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Opponent recognition and social status differentiate rapid neuroendocrine responses to social challenge

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ABSTRACT

Individual social status discriminates rapid neuroendocrine responses to non-social stress in male *Anolis carolinensis*, but whether such status-influenced reactions are retained in response to subsequent social stress is unknown. Dominant and subordinate males modify their behavioral responses to social challenge according to familiarity of the opponent, suggesting that accompanying neuroendocrine responses may differ according to opponent recognition despite social rank. We examined endocrine and neurochemical correlates of prior social status and opponent recognition during the opening stages of social challenge. Male pairs interacted and established dominant/subordinate status, followed by 3 days separation. Subsequently, subjects were paired with either the same opponent or an unfamiliar male according to rank (dominant with subordinate). After 90 s of social exposure, subjects were caught and brains and plasma collected for measurement of circulating corticosterone and limbic monoamines. Controls included pairs experiencing just one 90 s encounter plus a group of non-interacting subjects. Opponent recognition differentiated status-influenced responses, such that dominant lizards paired with familiar subordinate opponents had increased hippocampal dopamine and epinephrine, but showed increased plasma corticosterone and ventral tegmental area (VTA) norepinephrine when challenged with an unfamiliar opponent. Subordinate lizards encountering familiar opponents also had increased corticosterone, along with decreased hippocampal dopamine and increased VTA epinephrine, but showed no changes in response to an unfamiliar opponent. Such plasticity in status-influenced rapid neuroendocrine responses according to opponent recognition may be necessary for facilitating production of behavioral responses adaptive for particular social contexts, such as encountering a novel versus familiar opponent.

1. Introduction

Aggressive social encounters between male *Anolis carolinensis* lizards are stressful for both winners and losers and result in elevated plasma catecholamines and corticosterone, along with changes in limbic monoaminergic activity in both combatants [1,2]. However, the time course of neuroendocrine responses, along with the location, timing and specificity of changes in limbic monoaminergic activity, are predictive of eventual social status [3–6]. Typically, male *A. carolinensis* that go on to achieve dominance respond quickly to social challenge from a novel conspecific, and exhibit rapid increases in plasma catecholamines and corticosterone [1,7]. In contrast, male lizards that will become subordinate show a greater latency in aggressive responses and plasma corticosterone increases [5,7]. The faster physiological responses of putatively dominant males are believed to be adaptive in promoting aggression in future social interactions, and hence facilitating retention of dominant status, while the slower responses of subordinate males may serve to inhibit aggression and thus reduce the possibility of being injured by a stronger opponent [4,6].

Recently, we demonstrated that establishment of social status in male *A. carolinensis* also directs the nature and timing of rapid neuroendocrine responses to a brief non-social stressor (restraint), which differed from those elicited in males not exposed to social stress [8]. Prior to social rank formation, 90 s of restraint stress did not elicit immediate corticosterone increases, but rapid limbic monoaminergic responses were seen in the nucleus accumbens (NAc), hippocampus, amygdala, and locus ceruleus. In contrast, known dominant males exhibited heightened corticosterone release and raphe serotonergic activity immediately following restraint, while subordinate males showed decreased hippocampal dopaminergic activity but no increase in corticosterone [8]. The dissimilar neurochemical and endocrine responses of dominants and subordinates may reflect the differing behavioral profiles of these groups, enabling each to cope adaptively

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with changes in environmental conditions. In the current study, we investigated whether prior social status would also direct rapid neurochemical and endocrine responses to brief social challenge.

Like other lizards that establish and defend reproductive territories [9,10], male *A. carolinensis* show reduced aggression towards territorial neighbors with whom they have already established social relationships, a phenomenon termed the "dear enemy" effect [11]. In the laboratory, when male *A. carolinensis* are allowed to fight and establish a dominant–subordinate hierarchy, subordinate individuals exhibit dramatically reduced aggression if re-exposed to the same familiar dominant 3 days later, and retain their subordinate status [12]. In contrast, subordinates exposed to an unfamiliar dominant show increased aggression, and in some cases are able to win the second interaction and achieve dominance over the new opponent [12]. Recently, Yang and Wilczynski [13] reported that male *A. carolinensis* exhibit a habituated aggressive response towards repeated presentation of a simulated video opponent. However, these same males showed enhanced aggression towards subsequent exposure of a novel live opponent [13]. Memory of previous opponents also overrides artificial social rank reversal induced by manipulation of the autonomically-controlled postorbital skin darkening signal that conveys aggressive intent [1,14]. These studies suggest that for *A. carolinensis* opponent recognition plays a significant role in determining the appropriate level of aggressive response during social interactions, and in maintaining hierarchical relationships. As such, it may be expected that neurochemical and endocrine changes accompanying responses to social challenge will vary not only with prior individual status but also as a function of opponent recognition.

The aim of the current study was to determine whether rapid endocrine and neurochemical responses to a brief social challenge in male *A. carolinensis* are altered by previously established dominant or subordinate status, similar to the rank-differentiated responses to brief restraint stress [8]. We also examined differences according to familiarity of the second opponent, given that this appears to mediate behavioral response to social challenge regardless of prior rank [12]. We focused on rapid neuroendocrine changes occurring in the opening stages of the second social interaction, as previous studies have shown that neurochemical and endocrine differences according to perceived threat level and aggressive response are apparent in the first 90 s of the encounter [7,15]. In addition, this sampling time matched that used in our previous study demonstrating effects of social status on rapid corticosterone and limbic monoamine responses to restraint [8]. Limbic areas chosen for analysis comprised those showing rapid (90 s) monoamine responses to social and non-social stress in lizards of unknown status [8,15], and included those in which monoaminergic activity was further differentiated by attainment of dominant or subordinate status [8]. With regards to prior status effects on neurochemical and endocrine responses to a second brief social challenge, we predicted that known dominant males would exhibit faster reactions than subordinates, similar to our previous finding when males were exposed to restraint stress [8]. For examining effects of opponent recognition on these physiological variables, our experiment was designed on the premise that males defeated and made subordinate in the initial encounter remain subordinate when challenged by a familiar opponent, but respond aggressively towards an unfamiliar opponent [12]. Therefore, the accompanying neurochemical and endocrine responses during a second social challenge should differ according to opponent familiarity, despite the prior subordinate status of these males. We also hypothesized that rapid plasma corticosterone increases associated with reactions of dominant males to novel social and non-social stressors [7,8] would occur in dominants facing unfamiliar opponents, but not in familiar pairs, as a familiar opponent made subordinate in the first encounter should not be perceived as great a threat. Further, we predicted that brief exposure to unfamiliar opponents would be associated with differences in limbic monoamine concentrations distinct from those accompanying initial recognition of familiar opponents.

2. Methods

2.1. Subjects

Fifty-two adult male *A. carolinensis* (snout–vent length = SVL > 60 mm) were purchased from Thibodaux Live Supplies (Thibodaux, Louisiana, U.S.A), and were weighed and had snout–vent length measured on arrival. Lizards were housed in glass aquaria (51 × 25 × 25 cm) with fluorescent lighting (40 W, 3200 lumens) suspended 20 cm overhead. Each aquarium was divided by a removable opaque wall, providing an individual enclosure for each lizard (25 × 25 × 25 cm) that contained a diagonally upright wooden perch and water dish. Screens were also placed between aquaria to ensure that males in adjacent tanks could not see one another. Subjects were allowed to acclimate to their enclosure for 2 weeks before any experimentation took place [12,15], as previous studies have shown that opponent recognition is absent following >10 days of individual housing [12]. Artificial mating season conditions were maintained over this time and during testing (14 h/32 °C:10 h/21 °C light:dark cycle/temperature, 75% RH; [16]) to promote territorial behavior. Lizards were allowed free access to drinking water and were fed one live cricket (*Acheta* sp.) daily during this holding period and throughout the study. All males showed reproductive viability and hence territoriality by responding with courtship displays towards a female [17], presented 1 day before commencement of the study. The following experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, all efforts were made to minimize the number of animals used and to ensure high standards of welfare during experimentation.

2.2. Experimental design and procedure

Lizards were size-matched (within 0.2 g mass and 2 mm SVL) to an opponent [8,12]. Following the 2 week acclimation period to individual cages, pairs of size-matched lizards ($n = 15$ pairs) were allowed to interact for 10 min by removing the opaque divider between them. All subjects observed exhibited social signals normally produced during aggressive social interactions, such as ventrolateral swelling of the throat, darkening of postorbital skin and stereotyped motor pattern-based displays [15,18,19]. The number of stereotyped aggressive displays given by each subject over the 10 min interaction was also measured. Social status (dominant or subordinate) was recorded at the end of the interaction. Dominant males were characterized by lighter body color, attainment of a higher perch position, and continued expression of aggressive displays. In contrast, subordinate individuals were darker in color, hid from their opponent, and no longer displayed aggressively [5,12,14,17]. Subsequent analysis also showed that males who achieved dominance produced significantly more stereotyped aggressive displays than their subordinate opponents (dominants mean displays ± SEM = 31.7 + 7.5, subordinates mean displays ± SEM = 12.2 + 5.1; $t = 2.15$, $df = 28$, $P = 0.04$). Pairs were left together for an additional 2 h to ensure that social status remained stable [12], with behaviors indicating status confirmed again at this time.

The protocol for examining neuroendocrine correlates of opponent recognition was based on that developed by Forster et al. [12]. After 2 h of interaction, all lizards were removed from their respective testing area and put into separate plastic enclosures (30 × 7 × 12 cm) for 25 min. During this time, all testing areas were cleaned with white vinegar and rinsed twice with water in order to remove any chemical cues deposited during either the 2 week acclimation period or the first interaction. These temporary holding conditions did not appear to have any negative effects on the subjects, as none exhibited signs of stress such as body color darkening [17] or excessive scraping at the container walls, and feeding resumed immediately after return to test enclosures. Lizards were placed back into testing enclosures according to experimental group allocation.

Familiar opponent groups (FO; $n=8$ pairs) were paired with their opponent of interaction 1, but placed in opposite sides of the divided testing enclosure to eliminate spatial cues that may have been learnt during the first interaction [12]. Unfamiliar opponent groups (UO; $n=7$ pairs) were composed of novel pairs, such that known dominant males of interaction 1 were paired with different but size-matched subordinates. Three days later, lizards were allowed to interact by removing the dividing wall. Individual subject pairs were tested at same time of day as for interaction 1.

For interaction 2, opponents were only allowed to interact for 90 s before being caught and rapidly decapitated. Previous studies have shown that neuroendocrine responses differ at this time point during male *A. carolinensis* social encounters according to the degree of social threat and individual level of aggression [7,15]. In addition, lizards exposed to 90 s restraint stress exhibit rapid neurochemical and corticosterone responses that are differentiated by prior social status [8]. We also measured expression of visual signals that may facilitate rapid recognition of opponents, namely eyespot formation (postorbital skin darkening) and ventrolateral swelling of the throat. These two signals are commonly expressed in the opening stages of social interaction, before production of more dynamic signals such as stereotyped motor pattern-based displays, and indicate perception of social threat and potential aggression [15,20,21]. A third experimental group (social controls, $n=4$ pairs) consisted of opponents that had no previous social interaction and that were completely novel to each other. Social control opponents were also exposed to each other for 90 s before being caught and decapitated. Since interactions of such brief duration are not sufficiently long enough to allow formation of a dominant–subordinate hierarchy, social status of individuals in the social control group was not determined or recorded. Endocrine and neurochemical parameters (see below) of all subjects in this group ($n=8$ individuals) were pooled, so that neuroendocrine effects induced by a brief fight between naïve opponents interacting for the first time could be compared against those produced by opponent recognition and/or a second interaction. A second control group (non-interacting controls, $n=14$) consisted of individuals (social status unknown) that did not undergo a social interaction 3 days earlier, and were just caught and decapitated. Upon decapitation (mean time ± SEM for capture and decapitation across all treatments = 9.73 ± 0.65 s), trunk blood was collected using heparinized microcapillary tubes, centrifuged and plasma drawn off and stored at −80 °C. Brains were left intact in the cranium and rapidly frozen (−80 °C).

2.3. Analysis of plasma corticosterone

Measurement of plasma corticosterone was performed using a corticosterone enzyme-linked immunoassay kit (ELISA), as according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 10 µL of plasma and 0.25 µl steroid displacement reagent were diluted with 490 µl of assay buffer for a 50-fold dilution. Duplicates of 100 µl for each sample were added to assay plate wells coated with donkey anti-sheep polyclonal corticosterone antibody. Subsequently, 50 µl of corticosterone (200,000 pg/ml) conjugated to alkaline phosphatase and 50 µl of antibody solution (sheep polyclonal antibody to corticosterone) were added to each well, excluding control wells, and the plate was incubated at room temperature for two hours on a horizontal shaker (250 rpm).

The plate was washed after incubation using an automated plate washer (Bio-Tek Instruments, Winooski, VT, USA), and 200 µl of p-nitrophenyl phosphate substrate was added to each well. Samples were then incubated at room temperature for 1 h. Following this, 50 µl of trisodium phosphate solution was added to end the reaction, and the plate was placed into an automated microplate reader (Bio-Tek Instruments).

Plasma corticosterone levels were detected by absorbance of samples at 405 nm (wavelength correction set at 595 nm), using automated plate reader software (KinetiCalc Jr., Bio-Tek Instruments). Absorbance values were used to calculate maximum binding percent, which ranged between 16.25 and 16.32%, and percent of non-specific binding, which was 2.5%; both values were within the manufacturer's range. The sensitivity detection limit of this assay was 27.0 pg/µl.

2.4. Analysis of monoamines

Brains were left intact in the cranium, rapidly frozen (−80 °C), then sliced (300 µm) coronally in a temperature controlled cryostat at −10 °C. Sections were thaw-mounted on glass microscope slides, then refrozen and discrete brain regions microdissected using a 432 µm diameter cannula. Brain regions were identified using a stereotaxic atlas [22] and a map of central catecholamine staining [23] for *A. carolinensis*, and chosen based on regions previously shown to be involved in responses to different types of stress [15,24–26], and in which differential responses to restraint stress according to prior social status have been observed [8]. Regions chosen for analysis included the nucleus accumbens (NAc), the hippocampus, the central nucleus of the amygdala (CeA), the ventral tegmental area (VTA), the locus ceruleus (LC), and the raphé.

Norepinephrine (NE), epinephrine (Epi), dopamine (DA) and the DA metabolite: 3,4-dihydroxyphenylacetic acid (DOPAC); serotonin (5-HT); and its metabolite: 5-hydroxyindoleacetic acid (5-HIAA) were measured using high performance liquid chromatography (HPLC) with electrochemical detection [8,15]. Microdissected samples were expelled into 60 µl sodium acetate buffer (pH 5.0) containing 9.42 pg/µl of internal standard (dihydroxybenzylamine, DHBA) and frozen on dry ice. After thawing, 2 µl of a 1 mg/ml H_2O ascorbate oxidase solution (Sigma Chemical Co., St Louis, MO, USA) was added to each sample and the samples were centrifuged at 15 000 × *g* for 3 min. The supernatant was removed, 45 µl was injected into a chromatographic system (Waters Associates, Inc., Milford, MA, USA) and analyzed electrochemically with an LC-4B potentiostat (Bioanalytical Systems, West Lafayette, IN, USA). The electrode potential was set at +0.6 V with respect to an Ag/AgCl reference electrode. The tissue pellet remaining from each sample was dissolved in 110 µl 0.4 M NaOH and protein content assayed using the Bradford method [27].

2.5. Data and statistical analysis

Levels of plasma corticosterone were obtained using plate reader software as described above, while central monoamines were obtained and corrected for recovery using CSW32 v1.4 Chromatography Station for Windows (DataApex, Prague, Czech Republic). Corticosterone levels were expressed as ng/ml plasma. For central monoamines, neurotransmitter and metabolite levels were expressed as pg amine/µg protein.

Behavioral variables possibly associated with rapid opponent recognition were explored by comparing the proportion of subjects expressing the eyespot and/or throat swelling during interaction 2 within status (known dominant or subordinate from interaction 1) between FO and UO groups using separate Fisher's exact tests.

To examine the effect of opponent recognition according to social status, mean plasma corticosterone or brain monoamine concentrations after the 90 s interaction were compared across all treatment groups using separate one-way ANOVA, followed by Student-Newman-Keuls comparison of means analyses. The design of the experiment necessitated use of separate one-way ANOVA as it was not possible to determine social status for individuals in the social control group that underwent only one 90 s interaction. All analyses were performed using SigmaStat v. 3.5, with the α level set at 0.05.

3. Results

3.1. Aggressive signaling

Nearly all subjects in the FO and UO groups responded to their second interaction with ventrolateral throat swelling, indicating perception of

social threat. The proportions of dominant males expressing this signal in the FO (87.5%) and UO (85.7%) groups did not differ ($\chi^2 = 0.435$, $P = 1.0$). Likewise, there were no differences in the proportion of FO subordinate males showing throat swelling (87.5%) when compared to UO subordinates (71.4%; $\chi^2 = 0.017$, $P = 0.57$).

Very few FO or UO subjects exhibited maximum eyespot darkening during their second interaction. However, many showed partial eyespot formation, i.e., ~50% of postorbital skin darkened. There was no statistical difference in the proportion of dominant males within the FO group (87.5%) that developed partial eyespots when compared with UO dominants (50%; $\chi^2 = 1.164$, $P = 0.28$). Similarly, the proportions of subordinate males expressing partial eyespots in the FO (50%) and UO (50%) groups did not differ ($\chi^2 = 0.25$, $P = 1.0$).

3.2. Plasma corticosterone

Plasma corticosterone responses to a 90 s interaction differed according to both treatment group and status (Fig. 1; $F_{(5,49)} = 4.52$, $P = 0.002$). Further, increases in corticosterone were only seen in males undergoing a second interaction, with no change observed in social control males compared to non-interacting controls ($P = 0.91$). Dominant males facing an unfamiliar opponent exhibited significant increases in corticosterone levels compared to both control groups (highest $P = 0.039$), which were not seen in FO dominant males (lowest $P = 0.79$). However, corticosterone responses of UO dominants and UO subordinates did not differ ($P = 0.19$). Rapid increases in corticosterone were also shown by subordinate males challenged with a familiar dominant male as compared to FO dominants and both control groups (highest $P = 0.031$), and this increase was equivalent to that exhibited by UO dominants ($P = 0.91$). No differences in corticosterone levels were observed among controls, social controls, FO dominants and UO subordinates (lowest $P = 0.315$).

3.3. Limbic monoamines

Prior social status differentiated monoamine responses to a brief second interaction. However, status-mediated effects differed according to opponent familiarity, which also affected the location of neurochemical changes. For instance, facing a familiar opponent in a brief second interaction had specific effects on monoamine concentrations in the hippocampus. In contrast, rapid monoamine effects elicited by confrontation with unfamiliar opponents were primarily restricted to the ventral tegmental area (VTA). These changes differed from subjects that only had one 90 s interaction (social controls), which showed monoamine changes in the nucleus accumbens (NAc), locus ceruleus (LC) and central nucleus of the amygdala (CeA) but not in the hippocampus or VTA. However, no effects of either a single social interaction or opponent recognition in a second interaction were observed in the raphé, nor were differences in 5-HT levels detected for any of the brain regions analyzed (data not shown).

3.3.1. Nucleus accumbens

A short social interaction of 90 s stimulated an increase in the serotonin metabolite 5-HIAA (Fig. 2A, $F_{(5,50)} = 3.76$, $P = 0.006$), but only in social control subjects that had one brief interaction when compared with non-interacting controls ($P = 0.004$). Concentrations of NAc 5-HIAA did not differ among non-interacting controls and males undergoing a second interaction (lowest $P = 0.32$), nor were there any differences according to either prior status (lowest $P = 0.46$) or opponent familiarity (lowest $P = 0.356$). There was no effect of either a single 90 s social interaction or of a second social interaction between familiar or unfamiliar pairs on NE, Epi, DOPAC, DA or 5-HT levels in the nucleus accumbens (data not shown).

3.3.2. Hippocampus

Both prior status and opponent familiarity affected hippocampal responses to a second brief interaction (Fig. 3A; $F_{(5,49)} = 4.52$, $P = 0.002$). Dominant males in the FO group showed a significant increase in DA concentrations compared to non-interacting controls ($P = 0.041$), which was not exhibited by dominant males in the UO group ($P = 0.773$). In contrast, a significant decrease in DA concentration was observed in subordinate males facing a familiar opponent when compared to both social controls ($P = 0.043$) and FO dominants ($P = 0.004$). Hippocampal DA concentrations did not differ among non-interacting controls, social controls, UO dominants and UO subordinates (lowest $P = 0.08$). No effect of a single 90 s social interaction or a second social interaction between FO/UO pairs was seen on NE, DOPAC, 5-HT or 5-HIAA levels in the hippocampus (data not shown).

Status and familiarity of opponent also influenced hippocampal Epi concentrations (Fig. 3B; $F_{(5,44)} = 5.41$, $P < 0.001$), with dominant males in the FO group exhibiting significant increases in Epi concentrations compared to all other treatment groups (highest $P = 0.004$). There were no differences in hippocampal Epi concentrations among non-interacting controls, social controls, FO subordinates, UO dominants and UO subordinates (lowest $P = 0.82$).

3.3.3. Central nucleus of the amygdala (CeA)

Only individuals in the social control group that underwent a single 90 s social interaction exhibited any alteration in CeA monoamine concentrations, with changes restricted to DOPAC (Fig. 4; $F_{(5,50)} = 4.87$, $P = 0.001$). Social controls showed significant increases in DOPAC when compared to all other treatment groups (highest $P = 0.024$). Concentrations of CeA DOPAC did not differ among non-interacting controls, FO dominants, FO subordinates, UO dominants and UO subordinates (lowest $P = 0.326$). There was no

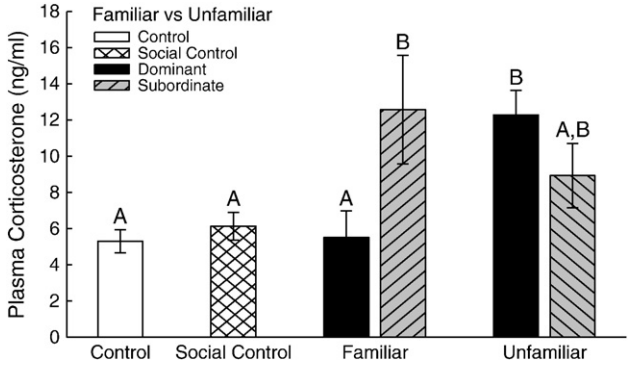

Fig. 1. Mean (±SEM) plasma corticosterone concentrations. Significant corticosterone increases after brief social challenge was exhibited by both subordinates facing familiar opponents and dominants challenged with unfamiliar opponents. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

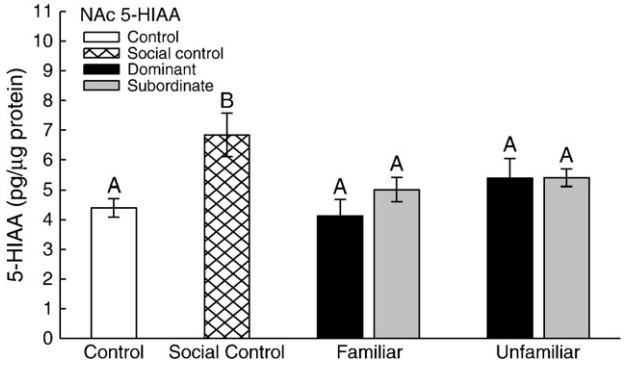

Fig. 2. Mean (±SEM) 5-hydroxyindoleacetic acid (5-HIAA) in the nucleus accumbens (NAc). Social control subjects engaging in a single 90 s interaction displayed elevated NAc 5-HIAA concentrations compared to all other treatment groups. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

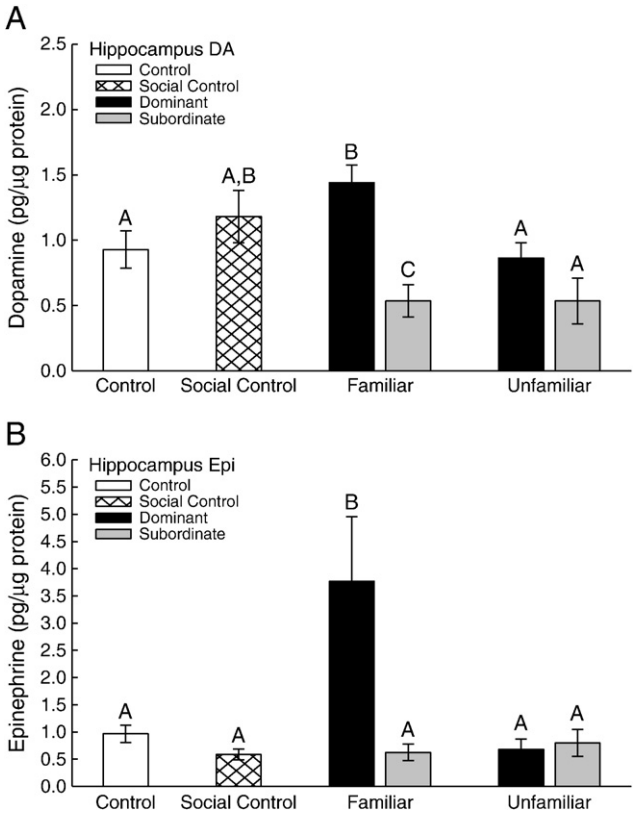

Fig. 3. Mean (±SEM) A) dopamine (DA) or B) epinephrine (Epi) in the hippocampus. A) Familiar subordinate subjects had reduced DA concentrations, while their dominant opponents showed increased DA concentrations when compared to non-interacting controls. No change was seen in hippocampal DA of unfamiliar opponents. B) Dominant males exhibited elevated hippocampal Epi concentrations when challenged with a familiar opponent, but not when faced with an unfamiliar subordinate male. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

effect of any experimental treatment on 5-HT, 5-HIAA, NE, Epi, or DA in the central nucleus of the amygdala (data not shown).

3.3.4. Ventral tegmental area

A second interaction between opponents of known social status elicited rapid alterations in VTA NE concentrations, which were not exhibited by social controls of unknown status that had one 90 s interaction (Fig. 5A; $F_{(5,50)}=7.31$, $P<0.001$). However, status-dependent VTA NE responses were differentiated by opponent recognition,

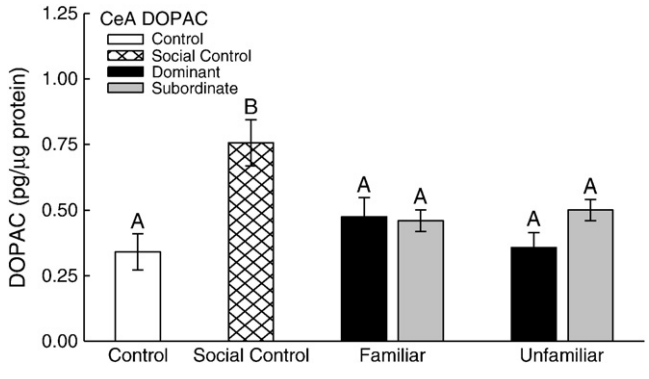

Fig. 4. Mean (±SEM) A) 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in the central amygdala (CeA). Social control subjects engaging in a single 90 s interaction exhibited elevated CeA DOPAC concentrations, which were not observed in males undergoing a second social challenge. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

such that subordinates challenged with a familiar opponent showed significant increases in VTA NE that were not seen in UO subordinates ($P<0.001$). Conversely, increases in VTA NE of known dominant males were elicited by facing an unfamiliar opponent but not by challenge with a familiar subordinate ($P=0.012$). This increase in UO dominants was equivalent to that exhibited by FO subordinates ($P=0.664$). Concentrations of VTA NE did not differ among non-interacting controls, social controls, FO dominants and UO subordinates (lowest $P=0.378$).

Similar to NE, rapid changes in VTA Epi concentrations were differentiated by both prior status and opponent recognition (Fig. 5B; $F_{(5,51)}=5.37$, $P<0.001$). However, in this case effects on Epi concentrations in the VTA were confined to subordinate males facing a familiar dominant opponent in their second interaction, with FO subordinates exhibiting increased VTA Epi compared to all other treatment groups (highest $P=0.033$). In contrast, VTA Epi concentrations in previously subordinate males exposed to unfamiliar dominants did not differ from both non-interacting and social controls (lowest $P=0.304$), nor were concentrations different from those of their unfamiliar dominant opponent ($P=0.387$). There was no difference in VTA Epi concentrations among non-interacting controls, social controls, FO dominants and UO dominants (lowest $P=0.24$). There was no effect of either a single 90 s social interaction or a second social interaction in FO/UO pairs on 5-HT, 5-HIAA, DA, or DOPAC levels in the VTA (data not shown).

3.3.5. Locus ceruleus (LC)

Rapid changes in LC monoaminergic activity were restricted to 5-HIAA concentrations (Fig. 6; $F_{(5,49)}=4.25$, $P=0.003$). Social

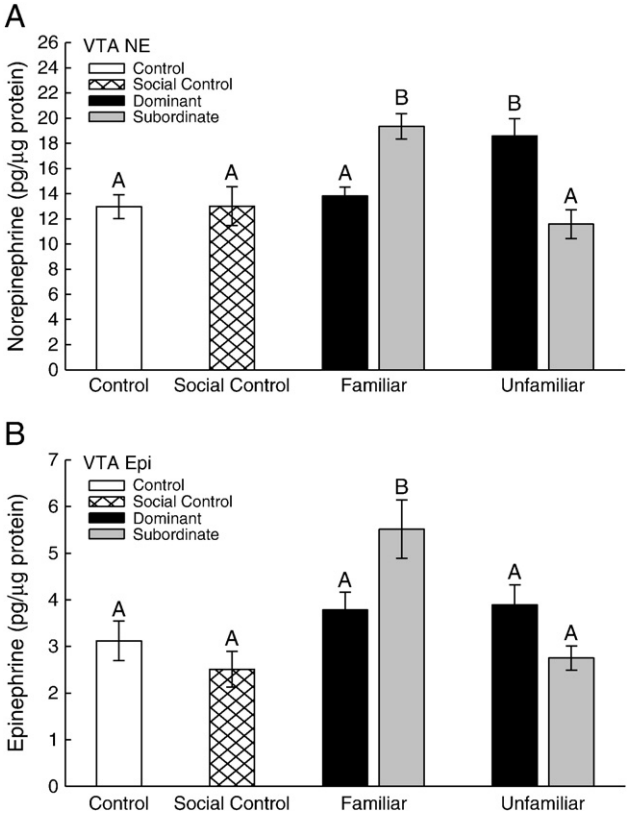

Fig. 5. Mean (±SEM) A) norepinephrine (NE) or B) epinephrine (Epi) concentrations in the ventral tegmental area (VTA). A) Elevated VTA NE concentrations were elicited both in subordinate males challenged with a familiar opponent, and in dominant males when facing an unfamiliar opponent. B) Subordinate subjects also exhibited increased concentrations of Epi in response to a familiar dominant, but not to an unfamiliar opponent. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

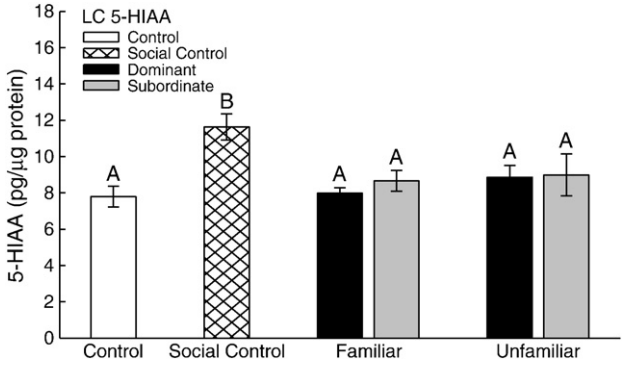

Fig. 6. Mean (±SEM) 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the locus ceruleus (LC). Only social control subjects engaging in a single 90 s interaction exhibited elevated LC 5-HIAA concentrations. Means with no common superscript letters (e.g., A and B) are significantly different ($P<0.05$).

controls undergoing a single brief interaction showed increases in LC 5-HIAA concentrations when compared to all other treatment groups (highest $P=0.028$). There was no effect of prior status or opponent recognition on LC 5-HIAA concentrations, with all other individuals undergoing their second social interaction showing no significant change in LC 5-HIAA relative to either non-interacting controls or each other (lowest $P=0.51$). No effect of any experimental treatment was seen on NE, Epi, DA, DOPAC, or 5-HT concentrations in the LC (data not shown).

4. Discussion

The effects of a brief second social interaction on corticosterone and limbic monoamines differ according to previously established status. As predicted, known dominant males showed rapid changes in both corticosterone and monoamine responses, but these were differentiated by familiarity of the opponent. For example, monoamine differences between known dominant subjects as a function of opponent familiarity were localized to the hippocampus and VTA, with unfamiliar opponent (UO) dominants showing increased VTA NE while familiar opponent (FO) dominants showed increased hippocampal Epi. Further, rapid increases in corticosterone were only seen in dominant males faced with an unknown opponent. However, rapid responses were not restricted to dominant males, as subordinates challenged with a familiar dominant also exhibited increased corticosterone along with changes to hippocampal DA and VTA adrenergic activity. In contrast, no rapid responses were seen in known subordinate males facing an unfamiliar opponent. We did not observe any difference in rapid expression of social signals, namely postorbital skin darkening and ventrolateral throat swelling, as a function of opponent familiarity. The mechanisms that enable recognition between opposing *A. carolinensis* are still unresolved. The perception of known opponents is not chemical, by means of olfaction or taste, nor is it visual, by means of physical displays or darkening of eyespots [12,14]. While the results suggest that memory must somehow play a role in opponent recognition, it is still uncertain as to what sensory cues are involved.

4.1. Corticosterone responses to social challenge are differentiated by rank and opponent familiarity

Familiarity of *A. carolinensis* opponents influences the level of aggression in subsequent encounters, with unfamiliar opponents showing greater aggression [12,14]. Males that go on to achieve dominance in an initial encounter with an unknown opponent also exhibit heightened plasma corticosterone levels, which facilitates enhanced aggression [7]. Therefore we hypothesized that greater familiarity during a second interaction between males would reduce corticosterone secretion, as dominant males would not perceive a known opponent that they have already beaten as threatening, and consequently would not mount the rapid aggressive response elicited by an unknown opponent. Our results did demonstrate that corticosterone levels of dominant males remained low, equivalent to those of both control groups, after a second brief exposure to a known subordinate male. In contrast with familiar opponent dyads, dominant males in unfamiliar pairs exhibit heightened plasma corticosterone concentrations responses to a second brief social challenge. In terms of perceived social threat, and perhaps also readiness to fight, elevated corticosterone may be appropriate for these dominant males when faced with an unfamiliar opponent [7]. Dominant males react more quickly to both restraint stress and social interactions than do subordinate males [3,4,8]. Corticosterone seems to be important for facilitating aggression during the early stages of a fight, between 1 and 7 min but not thereafter, as the glucocorticoid receptor antagonist mifepristone (RU486) only inhibits aggressive acts during this initial period of the interaction [7]. Males that are more aggressive in the early stages of social encounters also tend to achieve dominant status [5,28]. Dominant males that are unfamiliar with their opponent may perceive an elevated threat level compared to dominant males that are familiar with their adversary and his social rank. Higher plasma corticosterone levels may then assist socially dominant males in mounting a rapid aggressive response and hence to establish dominance over a new opponent.

Rapid corticosterone responses to social challenge were also shown by subordinate males in familiar dyads. In contrast to dominant males facing an unfamiliar opponent, the corticosterone response of familiar subordinates is unlikely to potentiate heightened aggression, because socially subordinate males do not respond aggressively to familiar dominant opponents [12,14]. As such, their increased plasma corticosterone most probably reflects a fearful response to a brief subsequent social challenge upon recognizing an opponent that has already beaten them once. Subordinate males facing an unfamiliar opponent did not exhibit any change in corticosterone. The absence of a rapid corticosterone response in these males may indicate hesitation in attacking before the new opponent's attributes are properly assessed. However, these same males are capable of being aggressive and assuming dominance against new opponents if the interaction is of greater duration [12].

4.2. Rapid responses differ between single and repeated brief social challenge

Social controls, which engaged in a single brief interaction with an unknown opponent, did not exhibit any changes in plasma corticosterone, and alterations to brain monoaminergic activity were seen in different regions from subjects undergoing a second interaction. A single 90 s social encounter stimulated increased 5-HIAA concentrations in the NAc and LC, along with elevations in CeA DOPAC. In contrast, dominants and subordinates re-matched with familiar or unfamiliar opponents did not exhibit any changes in these regions, with alterations to monoaminergic activity instead restricted to the hippocampus and VTA. Combined, these differences may indicate that brief encounters with unknown opponents before individual status has been established have a greater effect on dopaminergic and serotonergic activity in limbic regions associated with motivation, arousal and conditioned fear responses [15,29,30] than subsequent encounters with a familiar opponent whose social status is known. This result also highlights the effect of recent social experience regardless of opponent familiarity, as these changes were also absent in males that faced an unfamiliar opponent in their second interaction.

4.3. Possible functions of regional differences in limbic monoaminergic activity according to status and opponent recognition

Dominant males that were familiar with their opponents showed increased hippocampal epinephrine in response to a subsequent brief encounter, which was not evident in dominants of unfamiliar dyads.

Hippocampal activity is crucial for spatial learning in lizards, and also for mediating social recognition and anxiety-related responses in rats [31–33]. Social and spatial learning may be modulated by the *Anolis* hippocampus, where increases in NMDA receptors following social interactions suggest that individuals could be learning to recognize their status in relation to that particular opponent [34]. Hippocampal NMDA receptors are required in rats for associative memory recall [35], NMDA also improves social recognition [36], and in NMDA dependent LTP, stimulation of β-adrenergic receptors is necessary for attention and memory processes [37–39]. Epinephrine increases in dominant males facing familiar subordinates may play a role in stimulating hippocampal activity through β_2-adrenergic receptors, facilitating opponent recognition and limiting unnecessary expression of aggression towards a conspecific with whom a social hierarchy has already been established.

Rapid changes in DA activity were also seen in the hippocampus of both dominant and subordinate males in familiar dyads. Earlier studies in *A. carolinensis* have shown that previously established rank differentiates changes in hippocampal DA concentrations evoked by non-social stressors, with known subordinates exhibiting decreased DA levels in response to 90 s of restraint [8], similar to the response of FO subordinates reported here. Dopaminergic activity in the hippocampus during more prolonged aggressive *A. carolinensis* social interactions is also influenced by rank and social recognition [14,40]. In the current study, subordinate males showed decreased hippocampal DA after a second brief interaction, while their familiar dominant opponents exhibited increased DA concentrations. For subordinate males, the decrease in DA with no accompanying change in the metabolite DOPAC may reflect increased dopaminergic activity. Similarly, the increases in dominant male DA also without changes to DOPAC may be indicative of rapid pathway priming through increased synthesis, storage, terminal release and/or enhanced reuptake of neurotransmitter following initial opponent perception, prior to increases in catabolite levels that may occur as behavioral responses start being produced [15]. Increases in hippocampal dopaminergic activity in various species appears to be important for spatial memory formation and retrieval [41,42], and the hippocampus is also important for social recognition [32,43]. Similarly, increased dopaminergic activity in the hippocampus of male *A. carolinensis* may assist in recognizing a familiar socially dominant or subordinate opponent.

For previously subordinate subjects, a subsequent encounter with a familiar dominant opponent resulted in elevated Epi concentrations in the VTA. In contrast, VTA Epi concentrations did not change when subordinates were presented with an unfamiliar opponent. Norepinephrine in the VTA of subordinate males in familiar pairs was also elevated. Male *A. carolinensis* that respond submissively to sustained (10 min) presentation of a simulated high social threat situation have increased noradrenergic turnover in the VTA [44]. The combined rapid increases in VTA Epi and NE of familiar subordinate males found in the current study may contribute to the inhibition of aggressive behavior upon recognizing a socially dominant opponent [4,12,14]. However, VTA NE levels of dominant lizards paired with an unfamiliar opponent were also elevated relative to their subordinate opponents. Activation of adrenergic receptors in the VTA has been shown to facilitate attack behavior in cats [45]. The rapid increases in VTA NE concentrations of dominant *A. carolinensis* may similarly facilitate the aggressive response needed to achieve dominance over an unknown opponent. Together, our findings suggest that increased adrenergic activity in the VTA may play a bidirectional role in modulating aggressive response depending on the perceived level of social threat. Future experiments using specific adrenergic agents will be required to test this hypothesis.

5. Conclusions

Establishment of dominant or subordinate social status differentially affected rapid neuroendocrine responses to subsequent social challenge, similar to status effects on responses to non-social stress [8]. The relationship between social status and neuroendocrine responses to different stress types in male *A. carolinensis* bears many similarities with proactive and reactive stress coping styles identified in other vertebrates [46–48], with dominant and subordinate *A. carolinensis* exhibiting proactive and reactive profiles, respectively [49]. However, while these distinct profiles are retained following social status establishment in response to non-social stress [8], status-driven responses to social challenge in male *A. carolinensis* can be further differentiated by opponent familiarity. Exposure to an unfamiliar opponent generates a fast endocrine response by known dominant males that appears to mimic the proactive profile of rapid aggression, courtship, feeding and sympathetic activity shown by males that are predisposed to winning fights [49]. However, this same corticosterone response was not elicited when known dominants faced familiar opponents. Similarly, subordinate males challenged with a familiar dominant exhibit a rapid corticosterone response more reminiscent of a proactive profile, but show a reactive-type corticosterone response if exposed to either an unfamiliar opponent or a brief non-social stressor [8]. Moreover, the location and specificity of rapid neurochemical changes in both dominant and subordinate males also differed with opponent familiarity. These findings suggest that during social interactions, the degree to which these proactive or reactive stress coping styles will be expressed by dominant and subordinate males are mediated by opponent recognition. Such plasticity in neuroendocrine responses to subsequent social challenge according to prior rank and opponent familiarity may be necessary for facilitating the production of behavioral responses that are adaptive for a particular social context, such as exposure to a novel versus familiar opponent. For example, the ability to remember a previous opponent can be advantageous to both combatants, as subsequent encounters will not escalate to such a high degree in aggression. In contrast, the neuroendocrine responses shown by known dominant males when challenged with an unfamiliar conspecific may promote the rapid aggression necessary to win the fight and achieve dominance over the new opponent.

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