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Original Article

Social instigation and repeated aggressive confrontations in male Swiss mice: analysis of plasma corticosterone, CRF and BDNF levels in limbic brain areas

Instigação social e confrontos agressivos repetidos em camundongos Swiss machos: análise de corticosterona plasmática e dos níveis de CRF e BDNF em áreas cerebrais límbicas

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Abstract

Introduction: Agonistic behaviors help to ensure survival, provide advantage in competition, and communicate social status. The resident-intruder paradigm, an animal model based on male intraspecific confrontations, can be an ethologically relevant tool to investigate the neurobiology of aggressive behavior.
Objectives: To examine behavioral and neurobiological mechanisms of aggressive behavior in male Swiss mice exposed to repeated confrontations in the resident intruder paradigm.
Methods: Behavioral analysis was performed in association with measurements of plasma corticosterone of mice repeatedly exposed to a potential rival nearby, but inaccessible (social instigation), or to 10 sessions of social instigation followed by direct aggressive encounters. Moreover, corticotropin-releasing factor (CRF) and brain-derived neurotrophic factor (BNDF) were measured in the brain of these animals. Control mice were exposed to neither social instigation nor aggressive confrontations.
Results: Mice exposed to aggressive confrontations exhibited a similar pattern of species-typical aggressive and non-aggressive behaviors on the first and the last session. Moreover, in contrast to social instigation only, repeated aggressive confrontations promoted an increase in plasma corticosterone. After 10 aggressive confrontation sessions, mice presented a non-significant trend toward reducing hippocampal levels of CRF, which inversely correlated with plasma corticosterone levels. Conversely, repeated sessions of social instigation or aggressive confrontation did not alter BDNF concentrations at the prefrontal cortex and hippocampus.
Conclusion: Exposure to repeated episodes of aggressive encounters did not promote habituation over time. Additionally, CRF seems to be involved in physiological responses to social stressors.
Keywords: Aggression, social instigation, corticosterone, CRF, BDNF.

Resumo

Introdução: Comportamentos agonísticos ajudam a garantir a sobrevivência, oferecem vantagem na competição e comunicam status social. O paradigma residente-intruso, modelo animal baseado em confrontos intraespecíficos entre machos, pode ser uma ferramenta etológica relevante para investigar a neurobiologia do comportamento agressivo.
Objetivos: Analisar os mecanismos comportamentais e neurobiológicos do comportamento agressivo em camundongos Swiss machos expostos a confrontos repetidos no paradigma residente-intruso.
Métodos: A análise comportamental foi realizada em associação com medidas de corticosterona plasmática em camundongos expostos repetidamente a um rival em potencial próximo, porém inacessível (instigação social), ou a 10 sessões de instigação social seguidas de encontros agressivos diretos. Além disso, o fator de liberação de corticotrofina (CRF) e o fator neurotrófico derivado do cérebro (BNDF) foram medidos no encéfalo desses animais. Camundongos controles não foram expostos à instigação social ou confrontos agressivos.
Resultados: Os camundongos expostos a confrontos agressivos exibiram um padrão semelhante de comportamentos agressivos e não agressivos típicos da espécie na primeira e na última sessão. Em contraste com instigação social apenas, confrontos agressivos repetidos promoveram aumento na corticosterona plasmática. Após 10 sessões de confrontos agressivos, os camundongos apresentaram uma tendência não significativa de redução dos níveis de CRF no hipocampo, que se correlacionaram inversamente com os níveis plasmáticos de corticosterona. Por outro lado, sessões repetidas de instigação social ou confronto agressivo não alteraram as concentrações de BDNF no córtex pré-frontal e hipocampo.
Conclusão: A exposição a episódios repetidos de encontros agressivos não promoveu habituação ao longo do tempo. Adicionalmente, o CRF parece estar envolvido nas respostas fisiológicas aos estressores sociais.
Descritores: Agressão, instigação social, corticosterona, CRF, BDNF.

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Introduction

Social systems and social stressors differ between species but in general social animals form dominance-based hierarchies.[1] In this context, aggression is an adaptive behavioral trait important for establishing dominance as well as competition for mating partners, food, and territories.[2] While a certain level of aggression is supposed to be beneficial for survival, abnormal aggression can be detrimental. In fact, increase in aggressiveness is a common occurrence in a variety of psychiatric disorders.[3,4]

Laboratory models of social stress include the visible burrow system, crowding stress, and the resident-intruder paradigm.[5-7] Social stress models are particularly useful because they are considered ethologically relevant.[8] Furthermore, most of the stress faced by humans occurs in a social context.[9] Although preclinical research has produced important descriptions of aggression and provided a solid basis for analysis of the neurobiology of aggressive behavior, the degree of similarity across species, neural systems, behavioral expression, and outcomes of aggression remain unclear.

Limbic regions such as the prefrontal cortex (PFC) and hippocampus are involved in innate social behaviors and response to social stress.[10] These areas seem to be critical for emotional and cognitive functions such as social recognition, fighting, mating, fear, or motivated behaviors.[10] The PFC and hippocampus have been identified as particularly important in the modulatory control of subcortical circuits that mediate aggressive and impulsive behaviors[11,12]; the components of these circuits include the medial amygdala, hypothalamus and the periaqueductal grey.[13-22] Indeed, PFC lesions promote an increase in aggressive behavior in rats.[23] Similarly, lesions involving frontal and temporal brain areas have been demonstrated to dramatically increase aggressiveness in humans.[24]

It is already known that repeated episodes of social confrontation promote long-lasting neuroadaptation in rodents that are defeated in the resident-intruder paradigm.[25] However, less is known about functional, as well as neuroadaptive, changes that occur in the brain of aggressive residents exposed to repeated episodes of social interactions. In this study, male resident Swiss mice were repeatedly exposed to either a potential rival nearby, but inaccessible (social instigation),[12] or to 10 sessions of social instigation followed by direct aggressive encounters. Controls were exposed to neither social instigation nor aggressive confrontations. After the last session, we measured plasma corticosterone (CORT), the stress neuropeptide corticotropin-releasing factor (CRF) and brain-derived neurotrophic factor (BDNF) in limbic brain areas. CRF was measured in the hippocampus and hypothalamus, areas previously related to aggression and behavioral and physiological responses to stress.[26,27] BDNF was measured in the PFC and hippocampus, brain regions associated with behavioral planning and affective behaviors.[28]

The primary role of CRF is to activate the hypothalamic-pituitary-adrenal (HPA) axis by acting on receptors in the pituitary and promoting the release of adrenocorticotropic hormone (ACTH) into the portal blood system.[27] ACTH stimulates the release of CORT from the adrenal glands – CORT plays several roles in mediating appropriate responses to stress and also exerts a negative feedback control of the HPA axis.[29] Extrahypothalamic distribution of CRF includes neuronal populations in the amygdala,[30,31] hippocampus,[32] and locus coeruleus.[33] CRF and its related peptides exert central function and mediate several behavioral and physiological responses to stress,[34-38] including anxiety-like behavior and some aspects of aggressiveness.[35,39-43]

The present study tested the hypothesis that BDNF may underlie, at least in part, experience-induced neuroplasticity in resident mice exposed to repeated sessions of agonistic interactions. BDNF is a molecule involved in the regulation of diverse biological functions, ranging from neuronal survival and differentiation during development to synaptic plasticity and cognitive behavior in the adult[44]; it has also been demonstrated to be a critical mediator of changes in social motivation.[45] In both rodents and humans, BDNF disruption is associated with neurobehavioral alterations and psychiatric disorders.[44]

Methods

Subjects

Adult Swiss mice weighed 25-30 g (8 weeks old) upon arrival were housed in polycarbonate cages (30 × 19 × 15 cm) with pine shavings as bedding. Rodent laboratory chow and water were available ad libitum through stainless steel wire mesh lids. Male mice were assigned as residents (n = 24) or intruders (n = 20). Each resident mouse was pair-housed with a female Swiss mouse (n = 24), whereas intruders were kept in groups of 6 per cage. The vivarium of the Animal Experimentation Unit at Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil) was maintained on a 12h light/dark cycle, 22±2°C temperature, and 50-60% humidity. The experiments were performed during the light phase, between 09:00 and 12:00 a.m. Experimental procedures were conducted in accordance with Brazilian Federal Law no. 11.794/2008, which regulates the scientific

use of animals. The project was approved by the Ethics Committee on Animal Use of the Animal Experimentation Unit at Hospital de Clínicas de Porto Alegre.

Tubal-ligation surgery

Female mice were tubally ligated using antiseptic techniques and standard surgical procedure.[46] Briefly, mice were anesthetized with ketamine (120 mg/kg) + xylazine (30 mg/kg, intraperitoneally [i.p.]) and placed in the right lateral decubitus position. Then, a dorsal incision (approximately 1.0 cm) was made, the ovary was located, and the ends of the uterine horn were tied off using absorbable sutures. The oviduct was located and severed using a micro-scissor. All reproductive structures were repositioned back in the abdominal cavity, and the abdominal incision was closed with absorbable sutures and the skin with non-absorbable sutures.[47] The same procedure was performed on the left side. Mice were injected with tramadol (10 mg/kg, i.p.) immediately after the surgery and during the next 3 consecutive days (12/12h) to provide analgesia. Female mice were single-housed and allowed to recover for 7 days before being paired with a resident male. Upon termination of the experiment, females were euthanized with an overdose of ketamine (300 mg/kg) + xylazine (30 mg/kg, i.p.).

Experimental design

The experimental design is shown in Figure 1. Male resident mice were tested after being pair-housed with a female for 3 weeks. Before the sessions, the female cage mate was removed from the resident's cage and kept in a holding cage. Resident mice were divided into the following experimental groups: 1) controls (CT): an empty perforated acrylic tube (18 × 6 cm) was placed into the resident's cage for 5 min; 2) social instigation group (SI): a perforated acrylic tube (18 × 6 cm) containing an intruder mouse was placed into the resident's cage for 5 min. Mice had visual, auditory, and olfactory contact, but the resident had no direct access to the intruder; 3) social instigation + aggressive confrontation group (SI+AC): a perforated acrylic tube (18 × 6 cm) containing an intruder mouse was placed into the resident's cage for 5 min, followed by actual confrontation without any protection. The aggressive encounter was terminated 5 min after the resident initiated the first attack bite or at 5 min if the resident failed to attack.[48] Residents were exposed to 10 sessions of social instigation or social instigation + aggressive confrontation, twice in a week, with a minimum interval of 72 h between sessions. Mice that did not present bite attacks against the intruder in the first two sessions were excluded from the experiment.

Aggressive and non-aggressive behaviors exhibited by resident mice during the first (T1) and last (T10) agonistic encounters

T1 and T10 were video-recorded and later coded by two independent researchers using the Observer XT software (Noldus, v.9.0.436, Wageningen, The Netherlands). The measurements assigned by the two observers agreed with each other (r^2 = 0.94). The frequency of aggressive behaviors was measured, including attack bites, chasing, lateral threats, sniffing, and tail rattling, as well as the latency of the first attack bite. The frequency or duration of non-aggressive behaviors, including grooming, rearing, and walking, was also evaluated.

Plasma CORT analysis before the resident-intruder encounters (baseline) and after T10

Resident mice had blood samples collected from the submandibular vein 5 days before the resident-intruder encounters (baseline) and immediately after T10, using disposable sterile lancets. Samples were centrifuged for 10 min at 4°C and 4,000 revolutions/min. Blood plasma was extracted and frozen at -80ºC for subsequent dosages. Plasma CORT was assessed using commercial ELISA kits

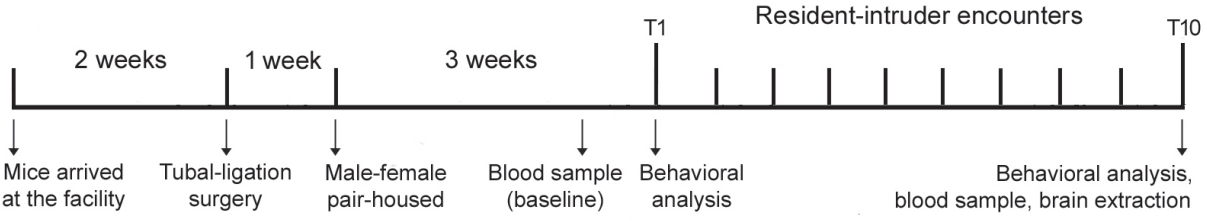

Figure 1 - Experimental design. Aggressive and non-aggressive behaviors were assessed during the first (T1) and last (T10) resident-intruder encounters. Blood samples were collected 5 days before the first resident-intruder encounter (baseline) and immediately after T10 for plasma corticosterone analysis. After the last blood collection, brains were extracted for CRF and BDNF measurements.

(Enzo Life Science, Farmingdale, NY, USA). Detection levels were 32-20 pg/ml, according to the manufacturer.

Brain CRF and BDNF measured after T10

After the last blood collection, resident mice were rapidly anesthetized with isoflurane and euthanized by decapitation. Brains were quickly removed, thoroughly washed in isotonic saline solution, and dissected on ice. The PFC, hippocampus, and hypothalamus were localized according to a brain atlas[49] and removed. Brain tissue samples were homogenized (weight/volume, 1:10) with ice-cold 0.1 M phosphate buffer (pH 7.4), with the addition of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Homogenates were centrifuged at 2,000 g for 5 min, and aliquots of supernatants were separated and stored at -80°C until further analyses. CRF in the hippocampus and hypothalamus were determined using a CRF mouse ELISA assay kit (My ELISA kits, St. Petersburg, FL, USA). BDNF in the PFC and hippocampus were measure by sandwich-ELISA, according to the manufacturer's instructions using specific monoclonal antibodies (R&D Systems Inc., Minneapolis, MN, USA).

Statistical analyses

Statistical analyses were performed using STATISTICA version 6.0. The data are reported as mean ± standard error of mean. Frequency and duration of aggressive and non-aggressive behaviors on T1 and T10 were analyzed with the Student *t*-test for dependent samples. Plasma CORT levels were analyzed using two-way analysis of variance (ANOVA) with repeated measures followed by post-hoc comparisons using the Newman-Keuls multiple-range test. CRF and BDNF concentrations were analyzed with one-way ANOVA. A linear least-square regression was conducted to determine the relationship between plasma CORT (ng/ml) and hippocampal CRF (pg/ml). Statistical significance was set at $p = 0.05$. For the behavioral experiments, each group contained eight animals, and six-seven were randomly used for hormonal and neurochemical measurements.

Results

The frequency or duration of aggressive and non-aggressive behaviors exhibited by male resident mice during T1 and T10 is shown in Table 1. The Student *t*-test for dependent samples revealed a significant decrease in the frequency of bites during T10 compared to T1 ($t = 3.11$, $p < 0.05$). No significant differences were observed between T1 and T10 in any other behavioral category evaluated (values of t varying from 0.05 to 2.21; $p > 0.05$ in all cases).

Plasma CORT measurements obtained at baseline and after T10 are shown in Figure 2. Two-way ANOVA with repeated measures revealed significant differences between groups ($F_{2,17} = 4.55$, $p < 0.05$), sessions ($F_{1,17} = 35.85$, $p < 0.0001$), and the interaction between these factors ($F_{2,17} = 4.36$, $p < 0.05$). Post-hoc analyses indicated a significant increase in plasma CORT in mice exposed to aggressive confrontations (SI+AC), compared to baseline levels, control animals, and mice exposed to social instigation only (SI).

CRF levels in the hippocampus and hypothalamus after T10 are presented in Figure 3. One-way ANOVA

Table 1 - Aggressive and non-aggressive behaviors exhibited by male resident mice during the first (T1) and the last (T10) social instigation + aggressive confrontation session.

	T1	T10
Aggressive behaviors		
Latency to first bite*	16.7 ± 6.2	7.1 ± 3.1
Bites†	21.4 ± 2.8	16.5 ± 3.6‡
Chasing†	11.7 ± 2.2	10.7 ± 1.8
Lateral threat†	4.3 ± 1.2	1.9 ± 0.3
Sniffing†	17.6 ± 3.6	12.0 ± 3.4
Tail rattle†	14.0 ± 3.2	9.9 ± 2.6
Non-aggressive behaviors		
Grooming†	5.1 ± 1.1	6.1 ± 1.5
Rearing*	49.8 ± 11.3	36.8 ± 7.8
Walking*	35.4 ± 7.1	25.5 ± 2.3

Data presented as mean ± standard error of mean.
* Duration in seconds; † frequency.
‡ $p < 0.05$ compared to T1 (n = 8).

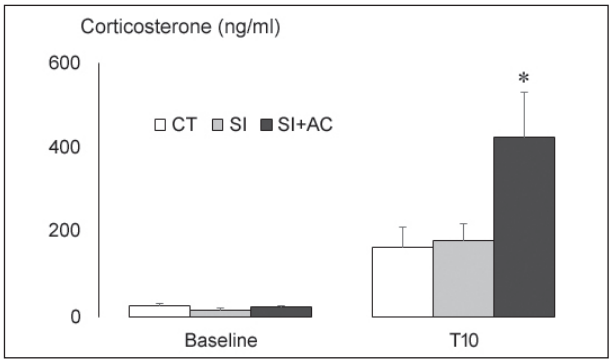

Figure 2 - Plasma corticosterone levels (ng/ml) measured in controls (CT), mice exposed to social instigation only (SI), or social instigation + aggressive confrontations (SI+AC). Blood samples were collected before the resident-intruder encounters (baseline) and after the last session (T10). Data presented as mean ± standard error of mean. * Compared to baseline, CT and SI in the same session. $p < 0.05$, n = 6-7 mice per group.

revealed no significant differences between the groups in both areas ($F_{2,17}$ = 1.66 and 0.37, respectively; p > 0.05 in both cases). However, in the hippocampus, the SI+AC group presented a decrease of 32% in CRF levels compared to control mice, and 42.5% compared to SI. Also, there was a significant negative linear relationship between plasma CORT and hippocampal CRF levels (r^2 = -0.52, p < 0.05, Figure 4).

BDNF levels in the PFC and hippocampus after T10 are presented in Figure 5. One-way ANOVA revealed no significant differences between the groups in both areas ($F_{2,17}$ = 1.07 and 1.23, respectively; p > 0.05 in both cases).

Discussion

In this study, male Swiss mice exposed to repeated aggressive inter-male confrontations exhibited a similar pattern of species-typical aggressive and non-aggressive behavior on the first and last sessions. Moreover, the current procedures showed that direct confrontations engendered an activation of the HPA axis during T10, suggesting that exposure to repeated episodes of aggressive encounters does not promote habituation over time. Additionally, after the last aggressive confrontation, mice presented a non-significant trend toward reducing hippocampal levels of CRF, which was negatively correlated with plasma CORT.

The activation of the HPA axis found in the present study is not a surprise, since aggressive encounters involve considerable risks for both resident and intruder. Either of the fighting parties can suffer injuries, and both loose energy reserves that may become crucial in a subsequent challenge.[50] In agreement with our neuroendocrinal results, male mice exposed to a paradigm of repeated experience of winning in a social conflict have been demonstrated to present increased levels of anxiety in the elevated plus-maze test.[26]

Increases in plasma glucocorticoids during a confrontation have been suggested to facilitate behaviors that are predominant for the animal in that specific context.[51] Indeed, brain mineralocorticoid receptor blockade during the first aggressive encounter inhibits subsequent propensity for violence in rats.[52] Thus, both offensive and defensive forms of aggression might respond to reductions in the glucocorticoid release normally associated with the stress of either challenge or conspecific attack, raising the possibility that treatments reducing the systemic activation of the HPA axis may affect aggressiveness.

Repeated sessions of social instigation, which is assumed to enhance the "aggressive arousal" of the resident male,[53] did not induce significant increases in CORT levels, suggesting that the mobilization of the HPA axis depends on direct confrontations, at least in the protocol used here. Importantly, the basal concentration

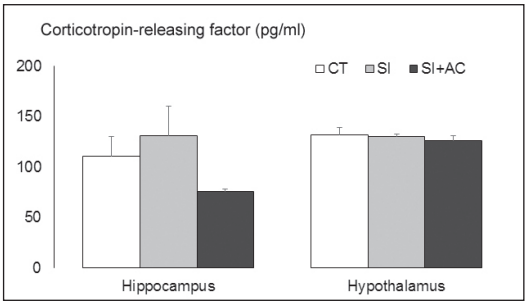

Figure 3 - Corticotropin-releasing factor levels (pg/ml) measured in the hippocampus and hypothalamus of controls (CT), mice exposed to social instigation only (SI), or social instigation + aggressive confrontations (SI+AC). Data presented as mean ± standard error of mean, n = 6-7 mice per group.

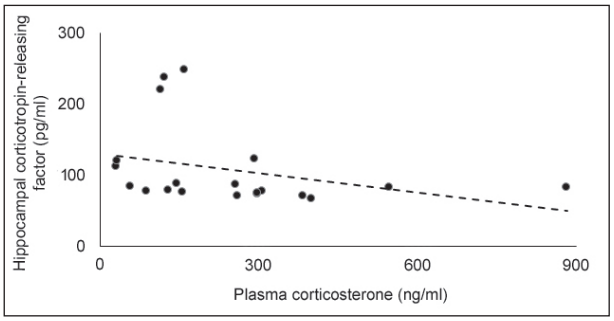

Figure 4 - Plasma corticosterone (ng/ml) plotted as a function of hippocampal corticotropin-releasing factor (pg/ml); the dotted line represents the linear fit (r^2 = -0.52). p < 0.05, n = 6-7 mice per group.

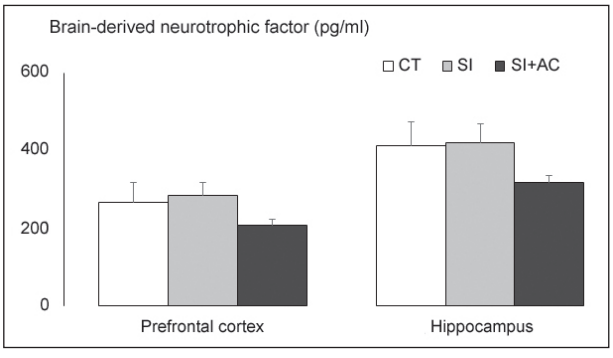

Figure 5 - Brain-derived neurotrophic factor levels (pg/ml) measured on the prefrontal cortex and hippocampus of controls (CT), mice exposed to social instigation only (SI), or social instigation + aggressive confrontations (SI+AC). Data presented as mean ± standard error of mean, n = 6-7 mice per group.

of plasma CORT in this study (mean = 31.3 ng/mL) is consistent with others.[54]

In a study using male rats defeated in the resident-intruder paradigm and measuring several neuropeptides in the brain, the authors found a decrease in CRF levels only in the hippocampus, suggesting a depressive-like state in submissive animals.[55] In the present study, even though aggressive confrontations did not promote a significant main effect on brain CRF, resident mice showed a trend toward reducing this neuropeptide in the hippocampus when compared to controls and mice exposed to social instigation only (reduction of 35.5% and 45.5%, respectively). Thus, it is possible that CRF in the hippocampus may be more involved in emotional and cognitive functions rather than in submissive or aggressive behavior. Interestingly, we found a negative correlation between hippocampal CRF and plasma CORT, suggesting that CRF may be involved in physiological responses to social stressors. Further investigation, however, is required before a mechanistic explanation of hippocampal CRF and plasma CORT can be proposed.

The hypothalamus, more specifically its paraventricular nucleus, is the origin of the HPA axis, whose activation culminates with the release of glucocorticoids from the adrenal glands.[27] Interestingly, in our experiment, after the last aggressive confrontation, mice presented an increase of plasma CORT without changing hypothalamic levels of CRF. A possible explanation for this apparent discrepancy could be that CRF and CORT present distinct temporal profiles of release. Thus, we may have collected the blood samples and extracted the brains at a time point when hypothalamic CRF had already stimulated ACTH release, but returned to basal levels. The stress response has classically been characterized by two temporal "waves" of stress mediator actions. The first one includes rapid actions of noradrenaline, serotonin, dopamine and CRF, promoting vigilance, alertness, appraisal of the situation and the choice of an optimal strategy to face the challenge. These events are followed by alterations of gene expression and cell function promoting sustained and adaptive stress responses attributed to glucocorticoids.[56]

Finally, increase in hippocampal levels of BDNF in resident hamsters has been suggested to evidence that behaviors associated with aggression and with winning a fight involve plastic mechanisms important to encode spatial representations.[57] Differently from our study, however, those animals were submitted to a single social interaction session. Thus, changes in BDNF levels may occur after the initial agonistic encounters, which may help to explain the lack of changes in BDNF in the present study. Moreover, aggression-induced cortical activation seems to be especially strong in mice selected for high aggressive behavior.[16,58] A limitation of this study is that we measured brain BDNF only after 10 resident-intruder sessions (social instigation only or social instigation + aggressive confrontation), when neuronal adaptations may be already stablished.

Conclusion

The present results extend our current knowledge about the neurobiology of aggressiveness by evaluating neurobiological mechanisms of species-typical aggressive behavior in male Swiss mice exposed to repeated sessions of agonistic encounters. The behavioral repertoire and the increase in plasma CORT after 10 aggressive confrontations indicate that resident mice do not present habituation over time. These findings support the idea that treatments reducing the systemic activation of the HPA axis may affect aggressiveness.[51] Contrary to our expectation, resident mice presented a non-statistically significant but substantial decrease of hippocampal CRF after the last confrontation, which correlated negatively with plasma CORT. Understanding the neuroadaptations that occur after successive episodes of social conflict may provide valuable insights into normal and abnormal forms of aggression and, ultimately, lead to effective approaches to control inappropriate aggressiveness in humans and other animals.

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Disclosure

No other conflicts of interest declared concerning the publication of this article.

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