Physiology & Behavior 146 (2015) 105–110

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Restraint stress and social defeat: What they have in common

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ARTICLE INFO

Article history:
Received 6 February 2015
Received in revised form 11 March 2015
Accepted 12 March 2015

Keywords:
Entrapment
Social defeat
Restraint
Hypothalamus
Hippocampus

ABSTRACT

Bob Blanchard was a great inspiration for our studies on the neural basis of social defense. In the present study, we compared the hypothalamic pattern of activation between social defeat and restraint stress. As important stress situations, both defeated and immobilized animals displayed a substantial increase in Fos in the parvicellular part of the paraventricular nucleus, mostly in the region that contains the CRH neurons. In addition, socially defeated animals, but not restrained animals, recruited elements of the medial hypothalamic conspecific-responsive circuit, a region also engaged in other forms of social behavior. Of particular interest, both defeated and immobilized animals presented a robust increase in Fos expression in specific regions of the lateral hypothalamic area (i.e., juxtaparaventricular and juxtadorsomedial regions) likely to convey septo-hippocampal information encoding the environmental boundary restriction observed in both forms of stress, and in the dorsomedial part of the dorsal premammillary nucleus which seems to work as a key player for the expression of, at least, part of the behavioral responses during both restraint and social defeat. These results indicate interesting commonalities between social defeat and restraint stress, suggesting, for the first time, a septo-hippocampal–hypothalamic path likely to respond to the environmental boundary restriction that may act as common stressor component for both types of stress. Moreover, the comparison of the neural circuits mediating physical restraint and social defense revealed a possible path for encoding the entrapment component during social confrontation.

1. Introduction

Newton Canteras first met the Blanchard couple in 1999 at a Society for Neuroscience Meeting, when he felt very honored to have the opportunity to discuss with Bob and Caroline his ideas on the hypothalamic circuits mediating anti-predatory fear responses. Newton and the Blanchard couple continued their interaction over subsequent years, and established a very fruitful collaboration. In 2001, Bob and Caroline invited Newton to visit their lab at the University of Hawaii, and during this visit, Bob introduced Newton to his data on social agonistic behavior. One afternoon during this visit, Bob spent quite a lot of time demonstrating what happens during social confrontation, and Newton was fascinated. Bob was inspiring enough to convince Newton to run a circuit analysis study for intruders exposed to social defeat, following the same line of the studies that Newton had previously performed in animals exposed to natural predators. After returning to Brazil, Newton started establishing the resident–intruder paradigm in his laboratory, and at that time, Simone Motta had just started her PhD in Newton's lab characterizing the neural circuits mediating social defense. We were able to show that social and anti-predatory defense are mediated by distinct hypothalamic circuits, and found that socially defeated animals recruited, in the medial zone of the hypothalamus, a circuit also engaged in other forms of social behaviors, the so-called social responsive circuit, composed by the medial preoptic nucleus, the ventrolateral part of the ventromedial nucleus and the ventral premammillary nucleus, in addition to recruiting a particular region of the dorsal premammillary nucleus (i.e., its dorsomedial part). We have further shown that dorsal premammillary nucleus (PMD) lesions block the passive components of social defense (i.e., freezing and sustained on the back position) seen during confrontation with the dominant aggressor [23].

Considering that social defeat represents an important stress, we have started exploring other forms of stress, and began to examine the pattern of hypothalamic activation in response to an acute restraint stress. The comparison between the pattern of hypothalamic activation found in physical restraint stress and social defense revealed interesting potential commonalities, which will be explored in the present publication. Briefly, we were particularly surprised to see that the dorsomedial part of the PMD showed a substantial Fos expression in both forms of stress, and that parts of the pathway relaying septo-hippocampal information to the PMD were also mobilized in both social defeat and restraint. Taking into account that the hippocampus provides a spatial map of the environment and that, in both situations, the animals are restricted to a certain location within the environment (either by the restraining apparatus or by a dominant conspecific), we hypothesized that this environmental boundary restriction would serve as a stressor component for both situations and, perhaps, processed by this common septo-hippocampal–PMD pathway, found to be recruited in both

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http://dx.doi.org/10.1016/j.physbeh.2015.03.017
0031-9384/

restraint and social stress. Curiously, during our last meeting with Bob, when he visited Brazil for the last time in 2012, we had the chance to discuss these ideas on the commonalities between social entrapment and physical constraint, and he said that the idea was very nice but hard to prove. We hope that the findings reported here start addressing these issues.

2. Materials and methods

2.1. Animals

A total of 20 adult Wistar male rats (three months of age) were used in accordance to the Ethical Guides of the Instituto de Ciências Biomédicas — Universidade de São Paulo. Animals were kept on a 12/12 h light cycle (lights on at 2 am and off at 2 pm) and had free access to food (Nuvilab®) and fresh water. Animals were isolated 24 h before the test in a cage measuring 30 × 20 × 19 cm and light, food and water were maintained under the same conditions as before. Animals were returned to this cage after the behavioral test. All tests were conducted during the first hour of the dark period under red light illumination.

2.2. Behavioral tests

2.2.1. Restraint

After 24 h isolation, animals were restrained for 30 min in an acrylic tube measuring 20 cm in length with an internal diameter of 5.3 cm (volume = 450 ml, Beiramar Ind. e Com. Ltda., Brazil) as previously described [9]. After this period, animals were returned to the same cage in which they had been previously been housed.

2.2.2. Resident–intruder paradigm

On the day of the test, subjects were placed singly in the home cage of a dominant, Long Evans rat. During the encounter, an initial investigation period was followed by the resident attacks. The dyad was separated 5 min after the first attack. Defensive behaviors were clearly observed in the intruders, i.e. upright and on-the-back postures, boxing, flight and freezing, for most of the time after being attacked [2,23]. Animals that did not display such submission were excluded from the analysis. As the restrained animals, after the social defeat, animals returned to their home cage. For a control group, rats were handled identically to the other two groups and left undisturbed in a cage.

2.3. Fos immunostaining

Ninety minutes after the behavioral test, animals were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with a solution of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4; the brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4 °C. The brains were then frozen and 5 series of 40-μm-thick sections were cut with a sliding microtome in the frontal plane.

One series was processed for immunohistochemistry with anti-Fos antiserum raised in rabbit (Ab-5; Calbiochem) at a dilution of 1:20,000. The primary antiserum was localized using a variation of the avidin–biotin complex system. In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) and then placed in the mixed avidin–biotin horseradish peroxidase (HRP) complex solution (ABC Elite Kit; Vector Laboratories) for the same period. The peroxidase complex was visualized by a 5-min exposure to a chromogen solution containing 0.02% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.3% nickel-ammonium sulfate in 0.05 M Tris buffer (pH 7.6), followed by incubation for 20 min, in chromogen solution with hydrogen peroxide (1:3000) to produce a blue–black product. The reaction was stopped by extensive washing in potassium PBS (KPBS; pH 7.4). Sections were mounted on gelatin-coated slides and then dehydrated and coverslipped with DPX (Sigma). An adjacent series was always stained with thionin to serve as a reference series for cytoarchitectonic purposes.

2.4. Quantification of Fos-labeled cells

Density of Fos-immunoreactive neurons were evaluated by an observer without knowledge of the animal's experimental treatment and were generated for selected brain regions using the 10× objective of a Nikon Eclipse 80i (Nikon Corporation, Chiyoda-Ku, Tokyo-To, Japan) microscope equipped with a Nikon digital camera DXM1200F (Nikon Corporation). For the quantification of the density of Fos labeling, we first delineated, in a given section, the borders of a region of interest, as defined in adjoining Nissl-stained sections, and Fos-labeled cells were counted therein. Only darkly labeled oval nuclei that fell within the borders of a region of interest were counted. The density of Fos labeling was determined by dividing the number of Fos-immunoreactive cells by the area of the region of interest. Both cell counting and area measurements were performed with the aid of a computer program (Image-Pro Plus, version 4.5.1; Media Cybernetics, Silver Spring, MD, USA). Cell densities were obtained on both sides of the brain and averaged for each individual. The brain regions examined in the present investigation were selected before the analysis following the criterions discussed below, and the employed parcellation followed *The brain maps: structure of the rat brain* [32]. The selection of the hypothalamic sites to be analyzed followed specific criterions. First, considering that both social defeat and physical restraint represent strong stressors, in the periventricular zone of the hypothalamus, we have focused our analysis on the paraventricular nucleus, both the parvicellular and magnocellular parts [9,27,28,34], and in the dorsomedial nucleus, which is a key component of a visceromotor pattern generator network, thought to control the neuroendocrine motor neurons [33]. In the medial zone, we analyzed the elements of the conspecific-responsive circuit, namely, the medial preoptic nucleus, the ventrolateral part of the ventromedial nucleus and the ventral premammillary nucleus, likely to respond to social cues [23], and the dorsal premammillary nucleus, a key site that integrates crucial threats that challenge the individual (i.e. social aggressor and predator, [8,23]). In the lateral zone, we focused on two specific regions, the juxtaparaventricular and juxtadorsomedial regions, which represent critical nodes to convey septo-hippocampal information to the dorsal premammillary nucleus [16].

2.5. Statistical analysis

A multivariate analysis of variance (MANOVA) was applied to the experimental data, followed by univariate analyses and Tukey HSD post hoc tests for pairwise comparisons. The significance level employed in the univariate ANOVAs was adjusted downward by a Bonferroni's correction (alpha = 0.005). In spite of possible departures of normality and homogeneity of variance assumptions by the present data set, our choice of a parametric analysis relies on the fact that ANOVA is relatively robust to such departures [36], thus preserving the statistical power of the analysis.

3. Results

During the social confrontation, we observed that all the resident rats, after a short latency (less than 30 s), started vigorously attacking the intruders. After the first attack, intruders were left for 5 min with the resident male, and remained passively frozen most of the time, usually presenting the typical 'on-the-back' position. During the attack, intruders also presented active forms of defense by trying to push the resident away, assuming an upright position with sparse boxing, and occasionally fleeing from the resident. During the acute restraint stress