in LG-ABN are not the exclusive product of spontaneous differences between dams; rather, maternal care is elicited by infant stimuli such that differences in maternal care are at least as likely to be driven by differences in infant genome and phenotype as they are by differences in maternal genome and phenotype. (An example of this was referred to in the Introduction, where we cited the careful and insightful work of Stern and colleagues in this area, including the observation that ABN is dependent on pup and litter size and therefore the frequency of this behaviour is clearly infant-driven [26].) Appreciation of the dynamics of the dam-litter relationship is crucial given the manner in which the Meaney group performs fostering, or more specifically, partial fostering. In their studies only two out of the average 12 pups are fostered meaning that in a situation where pups from a low LG-ABN dam are fostered to a

high LG-ABN dam, they are actually fostered to a high LG-ABN dam *and* litter, and *vice versa*. As such, the reported long-term effects that the Meaney group has attributed to be caused by the maternal care received could equally likely be caused by the environment provided by the foster littermates: that is, the pup behaviour that is stimulating differences in maternal care is stimulating differences in (foster) littermate neurobehavioural development. Indeed, the rationale given for partial rather than wholesale fostering of litters (e.g. [75, 82]) is the very fact that the latter has been reported to affect maternal behaviour [83] whereas the former has been reported not to [84]. In summary here, the Meaney group may well have provided some evidence that certain of the phenotypes yielded by EH are mediated by the postnatal social environment, but it has not provided evidence that these are mediated by maternal care. Clearly, these fundamental theoretical and methodological issues need to be carefully addressed.

The above criticisms of the interpretation of the existing evidence for the relationship between maternal care received and offspring neurobehavioural phenotype have been largely restricted to studies carried out with the EH-NH model and, extending from this, the Low-High LG-ABN model. The other postnatal manipulations that have been studied also provide extremely important evidence on this subject. We (e.g. [44]), and the Plotsky-Meaney group (e.g. [15]), have produced evidence that EH and AFR adults exhibit similar phenotypes in a number of (but by no means all (e.g. [45]) test situations. This, in line with the maternal behaviour theory, could at least in part be related to the similar levels of maternal care that they receive as pups [47]. An obvious prediction of the theory that maternal behaviour mediates the EH effect is that deprivation of maternal behaviour will lead to the opposite effects to those induced by EH, i.e. anxiogenesis and stress hyper-responsiveness. As we have seen above, in their EH-NH-MS model the Meaney-Plotsky group has demonstrated that relative to NH, 3-hour MS is largely without effect on behavioural, neuroendocrine and neurochemical measures of affective status. As an explanation for this we proposed in the previous section that the effects of both NH and MS (and therefore the absence of effect relative to each other) are mediated by under-stimulation relative to those levels that the developing rat CNS has evolved to expect [4, 31, 85]. According to this idea, the 3-hour MS litter is not separated from the dam for long enough to experience stress but is separated for long enough to be affected by the absence of any stimulation from the dam for 3 hours per day. With regards to what occurs when 3-hour MS litters are with the dam, although to our knowledge no data have been reported it has at least been described that, “the frequency of (MS) LG-ABN is reduced to levels typical of those observed in the mothers of NH litters” [33]. (Of course, given the high variation reported in NH dams (e.g. [55] see above), it is clear that published studies including these data are much needed.) Therefore, the 3-hour MS model does provide some support for the maternal behaviour theory of the mediation of the

effects of EH and other postnatal manipulations i.e. NH-MS similarity in maternal care received leads to neurobehavioural similarity in NH-MS adult offspring.

Our evidence for the effects of early deprivation is categorically not in line with the maternal behaviour theory. We have observed maternal behaviour in our EH-ED-AFR-NH model, but in the first three treatments only in accordance with the strict non-disturbance definition of NH in our laboratory. What we observed was that EH yielded an increase in ABN across several time points throughout the day but did not significantly affect any other behaviour [47] (Figure 8). ED stimulated a bout of maternal behaviour in all dams immediately following reunion such that LG-ABN was increased relative to AFR during the immediate post-reunion period. ED dams also exhibited increased LG-ABN at the beginning of the dark phase relative to AFR with pups aged PND 3-4 [47] (Figure 8). Otherwise there were no observed effects of ED on maternal care [47]. Thus, ED subjects were deprived of bouts of maternal care for 4 hours per day relative to EH and AFR and, with the exception of the post-reunion bout of care, there was little evidence for compensation for this deprivation. Despite these differences in maternal care received, we have obtained no evidence, in contrast to the predictions derived from the Meaney group maternal behaviour theory, that ED adults are more anxious, fearful or stress sensitive, or that they exhibit impaired cognition. In fact, as reviewed above, the opposite is the case. Furthermore, the subjects of our study of the effects of ED and EH on maternal behaviour relative to AFR [47] were also the subjects of the studies of the effects of ED and EH on physiology and behaviour relative to AFR and NH in adulthood [44, 45]. Clearly, the concept that quantitative differences in adulthood phenotypes are reliably predicted by the amount of specific forms of maternal care received, whether induced by specific manipulations or occurring spontaneously is inadequate to explain many findings to-date.

Some of the most persuasive evidence against the maternal behaviour mediation theory is provided by recent EH-NH research of the Meaney group, into the mechanism via which EH leads to increased GR expression in the hippocampus [77]. Previous studies had demonstrated, firstly, that the GR effects of EH are dependent on peripheral thyroid hormone release that stimulates serotonin (5-HT) activity at the level of the hippocampus, and second, that the 5-HT effect is mediated by a 5-$HT_7$-like receptor that is positively coupled to cAMP. In detailed *in vivo* studies conducted with EH and NH pups aged PND 7, evidence was obtained that EH: increases hippocampal cAMP formation, increases activity of the secondary messenger protein kinase A, and increases mRNA levels for cAMP-transducible transcription factors that are implicated in the regulation of GR expression during development [77]. Whilst this elegant study leads to the development of an epigenic-epigenetic model of how environment impacts on development via altered gene expression ([77]; p. 3933), it also provides evidence against the maternal behaviour theory. This is because the time course of

the response to EH by the various neurobiological factors, including protein kinase A and the transcription factors NGFI-A and AP-2, was studied, and what is clear is that each of these factors exhibits an acute and transient response to the EH manipulation. For example, in the case of protein kinase A, hippocampal levels are increased at 0 and 30 min after EH but by 240 min levels have returned to those present in NH pups. If LG-ABN maternal care, which is increased not just after completion of EH but throughout the daily cycle, were mediating these effects, then the neurobiological factors would be continuously elevated relative to NH and not only in a strict time window immediately post-EH.

As the evidence accumulates that many of the effects of postnatal manipulations cannot be accounted for via altered maternal care, attention shifts to the identification of additional and alternative mechanisms. Certainly in the case of ED and quite possible also in the case of EH, there are various lines of evidence that early life stress is an important factor [7]. Whereas PNDs 2-14 constitute the stress hyporesponsive period (SHRP) in the rat [86, 87], as described above, even a 1-hour ED can overcome the SHRP such that CORT is elevated during and after the daily ED procedure [43]. Furthermore, AFR pups exposed to high CORT during the SHRP via the maternal milk develop into adults that have reduced pituitary-adrenal endocrine stress responsiveness, increased hippocampal MR binding capacity, reduced behavioral aversive responsiveness, and enhanced spatial learning and memory [88-90]. Clearly, therefore, there is a great deal of important research to be conducted in the area of mediating mechanisms of the long-term neurobehavioural impact of the postnatal environment in rats.

**Conclusions**

Our goals with this review have been (i) to provide a timely analysis of the current evidence for the chronic effects and mediating mechanisms of specific experimental postnatal manipulations on neurobiological, physiological and behavioural phenotypes in adulthood in laboratory rats, and (ii) to provide insights into important future directions in this area of research. In attempting to achieve these goals we have focussed on the research of some of the most eminent and influential research groups in the field, to highlight the progress that has been made in describing and understanding the effects of specific postnatal experiences on long-term neurobehavioural status, and the extent to which this existing evidence has stimulated interesting hypotheses which now need to be rigorously tested across laboratories. Table 1 presents an overview of the research we have reviewed.

The study of the long-term neurobehavioural impact of the postnatal environment in rats is of fundamental importance. It is one of the major examples -- at the interface of the biological, social and medical sciences -- of animal models for understanding the interaction between the environment and the genome in both the acute and chronic regulation of the

phenotype. Furthermore, it has marked potential for the development of animal models for neuropsychiatric disorders, a theme that has not been addressed in this review. The animal model value has already been demonstrated within the EH-NH model, in terms of disruption of LI by NH in males and its restoration with neuroleptics as an animal model for LI disruption in schizophrenia [28]. The epidemiological and clinical evidence that early life stress markedly increases vulnerability to later development of stress-related disorders, most notably depression (e.g. [3]), will now doubtless lead to a focus on the utilisation of postnatal manipulations for the development of animal models of depression, both in rodents and in primates [46, 91, 92]. In terms of our own current research, given our theory that ED effects are mediated by prolonged and complete social deprivation, and given our findings that ED leads to emotionality-reducing and cognitive-enhancing effects relative to AFR, then we are clearly at a very interesting stage in our research programme. What we are now proceeding with is a large-scale study of the effects of ED under different conditions and in different strains. For example, what are the effects of performing ED during the light phase versus the dark phase, and at room temperature versus nest temperature? So far we have conducted ED specifically during the dark phase and at nest temperature [44, 45, 47]. And what are the relative effects of ED in different strains that differ in their stress sensitivity in adulthood? So far we have conducted ED in the Wistar strain and we are now investigating the Lewis CRF-deficient and Fischer stress-hypersensitive in-bred strains. The major aim of this ED research programme is to investigate the potential of ED as an animal model for one or more of the major symptoms of depression, stimulated in part by the evidence that ED exerts enduring effects on serotonergic activity in the limbic system [70].

In conclusion, for fundamental and applied reasons it is important that the study of the chronic neurobehavioural effects of postnatal manipulations is conducted using robust methodologies, paying due attention to issues such as the control group, that will allow for replication of findings across laboratories and the development of a sound theory of the effects of specific environmental experiences on neurobehavioural development and of the external and internal mechanisms that mediate these effects.

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Figure 1. Comparison of the effects of postnatal treatments on adult rat performance in fear-conditioned two-way active avoidance. The mean+SEM percentage of avoidance responses in 100 trials is given for six adult male Wistar rats per group. Each trial began with presentation of the conditioned stimulus (CS) in the form of a flashing light at 2 flashes/sec for 12 sec, with the final 2 sec of the CS contiguous with a 0.5 mA foot shock (UCS). Crossing a barrier to the opposite compartment during sec 1-10 of the CS resulted in termination of the CS and avoidance of the UCS (avoidance response). AFR and ED males demonstrated significantly more active avoidance than NH males, and EH males demonstrated a trend to significantly more active avoidance than NH. (For further details of the study see [45]).

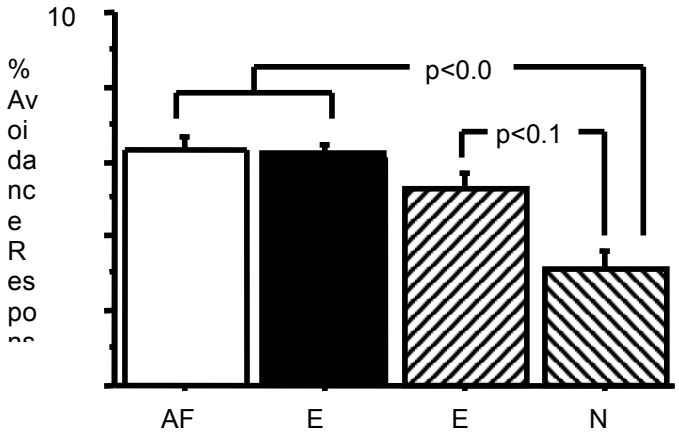


Figure 2. Comparison of the effects of postnatal treatments on adult rat performance in fear-conditioned freezing. Adult male Wistar rats, six per treatment group, were fear conditioned using 10 tone-foot shock (CS-UCS) pairings on day 1 (A), exposed to 8 min without CS or UCS but in the same environmental context on day 2 (B), and to 4 min continuous CS in a novel context on day 3 (C). The development of fear conditioning and its subsequent expression to context and CS were assayed in terms of freezing behaviour, measured used an automated system. There were no significant treatment group differences in fear conditioning (A) or in the expression of fear conditioning to context (B). AFR rats expressed significantly greater fear conditioning to the CS than EH and NH rats, and a trend to significantly greater fear conditioning to the CS than ED rats (C). Following the CS test on day 3, blood samples were collected at 10 and 100 min post-test and assayed for immunoreactive ACTH and CORT levels. Against a background of no treatment-group differences in basal levels for either hormone: For ACTH (D), AFR, ED and NH rats demonstrated a significantly increased ACTH stress response relative to EH at min 10 post-test, and AFR rats demonstrated a significantly increased ACTH response relative to EH and ED at 100 min post-test, as did NH rats relative to EH. For CORT (E), AFR rats demonstrated a significantly increased CORT response relative to ED, EH and NH rats at 10 min post-test as did NH rats relative to EH; at 100 min post-test AFR and NH rats continued to demonstrate significantly increased CORT responses relative to EH. Values depict the mean+SEM in all cases. (For further details of the study see [45]).
a: $p<0.05$ versus EH; b: $p<0.05$ versus ED; c: $p<0.05$ versus NH.

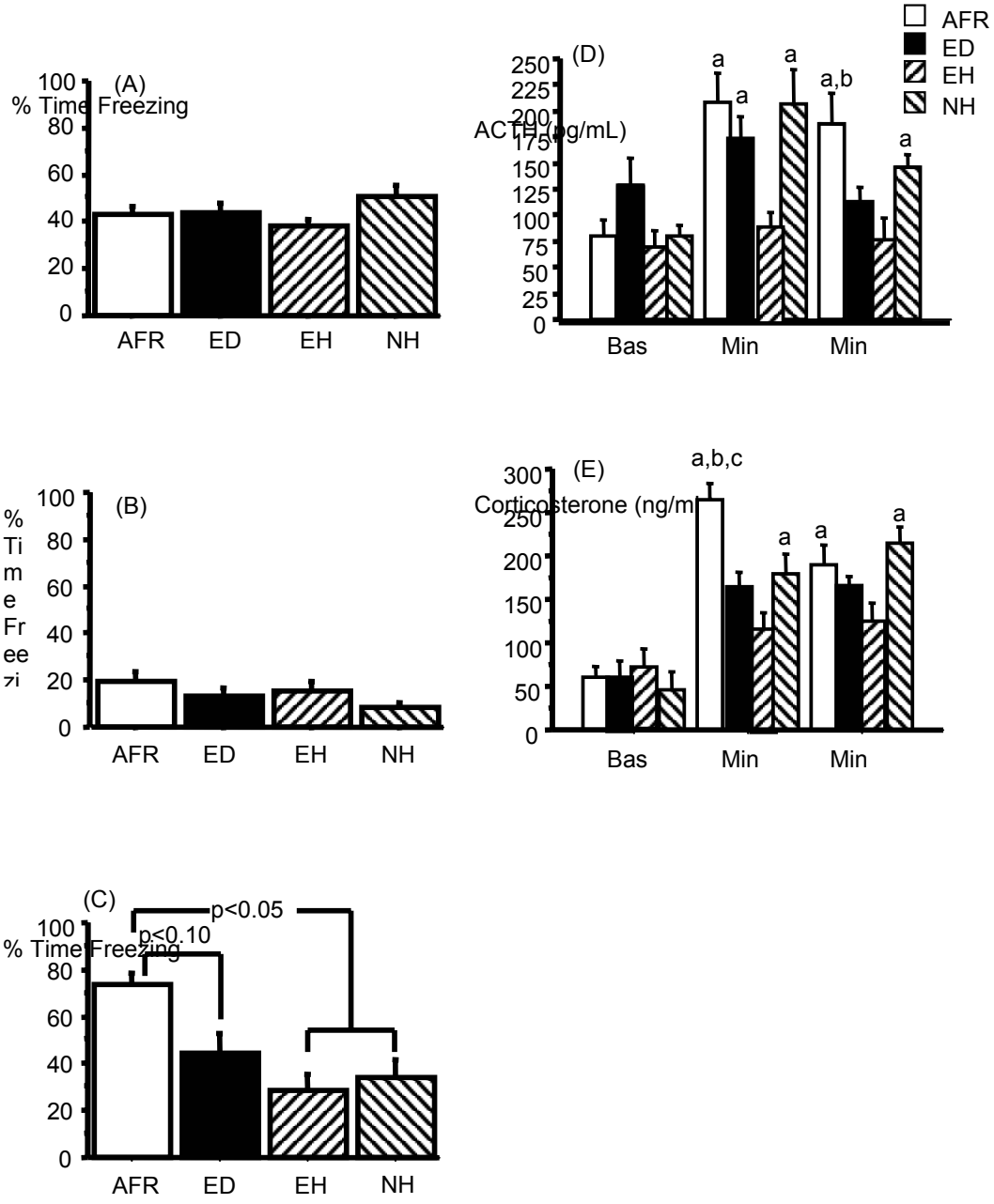

(A)
% Time Freezing
100
80
60
40
20
0
AFR
ED
EH
NH
(B)
% Time Freezing
(C)
% Time Freezing
p<0.05
p<0.10
(D)
ACTH (pg/mL)
250
225
200
175
150
125
100
75
50
25
0
a
a
a
a,b
a
Bas
Min
Min
(E)
Corticosterone (ng/mL)
300
250
200
150
100
50
0
a,b,c
a
a
a
Bas
Min
Min

Figure 3. Comparison of the effects of postnatal treatments on adult rat spatial learning and memory in the water maze. Adult female Wistar rats, eight per treatment group, were tested on their ability to acquire and utilise spatial information to locate a submerged platform in a water tank (Ø = 2m) surrounded by external visual cues. Testing was performed across six days, four trials per day from four different starting points, with the platform in a fixed position. (A) ED rats demonstrated a significantly reduced latency to locate the submerged platform relative to AFR and NH rats, and EH rats demonstrated a significantly reduced latency to locate the platform relative to NH rats and a trend to the same relative to AFR rats. As depicted in (B), the superior performance of ED and EH rats was most marked during days 1-3 of acquisition training. There were no treatment-group differences in swimming speed ($p>0.52$). Values depict the mean+SEM in all cases. (For further details of the study see [45]).

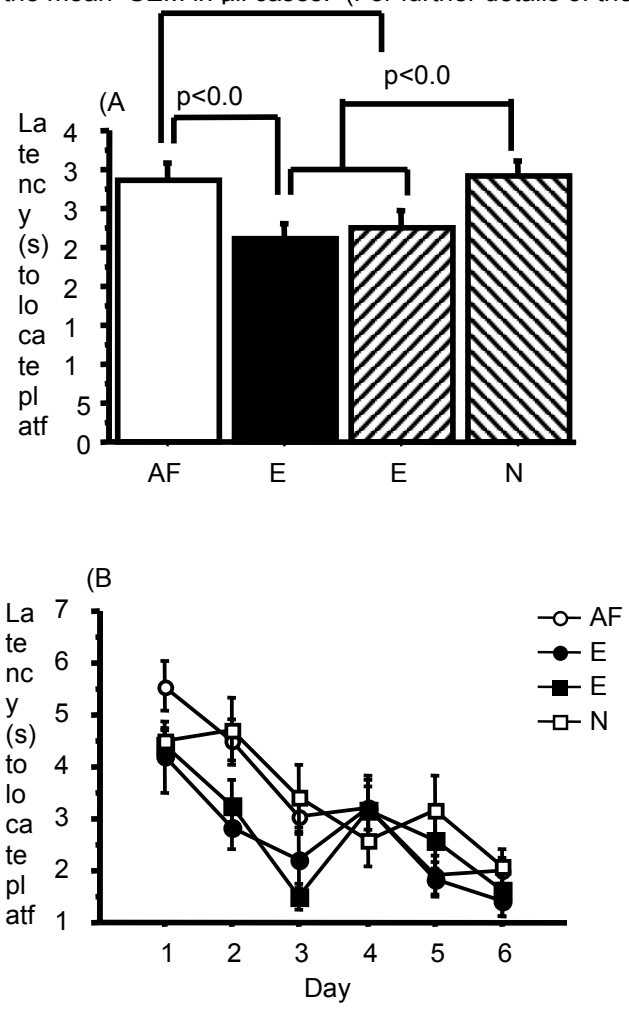


Figure 4. Comparison of the effects of postnatal treatments on adult rat latent inhibition to the CS in two-way active avoidance. Adult male and female Wistar rats, six per Sex (male, female) x Treatment (AFR, NH, ED, EH) x Preexposure group (CS preexposed, CS non-preexposed), were tested in terms of their two-way active avoidance performance (see legend to Fig. 1 for a brief description of methods). The CS-preexposure (PE) groups were placed in the active avoidance apparatus on days 1 and 2 and exposed 50 times per day to the flashing light that was to be used as the CS in the active avoidance test carried out on day 3. The CS-non-preexposure (NPE) groups were placed in the active avoidance apparatus on days 1 and 2 in the absence of the flashing light and tested in active avoidance on day 3. The active avoidance test comprised 10 bins of 10 CS-UCS trials each. Latent inhibition, analysed across individual bins, was defined as a significant reduction in avoidance responses by the PE group relative to the NPE group of the same postnatal treatment. (A) depicts AFR and NH Wistar adult rats, and (B) depicts ED and EH adult Wistar rats. LI was most pronounced in ED (avoidance significantly reduced in PE relative to NPE in 7 out of 10 bins), followed by AFR (3 out of 10 bins), EH (2 out of 10 bins), and was absent in NH (0 out of 10 bins). (For further details of the study see [45] and [66]). Values depict the mean in all cases. The SE is derived from the mean square of the overall analysis of variance (ANOVA) error term. * indicates that PE avoidance responses were significantly reduced compared with NPE avoidance responses in the same treatment group, with $p<0.05$ in the post hoc *t* test based on the ANOVA error term.

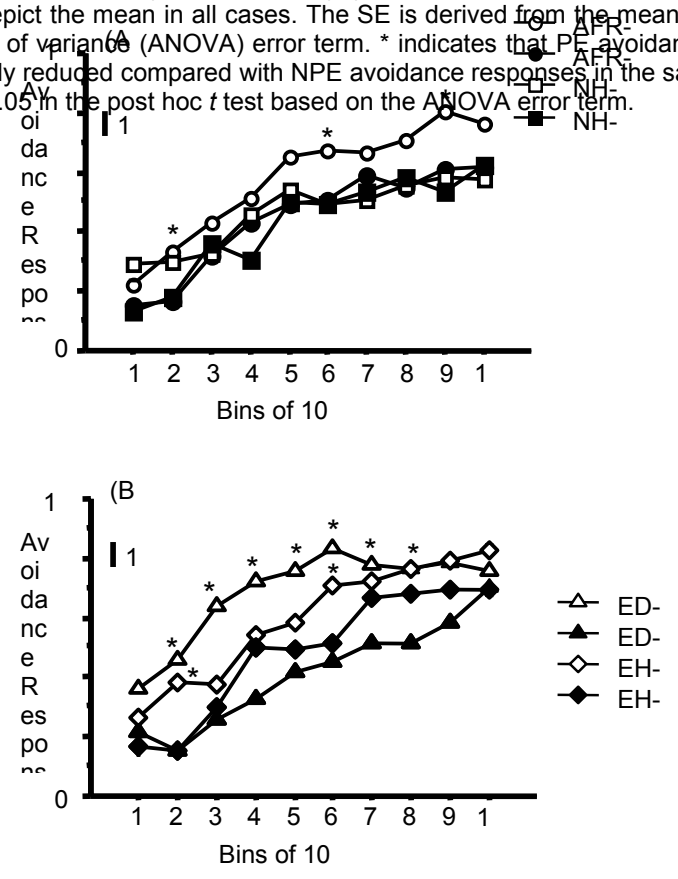


Figure 5. Comparison of the effects of postnatal treatments on corticosterone titres in adult male Wistar rats. Total plasma CORT titres as measured in the afternoon at rest (A) and following a 2-min novelty restraint and tail cut (B). NH males demonstrated significantly higher CORT titres at 30-min post-stress compared against AFR and ED males, and a trend ($p<0.07$) to significantly higher CORT titres at 30-min compared against EH males. (Adapted from [44] with permission; © 2001, American Psychological Association.)

* $p<0.05$ for NH versus AFR, ** $p<0.01$ for NH versus ED.

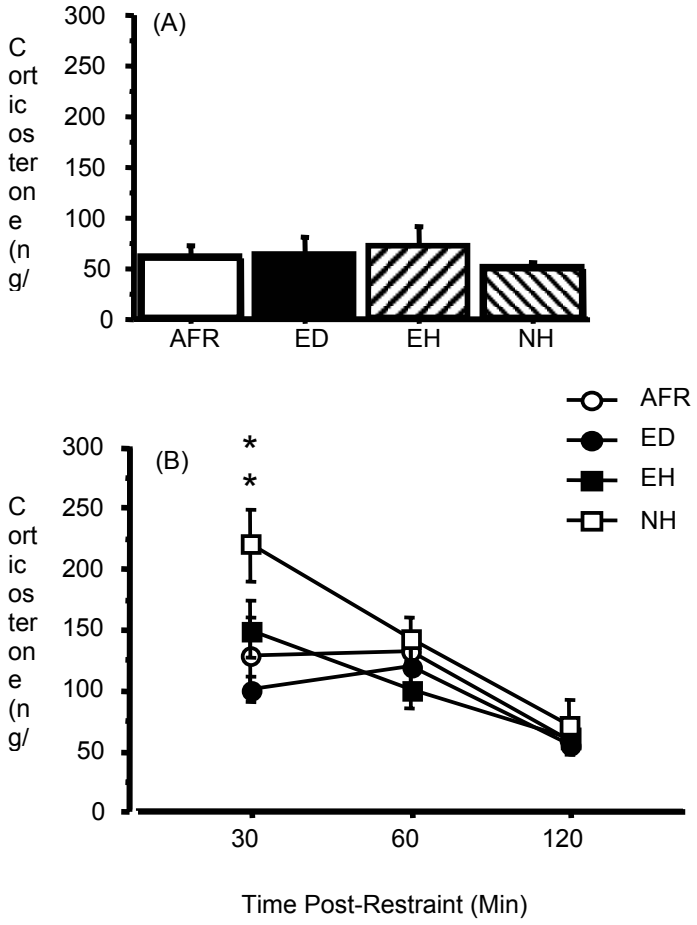


Figure 6. Comparison of the effects of postnatal treatments on locomotion in a novel open field. Adult Wistar rats, six males and six females per treatment, were tested for locomotor activity in a 76 L x 76 W x 49 H cm open field for 30 min. Values depict means+SEM for the entire 30-min period. ED subjects demonstrated significantly more locomotion than NH subjects. (Adapted from [44] with permission; © 2001, American Psychological Association.)

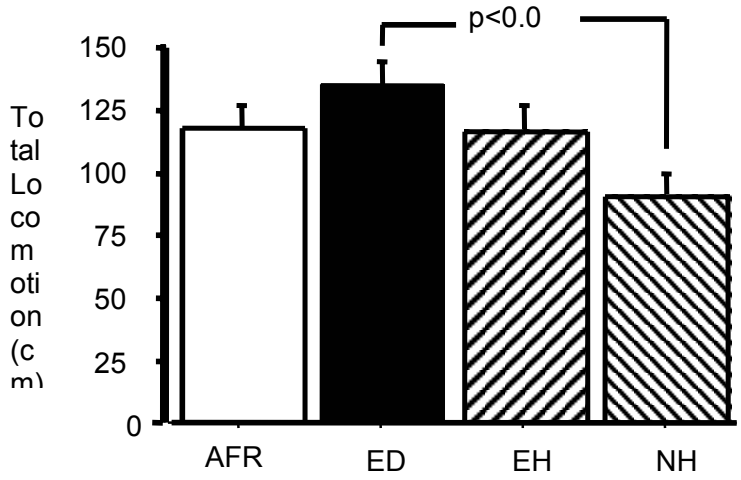



Figure 7. Comparison of the effects of postnatal treatments on acoustic startle responsiveness (ASR). Adult Wistar rats, eight per Sex x Postnatal treatment, were exposed to four acoustic startle pulses of 30-ms x 120 dB[93] white noise to determine their basal ASR in a purpose-built startle chamber. Values depict mean startle+SEM. NH female rats demonstrated significantly greater ASR relative to ED, EH and AFR females; NH male rats demonstrated significantly greater ASR relative to ED and AFR males. (Adapted from [44] with permission; © 2001, American Psychological Association.)
* p<0.05 versus NH.

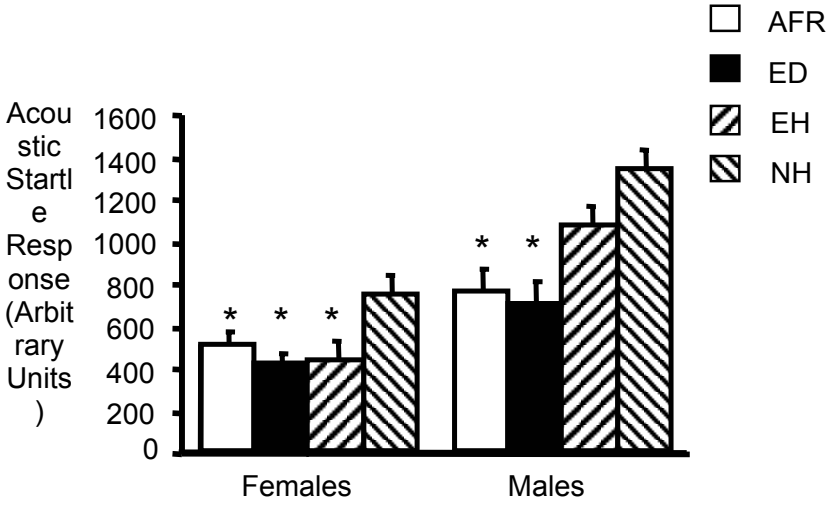
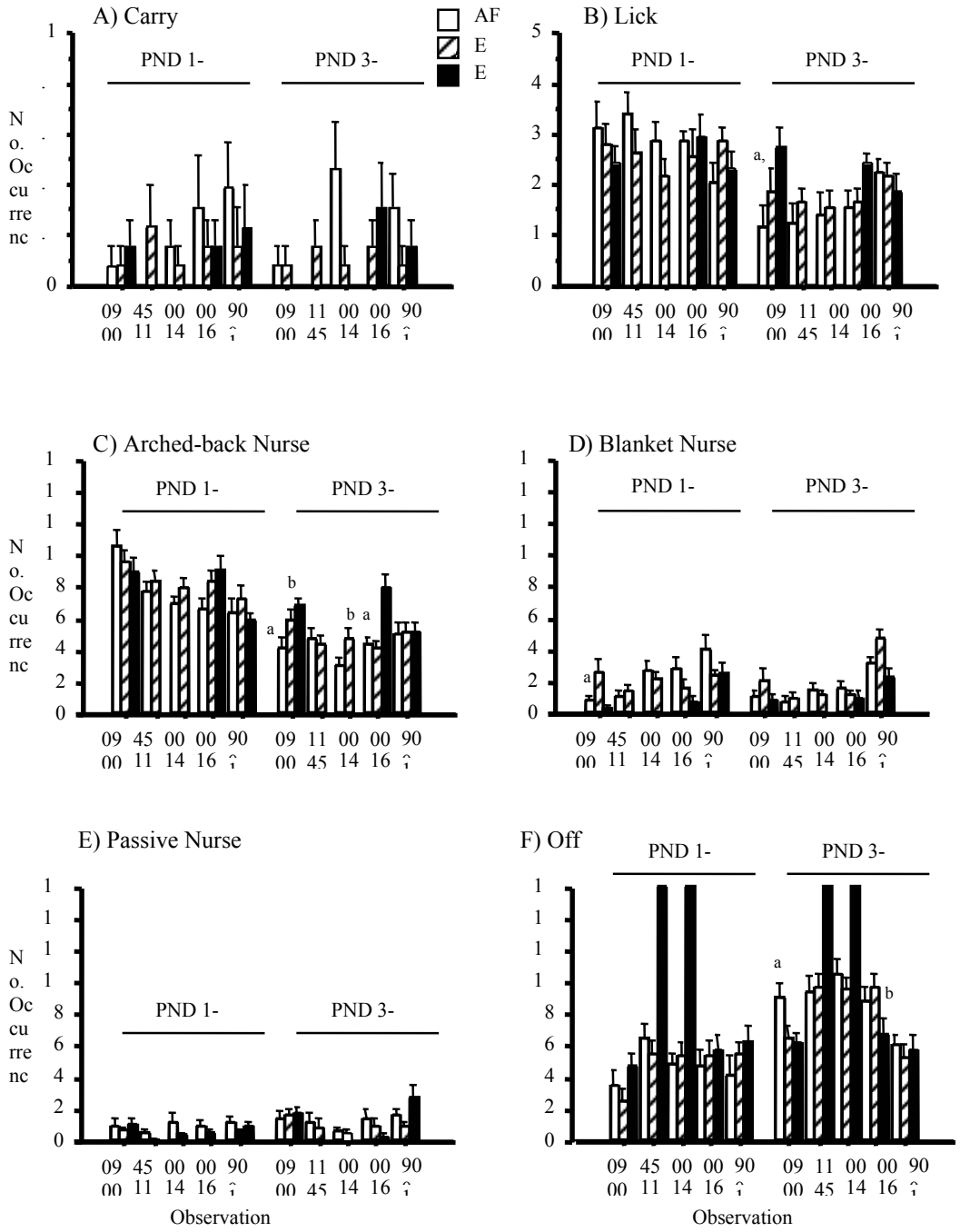


Figure 8. Comparison of the effects of postnatal treatments on maternal behaviour. Average (mean+SEM) number of observed occurrences for maternal behaviours obtained with dams with AFR, EH (15-min pup isolation beginning at 1100-1115) or ED (240-min pup isolation beginning at 1130-1145) litters. Pups were at age PND 1-2 or 3-4. The dark phase of the reversed L:D cycle began at 0700 and ended at 1900. Observation sessions began at 0900 (AFR, EH, ED), 1145 (AFR, EH), 1400 (AFR, EH), 1600 (AFR, EH, ED), and 1900 (AFR, EH, ED). The maximum possible score for any dam at each observation time was 16 (2 days X 8 samples per observation). In the case of graph F, dam off pups, ED dams have been depicted as being off the pups at all sample points at 1145 and 1400, i.e. these two observations were conducted during ED. (Reproduced from [47] with permission; © 2001, Developmental Psychobiology.)
a versus b denotes a significant effect of treatment on behaviour.


A) Carry
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D) Blanket Nurse
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PND 3-
a
E) Passive Nurse
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Observation

**Repeated Parental Deprivation in the Infant Common Marmoset (Callithrix jacchus, Primates) and Analysis of its Effects on Early Development**


## Abstract

This report describes a successful demonstration that repeated early deprivation of parental care (ED), as used to study effects of early life stress in rats, can be performed in a primate and that it constitutes an early-life stressor. Seven breeding pairs of marmoset monkeys each provided control twins (CON) and twins that were subjected to ED for 30-120 min/day on postnatal days (PND) 2-28. Urine samples were obtained to monitor the acute effect of ED on cortisol and catecholamine levels. Behavior samples were obtained in the home cage to monitor the effects of ED on infant and infant-parent behavior. ED caused acute increases in cortisol, epinephrine and norepinephrine. At PND 28, basal cortisol was reduced in ED compared with CON, and ED infants were smaller than CON. ED infants tended to spend more time in the suckling position than did CON. ED infants demonstrated more distress vocalization than CON infants, even though the parental care they received in the home cage was similar. ED infants tended to play less socially than did CON. To our knowledge this is the first demonstration that a repeated early-life stressor of the type developed in rats can also be applied to a primate species.



**Key words:** Early Deprivation (ED), non-human primate, cortisol, catecholamines, parental and infant behavior


## Introduction

There is growing evidence that adverse early life events constitute one of the major risk factors for the development of mood and anxiety disorders in adolescence and adulthood (Bernet and Stein 1999; Glaser 2000; Heim and Nemeroff 2001; Heim et al 1997; Wong and Licinio 2001). In interplay with the genome, the early environment regulates brain development and consequently the development of species-specific behavior as well as individual differences in behavior (Gottlieb 1998; Greenough et al 1987; Plomin 1994). In mammals, parental care is the major factor in the early environment, and there is compelling evidence that parental care commensurate with maintenance of the infant's homeostasis is important for the short-term and long-term development and functioning of neurobiology, physiology and behavior (Hofer 1987; Hofer 1994b). This rodent and primate evidence is provided by observational studies of the consequences of spontaneous differences in maternal care, and by experimental studies involving manipulation of the infant-mother dyad (Kraemer et al 1989; Liu et al 2000; Meaney et al 1996; Pryce et al 2001a; Rosenblum et al 1994). This approach in animals could yield models of the short-term and long-term consequences of abuse and neglect

in human parent-infant relationships. Models of the short-term consequences of early life stress will be directly relevant to child psychiatry. Studies of the long-term consequences can provide models of important symptoms of neuropsychiatric disorders including mood disorders and psychoses that can (but do not have to) include adverse early-life events in their etiology.

Most experimental evidence for the enduring effects of manipulation of the infant's maternal environment on its neurobehavioral development is based on rat studies. The effects of a number of different manipulation paradigms have been investigated. *Early handling* (EH) involves daily separation of pups from the mother, and in some laboratories also from the littermates, for a brief period (3-15 min), and its effects are studied typically in adulthood relative to mothers and pups that do not experience any environmental disturbance, so-called early n*on-handling* (NH) (Levine 1960). *Maternal* s*eparation* (MS) involves daily separation of the intact litter from the mother for a prolonged period (3-6 hr), and *early deprivation* (ED) or *isolation* involves daily separation of pups from the mother and littermates for a prolonged period (3-6 hr). The effects of MS and ED have been studied relative to NH or to mothers and pups that experience the environmental disturbance, including occasional brief handling, of typical *animal facility rearing* (AFR). As adults, EH offspring demonstrate reduced stress-related neuroendocrine responses (HPA axis) and reduced fearfulness in comparison with NH (Caldji et al 1998; Levine 1960, 2000; Meaney et al 1996; Pryce et al 2001a). ED adult offspring exhibit reduced (i.e. EH-like) stress/fear-related endocrine and behavioral responses relative to NH (Kosten et al 2000; Pryce et al 2001a) and AFR (Ogawa et al 1994).

In comparison to the large number of rat studies, the study of long-term effects of manipulation of the infant-mother dyad in nonhuman primates has, with some notable exception, received little attention. Primate studies could well provide an important complement to rodent studies in this research area. For example, the prefrontal and cortex amygdala are major brain areas in the regulation of emotion and motivation in nonhuman primates and humans, and have undergone considerable development in primates leading to marked primate-rodent differences (Drevets et al 2001; Rolls 2000). Second, the infant-parent relationship differs markedly between rodents and primates: For a rat pup, it is natural to be separated from the mother (but not the littermates) for periods of 15-30 min whilst the mother leaves the nest for foraging. In contrast, the 1-2 primate infants are in full and continuous body contact with the mother, or in some primates mother and father, throughout the first several weeks or life (Pryce 1996). Therefore, interrupting body contact for even brief periods is likely to constitute a severe stressor for a primate infant. Rats and primates also differ in their maturational state at birth, with rats being poorly developed at birth and undergoing rapid postnatal growth, and non-human primate infants being born well developed and undergoing relatively slow postnatal growth. Differences also exist in the postnatal state of the HPA

system. Postnatal days 2-14 constitute the stress hypo-responsive period (SHRP) of the rat HPA. Maternal care is an important regulator of the SHRP so that postnatal manipulations such as EH and ED might exert some of their long-term effects via SHRP disruption (Levine 2000). Primate infants do not demonstrate a SHRP, with neonates already capable of adult-like pituitary-adrenal stress responses (Bowman and Wolf 1965; Gunnar 1989). In the common marmoset monkey, infants actually demonstrate high basal levels of ACTH and cortisol relative to those of older conspecifics (Pryce et al 2002).

The majority of primate studies have been conducted with macaques and based on *maternal privation;* that is, the complete and continuous absence of the biological mother. Following separation from the mother within the first few days after birth, infants are either nursery-reared without physical contact to any other monkey during the first six months of life, or peer-reared in age-matched groups of maternally-prived monkeys. In contrast to rat ED, maternal privation constitutes a chronic absence of maternal care rather than the repeated stress of temporary deprivation of such care. Maternal privation has chronic physiological, neurochemical and behavioral effects. In comparison to mother-reared macaques, juvenile-adolescent maternally-prived peer-reared macaques exhibited either lower basal and stress-related HPA activity (Clarke 1993) or higher basal HPA activity (Higley et al 1992), and a phase shift in the HPA circadian rhythm has also been proposed (Boyce et al 1995). Nursery-reared macaques demonstrate reduced basal CSF norepinephrine relative to mother-reared macaques (Kraemer 1992; Kraemer et al 1989). Peer-reared macaques, in contrast, demonstrate increased basal CSF norepinephrine relative to mother-reared controls; stress-induced levels of CSF norepinephrine and metabolites were reduced, as were basal serotonin and dopamine metabolites (Clarke et al 1996; Higley et al 1992). The chronic behavioral consequences of maternal privation include self-clutching, stereotyped body rocking, deficiencies in social behavior, and affective flattening (Gandelman 1992; Kraemer 1992; Paul et al 2000).

Primate studies that have used repeated infant-mother separations have been performed with mature, semi-independent infants; for example 3-7 month-old squirrel monkeys (Coe et al 1983; Hennessy 1986) and 6 month-old rhesus macaques (Hinde and McGinnis 1977). These mature infants demonstrate a cortisol stress response to each separation whereas distress vocalization decreases markedly across separations. Reunited infants spend more time in contact with the mother and less time playing. Repeated deprivation of 5 hr per week in 3-5 month-old squirrel monkeys led to increased glucocorticoid feedback sensitivity in adulthood (Lyons et al 2000). Another approach used in primates is manipulation of maternal care received by mature infants via varying the feeding demands on the mother. When tested as young adults, bonnet macaque offspring of mothers exposed to unpredictable foraging demands exhibited elevated basal CRF levels in the CSF, increased noradrenergic and blunted serotonergic responses, and depression-like behavior including reduced social interactions

(Rosenblum and Andrews 1994; Rosenblum et al 1994). Using the correlational approach it has been demonstrated that the primate offspring of less responsive parents demonstrate higher stress reactivity (Dettling et al 1998; Gunnar et al 1981).

To our knowledge there has been no attempt to date to develop a repeated early deprivation procedure such as that conducted with rat pups, in a nonhuman primate. Here we describe a study in which we successfully conducted daily ED across the first month of life in the common marmoset (*Callithrix jacchus*). This is a small-bodied Neotropical primate that in terms of reproduction is characterized by twinning and biparental care (Pryce 1993). In contrast to rat pups, marmoset infants are in continuous body contact (i.e. being carried) with a caregiver for 24 h per day throughout the first 2-3 weeks of life (Ingram 1977; Pryce 1993), such that even a short period of ED is a non-biological event. Further to the description and validation of ED per se, we describe some of the early effects of this manipulation in terms of stress-related endocrine responses to ED and infant-parent behavior in the family group.

## *Methods and Materials*

### Subjects and Husbandry

This study was conducted under experimental permit in accordance with the Animal Protection Act (1978), Switzerland. Seven established breeding pairs of common marmosets each contributed two sets of twin offspring, which were the subjects of this study. Each study group comprised the breeding female, breeding male and the study twin infants only. No previous offspring were present and the first set of twins per group was euthanized (aged 1 year) prior to the birth of the second set. These breeding pairs and their offspring were maintained in colony rooms each holding 2 to 6 such groups, with groups in auditory and olfactory but not visual or tactual contact. The home cages measured 3-4 $m^3$ and were equipped with natural branches and with sawdust on the cage floor. Colony rooms were illuminated by artificial lighting on a 13:11 hr L:D cycle. Daily feeding comprised high-protein porridge containing vitamin and mineral supplements and crickets scattered in the sawdust, with commercial high-protein pellets and drinking water available *ad libitum*. Twice weekly monkeys were fed fruit and egg. All offspring were born during the dark phase and the following day was designated as postnatal day 1 (PND 1). The first set of twins of each breeding pair was allocated equally and at random (i.e. according to birth order) to the early deprivation (ED, N=7) or the control group (CON, N=7). The second set of twins of each breeding pair was assigned to the other treatment group. The seven control litters constituted 4 male-female, 2 male-male, and 1 female-female twin pairs (8 males, 6 females), and the seven ED litters constituted 6 male-female and 1 male-male twin pairs (8 males, 6 females).

**Repeated Early Deprivation**

In the case of all groups, on PND 2 at 09:00, the parent carrying the infants was caught and briefly restrained, the infants were removed to a procedures room and sexed, weighed, and one twin was marked by shaving some of the back and tail hairs. In the case of triplets, the least active or smallest infant was euthanized by sodium pentobarbital overdose. In the case of the seven sets of control infants, these were returned immediately to the parents, with the time from removal to return to the parent and the home cage being approximately 5 min. PND-2 ED infants were isolated for 30 min (see below) and then returned to the parents.

On PNDs 2 to 28 (4 weeks), ED was conducted daily according to a fixed schedule for between 30 to 120 min per day, beginning at different time points from 08:30 to 17:00. For each of weeks 1-4, a total of 9 hr ED was administered, comprising 2 x 30-min, 1 x 60-min, 2 x 90-min, and 2 x 120-min ED sessions. Variable durations as well as variable times of day of ED were chosen to add unpredictability to the manipulation. An additional condition of the manipulation protocol was that ED duration on PNDs 2 and 3 was set at 30 min per day. ED began with the infant being removed from the carrying parent's back while restraining the parent in the home cage. The infant was then taken to the procedures room where it was weighed. We then attempted to obtain a urine sample (0.1-0.5 ml) by gently stimulating the surface of the ano-genital region with a clean pipette tip. The infant was then placed alone in a plastic mouse cage (Macrolon, 25 x 19 x 14 cm) with an aluminium mesh top to which the infant could cling. This cage was placed in an isolation chamber (Coulbourn Instruments) fitted with a 4W light. Temperature within the isolation chamber was 23-25°C. The procedure room was sound-isolated from the colony rooms. ED was conducted consecutively with each infant in each pair, so that one infant remained with the parents at all times. At the end of the ED session, we attempted to collect a post-ED urine sample using the same method. The infant was returned to the sleeping box attached to the home cage, from where it was retrieved immediately by one of the parents. In the case of CON infants, the carrying parent was restrained briefly and then released, in order to simulate this component of the ED procedure. On PND 28, the CON infants were removed, weighed, a urine sample was obtained via ano-genital stimulation, and the infant then returned to the parents.

**Measurment of Urinary Cortisol and Catecholamines**

Urinary cortisol and catecholamine titers in samples obtained pre- (basal) and post-ED on PNDs 2-28, were measured for 5 of the 7 sets of ED twin subjects. Basal urinary cortisol titers were measured on PND 28 for ED and CON twin subjects. Immediately after collection, infant urine samples were stored at -25°C and transferred to -80°C within 48 h. Determination of total urinary cortisol values was performed within a single RIA following enzyme hydrolysis of 5μL urine, as described in Dettling et al. (1998) with the only protocol change being that $^{3}$H-

cortisol ([1, 2, 6, 7-$^{3}$H] cortisol, specific activity 80-105 CI/mmol; Amersham International, Amersham, UK) was used as tracer. Intra-assay precision was 3% (N=6). As a validation step, conducted with infants that were otherwise part of a separate study (Pryce et al 2002), matched blood and urine basal samples were obtained from eight infants aged between 2 and 28 days, one set of samples per infant. Plasma and urinary cortisol were determined in these samples to provide an estimate of the extent to which urinary cortisol values predict plasma cortisol values in this primate.

The urinary concentrations of epinephrine and norepinephrine were measured using a commercially available RIA kit for human plasma and urinary epinephrine and norepinephrine (BI-CAT-RIA, DLD Diagnostika GMBH, Hamburg, Germany; (Manz et al 1990), validated in-house for marmoset urine. Prior to RIA, the epinephrine and norepinephrine in 10µL urine sample were extracted using a cis-diol-specific affinity gel, and aceylated to N-acylepinephrine and N-acylnorepinephrine, respectively. Thereafter they were converted enzymatically as part of the kit procedure to N-acylmetaepinephrine and N-acylmetanorepinephrine. The RIA used rabbit antisera specific either to N-acylmetaepinephrine or N-acylmetanorepinephrine; N-metaepinephrine-[$^{125}$I]Bolton-hunter-reagent-conjugate or N-metanorepinephrine-[$^{125}$I]Bolton-hunter-reagent-conjugate, as tracers; and goat anti-rabbit-γ-globulin as secondary antibody. Intra-assay precision was 6% (N=4) for epinephrine and 5% (N=4) for norepinephrine.

Urinary concentrations of cortisol, epinephrine, and norepinephrine were expressed relative to urinary creatinine content to control for variation in urinary volume/concentration. Creatinine (Cr) was measured using a commercially available kit (Beckman Creatinine Kit 555A, Sigma-Aldrich Chemie, Schnelldorf, Germany) adapted in-house for use on microtiter plates. Intra-assay precision was 1%.

**Observation of Home Cage Behavior**

Behavior of subjects relative to their social and physical environments was measured in the home cage during postnatal weeks (W) 1-8. The ethogram used was based on those already published for the marmoset (Pryce et al 1995; Stevenson and Poole 1976). Observations, lasting 60 min each, were performed 3 times per week (3 hr / week / group) using a one-way viewing screen, with coded data entered into a handheld computer (Workabout, PSION, London, UK), running Observer mobile support package software (Noldus Information Technology, Wageningen, NL). The twin infants were the focal subjects, with behaviors recorded using all-occurrence sampling or 30-sec instantaneous sampling, and expressed as behavioral frequency/hr or behavioral duration in percent time, respectively. Relationships and behavior elements of interest, with the main examples of the latter given in parentheses, were *parent-infant* (retrieve, carry, lick ano-genital region, agonistic behavior); *infant-parent* (move around on parent, infant in suckling position, initiate carrying, terminate carrying (forced or

spontaneously), proximity); *infant-infant* (social play); and *infant alone* (distress vocalization, tail-piloerection, scratch, eat, explore, solitary play). Behavioral observations were scheduled so that they were evenly distributed between a.m. and p.m., and, for W 1-4, pre and post the ED session on that day. In the case of observations conducted post-ED, there was a minimum interval of 1.5 hr between the end of ED and the onset of the observation.

**Data Analysis**

The effect of ED on infant body weight was analyzed using 3-way ANOVA with within-pair, between-subject factors of infant treatment (ED vs. CON siblings) and sex (female vs. male), and a within-subject factor of age (PND 2 vs. PND 28). Effect of ED on mother and father body weights was analyzed using 2-way ANOVA with within-subject factors of treatment (ED vs.CON) and infant age (PND 2 vs. PND 28). For urinary cortisol and catecholamines values, studied in ED subjects only, male and female twins were treated as independent subjects. For each hormone, the mean value of all samples obtained between PNDs 2-28 was calculated for each subject for each of the following: a.m. pre-ED, p.m. pre-ED, a.m. post-ED, p.m. post-ED. These values were $\log_{10}$ transformed to normalize distribution and reduce heterogeneity of variance. Circadian changes in pre-ED (i.e. basal) urinary concentrations were analysed by means of paired t-tests on a.m. versus p.m. values. Basal a.m. and p.m. cortisol values were significantly different so that the effect of ED (i.e. pre-ED vs. post-ED) on cortisol values was analysed by paired t-tests for a.m. and p.m. samples separately. Pre-ED catecholamine values did not differ significantly between a.m. and p.m. so that an overall mean value was calculated per subject for analysis of pre-ED vs. post-ED values using paired t-tests. Basal a.m. urinary cortisol titers at PND 28 were compared in ED and CON siblings using a paired t-test.

In the case of behavioral analysis, individual infant subjects could often not be identified with high reliability during observations; therefore, mean values were calculated for each twin pair and used for statistical analyses. Behavioral scores were analyzed using 2-way ANOVA with a within-pair, between-subject factor of treatment (ED vs. CON siblings) and within-subject factor of age (W1-4 vs. W5-8). Behavior elements that were observed during a single age period only (for all elements where this was the case they were observed during W 5-8 only), were analyzed by means of paired t-tests. Paired t-tests were also used for *a posteriori* testing of significant two-way interactions. The significance level was set at $p<0.05$, with $p<0.10>0.05$ considered as a trend to a significant effect.

**Results**

Infants vocalized considerably during ED, primarily with the distress vocalization that they emit in the home cage in response to parental aggression and also with the long call that is emitted by all age groups in this species and functions to advertise location. We had the

clear impression that the amount of time that infants spent vocalizing during ED decreased with age and repeated ED exposure. ED infant vocalizations were not audible to marmosets in the main colony rooms including parents, and vice versa. Older infants also urinated and defecated during ED, in particular during ED sessions of longer duration (90-120 min). Piloerection of the body hair was also a frequent response to ED. All of these observations were qualitative.

**Effects of Early Deprivation on Body Weight**

Male and female infants did not differ significantly in body weight at either PND 2 or PND 28 as revealed by the absence of both a significant main effect of sex and interaction of age and sex ($p \geq 0.18$). Consequently, male-female twin weights were averaged and these data were analyzed by 2-way ANOVA. As given in Table 1, ED resulted in significantly lower body weight at PND 28 relative to CON siblings. This was supported by the significant interaction between age and treatment ($F(1,6)=20.6$, $p<0.004$) and the main effect of treatment ($F(1,6)=21.9$, $p<0.004$). At PND2 there was no significant difference in body weight between ED and CON infants ($t=1.01$, $df=6$, $p>0.35$), whereas at PND28, the last day of ED, ED infants were significantly smaller than CON infants ($t=5.00$, $df=6$, $p<0.003$). The reduction in body weight of PND-28 ED infants relative to their control siblings was 12% on average. The average ED duration per day was 1.3 hr and the average percent time spent by CON infants in the suckling position was 13% (see below; Table 2), so that ED infants were deprived of about 0.2 hr of suckling per day, or about 1% of the day.

Parental body weights were not affected significantly by the ED manipulation, as revealed by the absence of a main effect of treatment and age x treatment interaction, in the case of mothers ($p>0.18$) and fathers ($p>0.12$). At PND 2, maternal body weight was 440$\pm$10g for CON and 420$\pm$16g for ED, and at PND 28, 442$\pm$11g for CON and 437$\pm$16g for ED. At PND 2, paternal body weight was 415$\pm$20g for CON and 400$\pm$19g for ED, and at PND 28, 396$\pm$25g for CON and 400$\pm$20g for ED.

(Please insert Table 1 about here)

**Urinary cortisol and catecholamine responses to early deprivation**

Figure 1A presents the findings for urinary cortisol measures across PNDs 2-28 in ED infants. Basal a.m. cortisol/Cr titers were significantly higher than basal p.m. cortisol/Cr titers, as indicated by a paired t-test ($t=3.79$, $df=9$, $p<0.005$). When ED was performed p.m. then infants demonstrated a significant post-ED vs. pre-ED increase in their urinary cortisol/Cr titers ($t=-2.81$, $df=9$, $p<0.03$). This was not the case when ED was performed a.m., that is, when basal titers were high ($t=0.19$, $df=9$, $p>0.84$). Fig. 1B depicts the relationship between a.m. plasma and urinary cortisol titers in normally-reared marmoset infants: urinary cortisol/Cr values correlated significantly with the cortisol titers in matched plasma samples ($r=0.90$;

F(1,6)=25.95, $p<0.003$). In the case of basal a.m. cortisol/Cr titers at PND 28, these were significantly reduced in ED compared with CON subjects (t=-3.81, df=6, p<0.01, Fig. 1C). For urinary basal catecholamine titers, there was no significant circadian change for either epinephrine (t=0.66, df=6, p>0.54) or norepinephrine (t=0.82, df=6, p>0.44). Accordingly, a.m. and p.m. values were collapsed for further analysis. Infants responded to ED with a significant increase in epinephrine (t=-5.86, df=9, p<0.002, Fig. 1D) and norepinephrine (t=-7.49, df=9, p<0.0001, Fig. 1E).

*(Please insert Figure 1 about here)*

**Early deprivation and behavior**

All of the behavioral elements that occurred in both W1-4 and W5-8 (e.g. carrying, infant in suckling position, distress vocalization, play) demonstrated at least a trend to a significant main effect of age, reflecting maturation and development across weeks 1-8 of life in the marmoset. As given in Table 2, ED did not affect the percent time that subjects were carried by mothers and fathers, the frequency of ano-genital licking by mothers and fathers, the percent time subjects spent in proximity of the parents, or the frequency of parent-infant agonistic behavior such as aggression. The absence of such effects was reflected by the lack of (a trend to) either a significant main effect of treatment or an age x treatment interaction. ED did yield the following behavioral effects: When being carried by the mother, ED infants aged W1-4 tended to spend more time in the suckling position than did their CON siblings; this was supported by a trend to a significant age x treatment interaction (F(1,6)=5.12, $p<0.07$) (Table 2). ED subjects spent more time emitting distress vocalizations than did their CON siblings, as revealed by the significant main effect of treatment (F(1,6)=6.14, $p<0.05$, Table 2). Distress vocalizations are typically emitted in response to the parents' agonistic attempts to terminate carrying and also when the infant is alone on the substrate. Therefore what was particularly noteworthy here was that ED infants distress-vocalized significantly more than CON infants although they were neither aggressed against more nor carried less (Table 2). At W5-8 play behavior was observed in the form of both solitary and social play. ED infants spent relatively more time in solitary play and less time in social play than their CON siblings, as revealed by the trend to a significant interaction between play type and treatment (F(1,6)=3.71, $p=0.10$, Table 2).

(Please insert Table 2 about here)

Finally, all of the subjects included in this study survived to the end of the first year of life (young adulthood). During this time the effects of repeated ED were studied in terms of endocrine and cardiovascular parameters and spontaneous and conditioned behavior. At age 1 year, subjects were euthanized for the study of the neurobiological effects of repeated ED in this primate. These effects will be reported on elsewhere (e.g. Dettling et al, in press).

**Discussion**

The present study has demonstrated that repeated early deprivation, a postnatal environmental manipulation developed in the rat, can be applied to at least one non-human primate species, the common marmoset monkey. Primate studies based on repeated ED will represent an important complement to the rat ED studies, for investigation into the short-term and long-term effects of continuous disruption of the early infant-parent relationship on neurobehavioral maturation and development. Given the epidemiological evidence that early life stress, including neglect and abuse, represents an important short-term risk factor for infant physical and mental well-being, and an important long-term risk factor for the development of psychiatric disease, most notably mood disorders, then the availability of both rodent *and* primate repeated ED models represents an important advance in pre-clinical research in this area.

Using a counterbalanced cross-over design in which the same breeding pairs each contributed an ED and a control set of twin offspring enabled us to control for some of the genetic and environmental variation between groups - most notably the inter-group differences in parental care which are marked in the common marmoset (Pryce et al 1995) – and thereby increased the statistical power of the research design. Of course, this necessitated that the breeding pairs used remained in a stable physical and reproductive condition across the entire two-year period for which each was studied. Before discussing the effects of ED on infants it is important to note therefore that, based on the measure of body weight, mothers and fathers appeared to maintain a stable physical condition throughout the study period. Furthermore, these marmoset parents demonstrated behavior that was commensurate with survival of their ED infants; they demonstrated immediate retrieval of the infants at reunion following ED, and it was this retrieval that enabled the infant to cling onto the parent and initiate a carrying bout.

All of the marmoset infants survived the repeated ED procedure used in this study. Our procedure did include a process of selection in that, in the case of triplets, the smallest/weakest triplet was excluded from the study. Of course the demonstration that infants and parents can tolerate the ED procedure is a basic but essential validation of this original approach to inducing early-life stress in a non-human primate. Repeated ED had resulted in a robust reduction in body weight by PND 28, the final day of the early-life manipulation procedure. This is in contrast to a recent study in the rat in which we found that repeated ED for 4 hr at PNDs 1-21 did not affect pup body weight at PND 21 relative to controls (Pryce et al 2001b). Smaller-than-expected body size is a well-recognised phenomenon in pediatric medicine, and early-life stress might be important in its etiology (Boddy et al 2000). There are several potential explanations for this retardation in growth related to repeated ED in the marmoset,

including: Loss of homeostasis in the form of ED-induced stimulation of catabolic processes in infants; the reduction in the time available for suckling; and the reduction in milk quantity/quality due to stress experienced by ED mothers. With regard to reduced suckling time mediating the effect, as detailed in the Results, the reduction in body weight was markedly greater than can be accounted for by deprivation of suckling time per se. We were careful to distribute behavior observations throughout the day and to avoid observation for at least 1.5 hr following infant-parent reunion. Despite this, ED infants tended to be in the suckling position more than were CON infants, indicating that ED infants were able to compensate - in some cases possibly even over-compensate - for the deprivation of suckling opportunity. In the rat we observed that 4-hr ED pup-dam reunion is always followed by a bout of maternal behavior but then a rapid return to control levels of maternal care (Pryce et al 2001b). Of course, if differences in milk quality were responsible for the retarded growth in ED marmoset infants, then time spent in the suckling position would provide only a weak marker of nutritional intake, and analysis of the effects of ED on milk quantity/quality will be an important validation step. However, if stability of maternal body weight serves as a good predictor of stability of lactation, then we would not anticipate finding a marked impact of ED at the level of nutrition.

In contrast to the evidence against compromised milk intake being the mediator of the reduced weight gain in ED infants, the analysis of urinary levels of stress hormones provides supportive evidence for the hypothesis that stress-induced catabolic processes made an important contribution to this ED effect. We have recently described that infancy in the common marmoset is characterized by basal hyperactivity of the pituitary-adrenal system, with plasma basal levels of both ACTH and cortisol being markedly higher in weeks 1-4 of life than at older life stages including adulthood (Pryce et al 2002). Therefore, in terms of absolute basal values at least, this nonhuman primate represents the opposite extreme to the rat with its infantile HPA SHRP (Levine 2000). Despite the SHRP, the rat pup HPA system is sensitive to repeated ED: using a 1-hr ED at PNDs 2-9 resulted in no effect on basal plasma corticosterone levels but a marked potentiation of the plasma corticosterone response to the ED on PND 9 (McCormick et al 1998). In the present study, basal urinary cortisol levels reflected the high plasma cortisol levels of young infancy in this primate species. A cortisol response to ED was obtained in the afternoon and not in the morning; there was a circadian change in urinary cortisol levels and the lack of a morning urinary cortisol stress response may be attributable to a remarkable ceiling effect of morning adrenocortical function in young marmoset infants. By PND 28 basal a.m. urinary cortisol levels were reduced in ED infants compared with their CON siblings. This provides a very interesting and important parallel to the human evidence that neglected children with psychopathology exhibit low peak basal cortisol levels and that children with PTSD symptoms exhibit enhanced dexamethasone suppression of cortisol (Heim and Nemeroff 2001). Repeated maternal separation performed with older infants in the closely-

related squirrel monkey consistently induced a plasma cortisol stress response but did not alter basal cortisol levels or the magnitude of the stress response (Hennessy 1986); it did however lead to increased glucocorticoid feedback sensitivity in adulthood (Lyons et al 2000).

In the rat, ED has been well studied in terms of its acute and chronic effects on HPA activity whereas the sympathetic ANS has received relatively scant attention. This is despite the important role attributed to the ANS in influential models of the inter-relationships between maternal care, infant physiology and long-term development (e.g. Hofer 1994a). In the present study we were able to detect marked acute increases in the urinary levels of epinephrine and norepinephrine in response to ED. These increases provide robust quantitative evidence that repeated ED constitutes a repeated stress procedure in the infant marmoset monkey. Like the cortisol released from the adrenal cortex, epinephrine released from the adrenal medulla functions to promote conversion of nutrient reserves into glucose for energy synthesis. The ED-induced elevation of urinary epinephrine provides indirect evidence for activation of the sympathetic ANS, with the epinephrine conveyed in the blood stream to amplify the stress-induced neural activation of the sympathetic target organs. Norepinephrine is the major neurotransmitter at sympathetic ANS synapses and urinary norepinephrine, at least in the human case, is derived primarily from this source (Maas et al 1987). Therefore, our evidence suggests that repeated ED acts to activate the sympathetic ANS of infant marmoset monkeys. In rhesus macaques, maternal privation results in chronic alteration of basal and stress-related CSF norepinephrine activity (Clarke et al 1996; Higley et al 1992; Kraemer 1992). We are currently investigating the chronic effects of repeated ED in the subjects described in this study in terms of basal urinary, plasma and CSF levels of pituitary-adrenal hormones and catecholamines.

The study of the short-term effects of the repeated ED procedure on behavior within the infant-mother and infant-father dyads is of course extremely complex: Inherent to ED are periods of deprivation of parental care, deprivation of the opportunity to provide care, as well as, following reunion, altered status of the infant stimuli that interact with parental motivational factors to elicit parental care. Despite this dynamic series of consequences of ED, parental care was apparently quite unresponsive to ED. The amount of time spent carrying infants and the distribution of carrying between mothers and fathers was unaffected by ED compared with CON. Ano-genital licking of and aggression towards infants were also unaffected by ED. In rats, EH increases licking and arched-back nursing throughout the day relative to NH and AFR, whereas 4-hr ED in rats does not have this effect (Liu et al 1997; Pryce et al 2001b). Although we did not include a completely undisturbed parent-infant treatment in this study, the levels of all these parental behaviors in both CON and ED groups were very similar to those reported in previous studies conducted with undisturbed family groups (e.g. Ingram 1977; Pryce 1993).

ED infants did tend to spend more time in the suckling position on the mother and this might have been motivated by either increased hunger or, indeed, increased comfort seeking relative to CON infants. Furthermore, ED infants aged 1-4 and 5-8 weeks spent more time emitting distress vocalization even though they received similar levels of parental care to CON infants. This might indicate that the same amount of parental care was less effective in ameliorating distress in ED infants. Finally, in terms of behavior, the data suggest that ED infants spent relatively less of their total play time in social play and relatively more in solitary play, than CON. We are continuing to observe social behavior as the subjects mature, when we would normally expect their levels of social twin play behavior to increase markedly. A relative reduction in social play as a consequence of ED would provide a very interesting measure of a long-term effect of ED on emotional function in social situations.

It is important to comment on and compare the ED control group in rodent and primate studies. In the present study the control procedure comprised brief restraint of the carrying parent and infant and there can be little doubt that this is the most appropriate control for ED in the marmoset. In the case of rat postnatal manipulations, however, the form that the control group should take is a matter of considerable debate and controversy (Lehmann and Feldon 2000). NH is widely accepted as the control group for EH, although it does not allow for the separation of the effects of handling per se versus the effects of the brief separation. NH has been used as the control condition for 3-hr repeated MS (e.g. Plotsky and Meaney 1993) and has revealed that MS is without effect on a large number of behavioural and neurobiological parameters (e.g Caldji et al 2000; Plotsky and Meaney 1993). Perhaps related to this, some recent studies have abandoned the use of NH and have moved to AFR as the control condition for MS (Huot et al 2001). Our recent rat studies have included NH and AFR as control groups and found important differences as well marked similarities between the two, depending on the parameter under investigation (e.g. Lehmann et al 2001; Pryce et al 2001a; Pryce et al 2001b). There is no *a priori* reason why the brief restraint and handling of parent and infant, as performed here for the marmoset, should not also be used as a control condition for ED and other manipulations in the rat. Furthermore, there is no *a priori* reason why the crossover design used here should not also be used in rat studies.

In summary, with the present study we have demonstrated that the repeated ED can be performed successfully in a species of nonhuman primate, the common marmoset, thereby allowing for the study of the neurobehavioral effects of this early-life stressor in both rodents and primates. That repeated ED does indeed constitute an early-life stressor is demonstrated by its acute effects on growth and catabolic stress hormone levels, and by it leading to reduced basal urinary cortisol levels by the end of the ED protocol at PND 28. Behavioral effects of ED were observed, including at weeks 5-8 of life, that is, after the stressor had been terminated. The chronic physiological, behavioral and neurobiological effects of ED in the marmoset will

now be studied, with the ultimate aim of establishing the suitability of this manipulation as a primate model of human early-life stress and developmental psychopathology.

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**Intermittent deprivation of parenting disrupts development of homeostatic and reward systems in primate offspring**

**Parental care is essential for the survival and maturation of all mammalian infants; less obvious but extremely important is that care quality and quantity exert a marked impact on offspring development. In humans the clinical and epidemiological evidence indicates that receiving inadequate parental care markedly increases the likelihood of developmental and adulthood affective psychopathology [1,2]. Rat studies have described how intermittent separation of offspring from the mother for some minutes per day for the first few days of life ("early handling") actually reduces offspring emotionality in adulthood [3,4]. Rat early handling also increases specific maternal behaviours, and receiving spontaneously high levels of these same behaviours in an undisturbed environment is associated with reduced emotionality in adulthood [4,5]. Here we describe, for the first time in a primate, how intermittent deprivation of parental care in very young infants, a procedure that beyond the deprivation per se is without effect on parental care [6], leads to abnormal physiological and behavioural functioning of stress and reward systems across the first year of primate life.**

The common marmoset is a small-bodied primate characterized by biparental care and twin births. Captive breeding pairs of such monkeys contributed both early deprivation (ED) and control (CON) twin offspring to this longitudinal study. The ED procedure was performed daily across days 2-28 of life, with marmoset infants being exposed to intermittent "neglect" in terms of interruption of the continuous parent-infant thermal, kinaesthetic, visual, olfactory, auditory and tactile stimulation that is otherwise typical for this primate and that was experienced by the CON infants [6]. ED was unpredictable in terms of time of day and

duration and it elicited acute endocrine stress responses from the hypothalamic-pituitary-adrenal (HPA) system and sympathetic autonomic nervous system (ANS) across days 2-28 [6]. In the home cage, parental care was unaffected by ED such that neither mothers nor fathers compensated for ED by increasing care relative to that demonstrated with CON infants. ED infants were of lower body weight than CON infants and they emitted more distress vocalisations in the presence of their parents than did CON infants [6]. Basal levels of the HPA hormone cortisol were reduced in ED infants (months 1-2) [6] and juveniles (month 4) [7] relative to their CON siblings, a physiological state analogous to that exhibited by children exposed to early life stress and suffering from posttraumatic stress disorder (PTSD) [8].

Noradrenaline (NE) is both a central and sympathetic ANS neurotransmitter and an ANS hormone. Early-deprived marmosets exhibited abnormally high basal urinary levels of noradrenaline (NE) across infancy to adolescence relative to their CON siblings, as reflected by the significant main effect of treatment ($F(1,8)=19.20$, $p<0.002$) (Figure 1A). The average developmental profile and indeed also the absolute urinary NE levels of CON marmosets are similar to the human equivalents [9,10]. However the most striking analogy with the human situation is the marmoset's long-term response to ED in terms of basal NE hyperactivity: children suffering from PTSD and co-morbid depressive symptoms secondary to past maltreatment experiences demonstrate basal NE hyperactivity [11]. In human adulthood, PTSD and depression both present with elevated basal NE levels in urine, cerebrospinal fluid and blood [9]. The elevated urinary NE induced in ED marmosets could reflect an increase in the set-point for ANS adrenal and synaptic basal activity [12]. Corroborative evidence for this was provided by our demonstration of chronically elevated basal blood pressure in ED marmoset juveniles (months 4-5). Basal blood pressure was measured in freely-moving monkeys whilst in their family group and home cage using radio-telemetry [13]. ED marmosets demonstrated a significant elevation in systolic blood pressure relative to their CON siblings throughout the day and night (treatment main effect: $F(1,5)=12.03$, $p<0.02$) (Table 1; Figure 1B). Average diastolic blood pressure, in particular the nocturnal diastolic, was consistently higher in ED versus CON marmosets but this did not reach statistical significance ($F(1,5)=4.22$, $p<0.09$) (Figure 1C). To summarize the observed chronic effects of ED on stress-sensitive homeostatic systems in this primate, noradrenergic basal hyperactivity and basal systolic hypertension are both consistent with an altered set-point in ANS functioning and with chronic allostatic load [12].

Such abnormal functioning of physiological stress systems is often associated with disruption of neuropsychological processes as measured using behavioural end points. This has been demonstrated in rodents [5,14], monkeys [15] and humans [8], and is testimony to the dialogue between brain and physiology including mutual chemical messengers (e.g. NE, corticosteroids) and receptors. In this context, the regulation and representation of the

emotional significance of environmental stimuli (rewards and punishers) is of marked importance [16]. We have recently described that ED juvenile marmosets exhibit greater aversion to the challenge of social isolation in an unfamiliar environment compared with their CON peers [7]. In the present study we focussed on the effects of ED on emotional processing and behaviour relative to reward, as studied in the home cage in the presence of the family group. Computerised neuropsychological testing of stimulus-reward association using a touch-sensitive computer screen allowed for the quantification of motivation to obtain innately palatable reward, visual discrimination learning and visual reversal learning [17]. To assess motivation to obtain innate reward (banana milkshake), and presumably therefore to assess the incentive or hedonic value of the latter, adolescent (9-12 month) ED and CON offspring of seven breeding pairs were tested on a progressive ratio schedule of reinforcement (PRS). To obtain successive reinforcers marmosets had to perform successively more responses to a large single stimulus on the touch screen. ED marmosets performed significantly less PRS responses than did CON (treatment main effect: $F(1,12)=8.17$, $p<0.02$) (Figure 2, inset). There was also a significant effect of twins within treatment ($F(12,12)=2.90$, $p<0.04$), indicating that the effect of ED on PRS differed between families. As given in Figure 2, a posteriori comparison of ED and CON siblings revealed that motivation to obtain reward was significantly reduced in ED relative to CON monkeys in four families and was not significantly affected in either direction in the other three. That this effect of ED was attributable to reduced interest in obtaining reward and not to reduced ability to taste the reward was supported by providing the same subjects with direct access to an excess of the banana milkshake reward: similar amounts of milkshake were consumed by ED ($13.9\pm0.9$ml) and CON ($13.6\pm4.5$ml) adolescents ($F(1,6)=0.57$, $p>0.48$). Thus, ED led to adolescent monkeys being anhedonic in some family groups, which is of particular interest given that anhedonia is a major and diagnostic symptom of depression.

In addition to the chronic disruptive effect of early life stress on emotional processing of the significance of innate rewards, we considered it important to assess its impact on the cognitive processes that underlie the formation of associations between environmental stimuli and innate reinforcers, particularly in the changing environmental circumstances with which nonhuman and human primates are often confronted [18]. Using the most motivated subjects from the previous study (ED and CON siblings from five families), adolescent monkeys were trained to make visual discriminations between two blue visual stimuli that differed in shape; responding to one shape resulted in milkshake reward and to the other resulted in punishment timeout. Simple discrimination learning was defined as eight consecutive correct responses, and subjects were trained to this criterion on three pairs of novel stimuli. Testing began with subjects being presented with a fourth novel stimulus pair and simple discrimination learning was measured as the number of errors to criterion (SD) (Figure 3). Then the stimulus-reward

association was reversed such that monkeys were now reinforced for responding to the previously negative stimulus (SR1); the number of errors to criterion was again the measure used and now included a component of perseveration, that is, responses made to the stimulus rewarded at SD. SR1 was followed by re-reversal to the original association (SR2). Both ED and CON marmosets made more errors when performing SR than SD, indicative of perseveration; this was confirmed by the significant main effect of discrimination type ($F(1,8)=26.31$, $p<0.001$) and by a posteriori paired t tests that revealed significantly more errors to reach criterion at SR1 and SR2 than at SD ($t\geq5.3$, $df=17$, $p\leq0.0001$). There was a significant main effect of treatment ($F(1,8)=9.33$, $p<0.02$) and the absence of a significant effect of twins within treatment indicated that this was consistent across family groups ($F(8,8)=1.98$, $p>0.17$). Stage-by-stage analysis revealed that ED marmosets did not make significantly more errors than CON when performing SD ($p>0.34$; Figure 3), indicating that their ability to form novel stimulus-reward associations was unaffected by their early life experience. However, ED marmosets made significantly more errors than CON at SR1 ($p<0.05$) and SR2 ($p<0.02$), indicating that this abnormal postnatal experience chronically impaired their ability to either acquire or at least utilise information on the altered emotional significance of conditioned stimuli [18].

The present nonhuman primate model of parent-infant neglect provides strong evidence that early life stress leads to chronic alteration of the functioning of vital homeostatic and reward systems. As such it represents a major advance in our potential to understand the importance of the early environment and the epigenic and epigenetic mechanisms via which it impacts on the phenotype in both the short- and long-term. Human infants that experience neglect or abuse within the primary caregiver relationship are at high long-term risk for PTSD and major depression disorder (MDD), which further emphasises the importance of such models [2]. The next step will be to investigate the neurobiological correlates of the physiological and behavioural effects described. In this respect it is noteworthy that surgical lesioning of the marmoset orbitofrontal cortex leads to the same perseveration deficit that we induced with ED ("environmental lesion") [19], and that human MDD is associated with orbitofrontal hypoactivity [20].

**Methods**

**Study design and early deprivation**

Ten established breeding pairs of common marmosets living in large cages that could be entered by personnel each contributed two sets of twin offspring to this study, with an inter-birth interval of 12 months. In the case of five pairs, the first set of twins was allocated to the ED treatment and the second set to CON, and in the other five pairs the reverse was the case. All subjects developed in a family group that included the twin and the biological parents, only. ED was conducted for 30, 60, 90 or 120 min per day and began at different

times of day, as early as 0800 and as late as 1700; with a total weekly duration of 9 hours. ED was performed with one infant at a time so that parents of ED infants always had one infant to care for. CON comprised brief restraint of the parent and the infant it was carrying followed by release [6]. ED and CON subjects from three families were surgically fitted with pressure-sensitive transmitters that allowed for monitoring of cardiophysiology in the home cage (fitted with receivers). ED and CON marmosets from the other seven families were the subjects of the conditioned behaviour study.

**Statistical analyses**

A first stage analysis of variance (ANOVA) was performed with between-subject factors of treatment and sex. There were no significant ($p<0.05$) main or interaction effects of sex ($p\geq0.25$) and this factor was excluded from subsequent analyses. ANOVAs were performed with the between-subject factor of treatment and the factor of twins within treatment, the latter allowing for identification of variables where the impact of ED differed significantly between families [21].

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