Daily maternal separations during stress hyporesponsive period decrease the thresholds of panic-like behaviors to electrical stimulation of the dorsal periaqueductal gray of the adult rat

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ABSTRACT

The present study examined whether early life maternal separation (MS), a model of childhood separation anxiety, predisposes to panic at adulthood. For this purpose, male pups were submitted to 3-h daily maternal separations along postnatal (PN) days of either the 'stress hyporesponsive period' (SHRP) from PN4 to PN14 (MS11) or throughout lactation from PN2 to PN21 (MS20). Pups were further reunited to conscious (CM) or anesthetized (AM) mothers to assess the effect of mother-pup interaction upon reunion. Controls were subjected to brief handling (15 s) once a day throughout lactation (BH20). As adults (PN60), rats were tested for the thresholds to evoke panic-like behaviors upon electrical stimulation of dorsal periaqueductal gray matter and exposed to an elevated plus-maze, an open-field, a forced swim and a sucrose preference test. A factor analysis was also performed to gain insight into the meaning of behavioral tests. MS11-CM rather than MS20-CM rats showed enhanced panic responses and reductions in both swimming and sucrose preference. Panic facilitations were less intense in mother-neglected rats. Although MS did not affect anxiety, MS11-AM showed robust reductions of defecation in an open-field. Factor analysis singled out anxiety, hedonia, exploration, coping and gut activity. Although sucrose preference and coping loaded on separate factors, appetite (adult weight) correlated with active coping in both forced swim and open-field (central area exploration). Concluding, whereas 3h-daily maternal separations during SHRP increased rat's susceptibility to experimental panic attacks, separations throughout lactation had no effects on panic and enhanced active coping.

1. Introduction

Existing evidence suggests that panic disorder (PD) is either "respiratory" or "non-respiratory" depending on the prominence of respiratory symptoms [1,2]. In particular, whereas the respiratory panic is characterized by dyspnea, hyperventilation and feelings of choking and "hunger for air", non-respiratory panics are marked by fear-like symptoms of palpitations, tremors and sweating. Respiratory panics are also precipitated by administration of the respiratory metabolites carbon dioxide (CO_2) and sodium lactate [3]. Accordingly, the major panic theories suggest that clinical panics are either a suffocation false alarm [3,4] or a false alarm to a proximal threat [5].

Although the neurobiology of PD remains largely unsolved, clinical studies showed that fear-unresponsive Urbach-Wiethe disease patients with bilateral extensive calcifications of the amygdala develop panic attacks either spontaneously [6] or experimentally upon the inhalation of a vital capacity of 35% CO_2 [7]. Accordingly, Feinstein et al. [7] suggested that panics are mediated "at the brainstem" in spite of the

established role of the amygdala and ventral hippocampus in fear and anxiety of both animals and humans. As it happens, plenty of evidence suggests that the periaqueductal gray matter (PAG) is crucial in panic attacks [5,8–12]. In particular, electrical stimulations of the dorsal half of the PAG (DPAG) of humans produce panic-like symptoms, including intense anxiety, urge to escape, dyspnea, palpitations, chest pain and sensations of choking and "hunger for air" [13–16]. The DPAG was also markedly activated in volunteers experiencing either breathlessness/air hunger to 8% CO_2 [17] or fear to an imminent attack of a virtual predator that was otherwise able to inflict real shocks to the subject's finger [18].

In rats, electrical, chemical and optogenetic stimulations of the DPAG produce freezing and flight behaviors along with marked cardiorespiratory responses that are reminiscent of panic [19–23]. Most importantly, DPAG-evoked defensive behaviors were markedly attenuated by both tricyclic antidepressants (TCA) and serotonin-selective reuptake inhibitors (SSRI) given in doses and regimens alike to those of the therapy of PD [8]. Remarkably, as well, de Souza Armini et al. [24] showed that DPAG-evoked panics are not accompanied by activations of hypothalamus-pituitary-adrenal axis (HPA), as it actually occurs in clinical panic [25,26]. The latter data make unlikely cognitive theories that equate panic to anxiety [27]. Finally, recent studies showed that the PAG harbors a hypoxia-sensitive alarm system which is both sensitized by hypercapnia and inhibited by clinically-effective treatments with the panicolytics fluoxetine, clonazepam and alprazolam [28–32]. Overall, these studies support the PAG mediation of both respiratory and non-respiratory panic attacks [9,11,12].

In turn, the childhood separation anxiety (CSA) is a condition in which children show excessive, recurrent distress in anticipation of, or during, or immediately following, separation from a major attachment figure [33]. Most pertinent to the present study, clinical and epidemiological evidence suggests that CSA not only predisposes to panic [34–39] but also shares a common genetic diathesis, as indicated by twin-based clinical studies [40,41]. Although the latter studies did not find any correlation between childhood environment adversities and adult-onset panic, Spatola et al. [38] reported that the sensitivity to 35% CO_2 is only increased by stressful experiences occurring before 19 years of age. Similarly, preclinical studies showed that respiratory responses to hypercapnia are facilitated in both rats and mice exposed as pups to maternal separation [42–46] or unstable maternal environment [47], respectively. There is also evidence that early-life stress predisposes to both anxiety and depression [48]. As a matter of fact, PD is very often comorbid with the latter conditions [49,50].

Although the PAG appears to mediate both respiratory and non-respiratory panics, the dorsolateral PAG (DLPAG) has been specifically related to panic-like defensive reactions to predators [9]. Predation is in turn a major threat to an isolated pup. Therefore, a previous study of our group examined whether neonatal social isolations from both mother and siblings facilitates DLPAG-evoked 'panic attacks' at adulthood [51]. As hypothesized, 3-h daily neonatal social isolations throughout lactation produced robust facilitations of DLPAG-evoked panics of the adult rat. Notably, as well, separations had no effects or even reduced standard measures of anxiety and depression, respectively.

Current knowledge also suggests that the late effects of maternal separation (MS) result from a time-dependent disruption of the normal development of HPA axis. Indeed, whereas the HPA axis is quiescent in the first two weeks of life (the "stress hyporesponsive period", SHRP), it becomes fully responsive following a 24-h maternal deprivation at the 3rd or 10th postnatal day (PN) [52–57]. Daskalakis et al. [58] showed in addition that HPA axis responses of pups undergo rapid habituation to daily maternal separations if the pups stayed in the home cage. Therefore, it remained unclear whether the DPAG-evoked panics could be further facilitated if separations were carried out during only the SHRP. The influence of mother-pup interaction at the moment of reunion also can have an effect [59,60]. Accordingly, here we examined the effects of separation period (PN4-PN14 vs PN2-PN21) and mother-

pup interaction during reunion (anesthetized vs non-anesthetized mothers) on DPAG-evoked panic attacks and other behaviors of the adult rat (open-field, elevated plus-maze, forced swim, sucrose preference and eating/appetite).

2. Methods

2.1. Animals

Male albino Wistar rats ($n = 104$) were bred in a stress-controlled animal facility. The facility was a restricted room with periodic renewal of the air and controlled conditions of sound (46 dB white noise), temperature (24–25 °C) and light (12-h light/dark cycle, lights on at 6:00 am). About 10 to 15 days after crossings, the pregnant females were housed individually in polypropylene nest cages (30 cm × 20 cm × 12.5 cm) which were cleaned once a week and had water and food *ad libitum* and floor covered with wood shavings. Female pups were sacrificed in the 2nd postnatal day (the birthday was designated PN0). Thereafter, male pups were either subjected to 3-h daily separations or kept undisturbed until weaning (PN21). This study was approved by the local Committee on the Ethical Use of Animals (CEUA-UFES 032/2013) for scientific research and complied with EU Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_ en.htm).

2.2. Maternal separation

Pups were subjected to 3-h daily MS (1:00–4:00 p.m.) by placing the whole offspring in a 'separation cage' (30 cm × 20 cm × 12.5 cm) with clean wood shavings. Of 104 pups employed, 42 were subjected to MS between PN2 and PN21 (MS20), 42 were subjected to MS between PN4 and PN14 (MS11) and 20 were subjected to brief daily handling between PN2 and PN21 (BH20) in which the whole litter and the dam were moved to a separation box for about 15 s and returned to nest cage immediately thereafter. During separation, mother-pup communication was much reduced or even suppressed by placing the separation cages inside an incubator at room temperature. The incubator had a 10-cm^2 roof opening for air renewal and was placed in a room distinct from that of mother. At the end of separation, pups and dams were reunited in nest cage. Yet, whereas 42 pups were reunited to conscious mothers (CM), 42 did it to anesthetized mothers (AM) which were administrated with chloral hydrate (400 mg/kg I.P.) 20 min before reunion. After weaning (PN21), male rats were housed in individual polypropylene cages (30 cm × 20 cm × 12.5 cm) with wood shave bedding and food and water *ad libitum*. Cages were kept in a temperature-controlled (21–23 °C) sound-attenuated (46 dB) room under a 12-h light/dark cycle (lights on at 6:00 a.m.).

2.3. Electrode implantation

In PN52, rats (250–300 g) were anesthetized with ketamine (80 mg/kg, I.P.) and xilazine (6.5 mg/kg, I.P.), treated with ceftriaxone (30 mg/kg, I.M.) and diclofenac sodium (1 mg/kg, I.M.), fixed on a stereotaxic instrument (David Kopf, Tujunga, USA) and implanted with an electrode as previously described [24].

2.4. DPAG stimulation

The DPAG stimulation was carried out at PN59 in a sample of 89 rats from both control and separated groups (BH20, $n = 17$; MS11-AM, $n = 12$; MS20-AM, $n = 16$; MS11-CM, $n = 15$; MS20-CM, $n = 19$). Of these, 42 rats were also subjected to behavioral tests. DPAG stimulations were carried out in a Plexiglas transparent cylindrical open-field (60 cm wall height and diameter) placed in a sound-attenuated (63 dB white-noise) temperature-controlled (22-23 °C) room. Stimulation was performed through a sine-wave constant current stimulator (Flávio Del