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Early experience of a novel-environment in isolation primes a fearful phenotype characterized by persistent amygdala activation

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Summary Prolonged maternal separation (MS) activates the neonate's hypothalamus—pituitary—adrenal axis causing elevated basal and stress-induced corticosterone levels that may initiate amygdala-dependent fear learning. Here we test the hypothesis that the adult fearful phenotype is programmed by the pup's stressful experience during prolonged MS rather than by prolonged maternal absence per se. For this purpose, Wistar rat pups were exposed, on postnatal-day (*pnd*) 3, to: (i) repeated-MS in home-environment (HOME-SEP), 8h-MS daily for three days with the pups remaining together in the home-cage; (ii) repeated-MS in a novel-environment (NOVEL-SEP), with the same separation procedure, but now the pups were individually housed in a novel-environment during the 8 h dam's absence; (iii) repeated handling, which consisted of daily brief (15 min instead of 8 h) MS in the home-altogether or in a novel-environment individually (HOME-HAN and NOVEL-HAN, respectively); (iv) no-separation/no-handling (NON-SEP/NON-HAN) control condition, in which pups were left undisturbed in their home-cage. Compared to

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HOME-SEP rats, the NOVEL-SEP rats showed one day after the last MS enhanced stress-induced amygdala c-Fos expression and ACTH-release, despite of reduced adrenal corticosterone secretion. The higher amygdala c-Fos expression, ACTH-release and reduced corticosterone output observed postnatally, persisted into adulthood of the NOVEL-SEP animals. Behaviorally, NOVEL-SEP juvenile rats displayed deficits in social play, had intact spatial memory in the peri-pubertal period and showed more contextual fear memory compared to HOME-SEP in adulthood. Finally, NOVEL-HAN, compared to HOME-HAN, displayed increased stress-induced corticosterone output, no deficits in social play and reduced contextual fear. In conclusion, programming of an adult fearful phenotype linked to amygdala priming develops if pups are repeatedly isolated from peers in a novel-environment, while away from the dam for a prolonged period of time.

1. Introduction

Rodents deprived as pups from their dam have been widely used as a model for early-life adversity (Levine, 2005; Oitzl et al., 2010). During early-life, they display a strongly reduced ability to respond to mild stressors with elevation of corticosterone (CORT), what is known as stress-hyporesponsive period (SHRP; postnatal-days (*pnd*): 3—14 for rats, 1—12 for mice) (Sapolsky and Meaney, 1986; Rosenfeld et al., 1992a; Schmidt et al., 2003). At that time, a single period of prolonged maternal absence (8 h or longer) increases the neonate's basal adrenal CORT secretion, and enhances its neuroendocrine stress- and behavioral fear-responsiveness (Levine et al., 1991; Schmidt et al., 2004; Fenoglio et al., 2006; Moriceau et al., 2006). Remarkably, already from the second (Enthoven et al., 2008) or third episode of prolonged MS (Rosenfeld et al., 1992b; Enthoven et al., 2008; Daskalakis et al., 2011), basal CORT levels are no longer elevated, suggesting that the pups have learned to predict the return of the dam. The rodent's adaptation to repeated and prolonged maternal absence does not depend on metabolic factors and occurs irrespective of genotype (strain or species) or the extent of post-reunion maternal care (Rosenfeld et al., 1992b; Enthoven et al., 2008; Daskalakis et al., 2011). This rapid adaptation to repeated-MS occurs even if the pups are individually placed in a novel-environment (Daskalakis et al., 2011). Interestingly, human infants experiencing the transition to the novel-unfamiliar setting of the day-care show a habituation of their cortisol response to MS after approximately 9 days (Ahnert et al., 2004).

The separation context has profound consequences for the CORT stress-response (Enthoven et al., 2008; Daskalakis et al., 2011). If the dam is removed and the pups either remain in the home-cage or get transferred as a group to a novel-environment (Rosenfeld et al., 1992b; Daskalakis et al., 2011), their adrenals become hyper-responsive to a subsequent stressor, in parallel with enhanced expression of adrenal melanocortin receptor type 2 (MCR-2) for adrenocorticotropic hormone (ACTH) (Daskalakis et al., 2011). However, if the pups are placed in a novel-environment individually, they display lower CORT-secretion in response to the novelty stressor (Daskalakis et al., 2011). This indicates that the combination of novel MS-context and peer isolation alters the pattern of stress-responsiveness. Similarly, in monkeys, enhanced CORT response to novelty was observed in maternally separated infants that were group reared, but not in the ones reared in isolation (Wiener et al., 1987). These findings are reminiscent of human twin studies, where variance in childhood cortisol-reactivity in conditions of increased early-life adversity was accounted for by environmental, rather than genetic factors (Ouellet-Morin et al., 2008).

Short periods of maternal absence ($\leq$3 h) are insufficient to increase basal CORT-secretion in the pups (Levine et al., 1991; Schmidt et al., 2004), but are capable, when repeated, to induce sensitization of the CORT stress-response (D'Amato et al., 1992; McCormick et al., 1998; Huot et al., 2002). Adult rats having experienced as pups short-MS periods of 3—15 min in a novel-environment for the first two or three weeks of life — the so-called handling procedure — display an attenuated stress-induced HPA-axis response, improved cognitive performance and reduced emotional arousal (Levine, 1957; Meaney et al., 1988). The opposite outcome is observed, however, if the handling procedure is applied in the absence of novelty exposure by maintaining the pups altogether in their home-cage (Tang, 2001; Tang et al., 2003, 2006; Daskalakis et al., 2009).

The mechanism underlying the immediate and long-term outcome of MS procedures is not completely understood. In this context, it is notable that during the SHRP elevated CORT leads to activation of the pup's amygdala fear pathway in a context-dependent manner. This action exerted by CORT on the amygdala allows the development of fear learning, with persistent consequences for adult contextual fear-responses (Moriceau et al., 2006, 2009; Sevelinges et al., 2007, 2011). Based on this, in the current study we hypothesized that the combination of prolonged maternal absence and pup's separation context dictates the programming of fear-related phenotypes in adulthood. In particular, we reasoned that the rise in endogenous CORT, induced by the first episode of maternal absence, is a critical factor in priming amygdala fear circuit for the effect of an early adverse experience, precipitating a persistent fearful phenotype.

To demonstrate this we have performed two experiments. In the first experiment we asked whether in early postnatal life the MS together with the MS context can affect the amygdala and neuroendocrine components of the stress-response circuitry. One group of pups was exposed once to 8h-MS on *pnd* 3 and another group twice to repeated 8h-MS on *pnd* 3 and 4. During MS, pups remained either in the home-context (home-separation), or they were removed from the home-cage and housed in isolation from peers (novel-separation) in a novel-context. We chose these two distinct MS procedures as they are predicted to have the most robust