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HOUSING ENVIRONMENT INFLUENCES ON CHRONIC STRESS-INDUCED CHANGES IN BEHAVIOR AND NEUROCHEMISTRY: SEX DIFFERENCES IN SPRAGUE-DAWLEY RATS

by

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A dissertation submitted to the Graduate Faculty in Psychology (biopsychology subprogram) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

HOUSING ENVIRONMENT INFLUENCES ON CHRONIC STRESS-INDUCED CHANGES IN BEHAVIOR AND NEUROCHEMISTRY: SEX DIFFERENCES IN SPRAGUE-DAWLEY RATS

by

Kevin D. Beck

Advisor: Professor Victoria N. Luine

Wade and Maier's classic study (1986) showed that modeling behavioral impairments due to stress could be greatly influenced by subjects' housing conditions. The work described here sough to determine the extent to which similar manipulations of housing conditions could moderate assessed behavioral and neurochemical measures following chronic stress. Using a 21-day (6-hour) restraint paradigm, housing conditions (food restriction and living alone or with a cage-mate) were manipulated in Sprague-Dawley rats. Both sexes were tested under these conditions to ascertain whether a different pattern of effect on behavior or neurochemistry would immerge after being stress and / or housed in a particular manner. Male rats were most affected by chronic restraint in the object recognition test exhibiting a failure to explore a novel object more so than a previous sample object at delays 2.5-hours or greater. This effect in males was buffered if they were food deprived subsequent to the stress (during behavior testing). Pair housing males was also detrimental to object recognition performance, regardless of stress condition. Double housing during the 3-week restraint period appeared to be a necessary condition for stress-induced object recognition impairments in males. Single-housed stress males were selectively impaired on object placement recognition following a 2.5-hour delay. Stress males, regardless of housing condition, also entered and explored objects in a free open-field quicker than naïve males. Females did not show any housing or stress-induced changes in object

recognition performance. Stress enhanced object placement recognition above that of naïve females who did not show a preference for exploring a newly located object. Double-housed stress females also took significantly longer time to enter a free open-field. Norepinephrine, dopamine, and histidine levels showed the most consistent changes due to stress in prefrontal cortex and hippocampus, but these changes were sex-dependent. Serotonin, glycine, and GABA also showed sex-dependent changes based on housing conditions. These results show that the housing environment of subjects influences both the behavior and underlying neurochemistry of naïve and chronically stressed rats, but the pattern differs across sex. Thus, the basic housing environment must be considered a critical variable when modeling behavioral or neurochemical changes across sex.

Preface

This work is the result of many years trying to understand and model a widespread psychological phenomenon, occurring in every single individual at some point in his/her life, which, all to often, becomes pathological. Generally, we have labeled this condition – stress. The problem with studying this psychological condition or event (i.e. "being under stress") is that the physiological events, that are believed to be the "stress response", do not uniformly occur in all individuals (human or animal) in the same situations. Here, I have attempted to illustrate this fact by showing that housing the animals in different environments can modify both the behavioral and neurochemical results of a chronic stress paradigm. A second goal of this work was to show that an established male model of chronic stress should not be simply assumed to apply to females. Thus, by studying males and females under different environmental conditions, I hope to show that the effects of "being stressed" are both diverse and malleable. This idea or general approach is a hallmark of a more environmental view of psychology and neuroscience that considers individual differences are, in part, shaped by the environment and should be given proper status in theory. Thus, the current work serves to present new behavioral and neurochemical data on the effects of chronic stress but also is a reminder that sometimes the most basic variables teach us that nothing is simple. There is always a level of the unknown in our most tested paradigms for "there is structure underlying the most accessible levels of things that fill us with awe" (Sapolsky, 1997).

Acknowledgments

First, I must state that the list here is not complete, by a long shot; therefore, I apologize profusely to the people I have, either unknowingly or due to a lack of space, failed to properly recognize here. (I'll make it up to all of you in my next book.) Now, having covered myself at this point, I want to thank the "committee of five" for, hopefully, finding this work both entertaining and thought provoking. I especially have to recognize and thank my advisor Vicky Luine. The title of "advisor" does not justly describe the relationship we have forged in the last six years (yes, it has been that long). As one of a select handful of people, who saw all sides of my graduate experience, I have always felt your presence in supporting both my professional and personal development. Most of all, thank you for giving me "space", letting me explore avenues probably no one in their right mind would want to explore (most of the time), and allowing me to study variables I found to be important.

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Table of Contents

1. Abstract iv
2. Preface vi
3. Acknowledgments vii
4. Figure Captions x
5. General Introduction 1
6. Experiment 1: Food Deprivation Modulates Chronic Stress Effects on Object Recognition in Male Rats: Role of Monoamines and Amino Acids 10
7. Experiment 2: Chronic Restraint Stress and Food Deprivation Affect Female Monoamine Levels without Causing Behavioral Deficits in Object Recognition 43
8. Experiment 3: The Influence of Housing Status and Chronic Restraint on Male Object Memory and Neurochemistry 61
9. Experiment 4: Chronic Stress and Housing Condition Differentially Affect Limbic Neurochemistry, Object Memory and Open-Field Behaviors in Females 91
10. General Discussion 115
11. References 125

Figure Legends

Figure 1: A summary of some of the known interconnections between the limbic system: hippocampus (DG, CA1, CA3), thalamus (MDth), amygdala (AMYG); basal ganglia: striatum (STR), nucleus accumbens (NAC); cortical regions: medial prefrontal cortex (mPFC), entorhinal cortex (EC); and brainstem-midbrain nuclei: ventral tegmental area (VTA), locus ceruleus (LC), raphe nuclei (RapNuc) in relation to the hypothalamic-pituitary-adrenal axis (HYP, PIT, and Adrenals).

Figure 2: The sequential order of group manipulation (restraint and food restriction), behavior testing, and sacrificing of subjects.

Figure 3: Object recognition in naïve-control (NC), naïve-food-deprived (FD), chronic stressed (STR), and chronic stress-food-deprived (STFD) rats. Bars represent the mean time (± SEM) groups spent exploring the novel object (open bars) and the sample object (shaded bars). An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group.

Figure 4: Combined exploration times of objects in sample (T1) and test (T2) trials across groups. Group designations are the same as in figure 2. An asterisk (*) represents a main effect of increased exploration of objects over trial ($p < .05$) and a cross (†) represents a main effect of food deprivation increasing combined exploration times (T1 & T2).

Figure 5: Regional serotonin activity as indexed by mean 5HIAA/5HT ratio (± SEM). Group designations are the same as presented in figure 2. An asterisk (*) indicates a significant main effect of food deprivation ($p < .05$) increasing serotonin activity in prefrontal cortex and hippocampus CA1.

Figure 6: Prefrontal, CA3, and nucleus accumbens regional changes in aspartate, GABA, histidine, and glycine across group condition (same designation as figure 2). The left scale reflects amounts of aspartate and GABA while the right scale reflects amounts for histidine and glycine (ng / ug protein). An asterisk (*) represents a main effect of food deprivation ($p < .05$). A cross (†) represents a main effect of stress ($p < .05$). Double-crosses (‡, $p < .05$; ‡‡, $p < .01$) designate food deprivations X stress interactions.

Figure 7: Mean rears and wall climbs (± SEM) occurring in the first 3 and second 3 minutes of the 6-minute open field trial. Group designations are the same as figure 2. An asterisk (*) indicates a greater number of rears by STR-FD subjects over N-FD and STR subjects (F interaction, $p < .05$, post-hoc LSD $p < .05$). A cross (†) designates a main effect ($p < .05$) of decreasing wall climbs over time across groups.

Figure 8: Object recognition in a large arena (using the same group designations as figure 2) show significant discriminations (correlated t-test) between the novel (open bars) and sample (shaded bars) objects (mean time ± SEM). An asterisk (*) represents significant discriminations ($p < .05$) and a cross (†) represents a marginal difference ($p = .07$). Only N-C subject consistently exhibited preference for the novel object following all delay lengths. STR subjects did not discriminate beyond a 1-hour delay. Food-deprived groups did not discriminate beyond a 2.5-hour delay.

Figure 9: Combined exploration times of objects in sample (T1) and test (T2) trials across groups (± SEM). Group designations are the same as in figure 2. Asterisks (*, $p < .05$; **, $p < .01$) represent a main effect of increased exploration of objects due to food deprivation, and a cross (†)

represents a main effect of increased exploration over trials ($p < .05$). A double-cross (‡) represents a food X stress X trial interaction ($p < .10$).

Figure 10: Norepinephrine tissue levels (± SEM) in prefrontal cortex (mPFC), hippocampus CA1 and CA3, and basolateral amgydala. Groups are designated the same as figure 2. An asterisk (*) represents a main effect of food deprivation ($p < .05$). A cross (†) represents a main effect of stress ($p < .07$), and a double- cross (‡) represents a food deprivation X stress interaction ($p < .05$).

Figure 11: Mean open field sector visits (± SEM) occurring in the first and second 3 minutes of the trial. Groups are designated the same as figure 2. An asterisk (*) reflects a main effect of time in outer sector visits ($p < .001$).

Figure 12: Object recognition performance in stress and food deprived subjects. Groups are designated the same as figure 2. Bars represent the mean time (± SEM) groups spent exploring the novel object (open bars) and the sample object (shaded bars). An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group.

Figure 13: Combined mean exploration times (± SEM) of objects in sample (T1) and test (T2) trials across groups. Group designations are the same as in figure 2. There are no significant differences in time measures across groups or trials.

Figure 14: Prefrontal monoamine activity as indexed by DOPAC / DA, HVA / DA, and 5HIAA / 5-HT ratios (± SEM). Group designations are the same as in figure 2. An asterisk (*) represents a main effect of stress ($p < .02$) and a cross (†) represents a main effect of food deprivation ($p < .03$) on dopamine activity.

Figure 15: Mean prefrontal and hippocampal CA1 amino acid levels of aspartate, asparagine, glycine, and histidine (± SEM). Group designations are the same as in figure 2. An asterisk (*) represents a main effect of stress on aspartate levels ($p < .02$). A cross (†) represents a significant ($p < .05$) stress X food deprivation interaction effect on prefrontal glycine and histidine and CA1 glycine and asparagine.

Figure 16: The sequential order of group formation (housing), manipulation (restraint), behavior testing, and sacrificing of subjects through the experiment.

Figure 17: The growth curve (mean weights ± SEM) of single-housed naïve, double-housed naïve, single-housed stress, and double-housed stress groups through the duration of the experiment. An asterisk (*) reflects a significant difference in the mean weight of the double-housed stress group from all other groups ($p < .05$).

Figure 18: Mean number (± SEM) of forced open-field rears and wall climbs recorded in either the first or second 3 minutes of the trial. Group designations are by housing status and prior exposure to chronic stress (restraint). An asterisk (*) represents a main effect of stress ($p < .01$) on the total number of rears in the 6-minute trial. An increase in wall climbing also occurred in all groups over time (main effect of time, $p < .02$).

Figure 19: Mean latency to enter a free open-field, mean latency to first approach one of two novel objects in the field, and mean total time spent in the field in the first 3 and second 3 minutes of the trial. Error bars reflect (±) SEM. A single asterisk (*) represents a marginal main effect of stress on field entrance latency ($p < .08$). A double asterisk (**) represents a main effect of stress on object approach latency ($p < .05$). A cross (†) represents a significant difference ($p < .05$)

between the single-housed stress group and all other groups in the mean total field time (first 3 minutes). A double cross (‡) represents a significant difference ($p < .05$) between single-housed stress and double-housed naïve group mean total field times in the second 3 minutes of the trial.

Figure 20: Object recognition performance in differentially housed and stressed subjects. Bars represent the mean time (± SEM) groups spent exploring the novel object (open bars) and the sample object (shaded bars). An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group.

Figure 21: Combined exploration times of objects in sample (T1) and test (T2) trials across groups (± SEM) in the object recognition trials. Group designations are as follows: S-N, single-housed naïve; D-N, double-housed naïve; S-S, single-housed stress; D-S, double-housed stress. An asterisk (*) represents a main effect of housing on total object exploration in the 4-hour delay trial ($p < .02$). A cross (†) represents a main effect of housing on total object exploration in the 2.5-hour delay trial ($p < .006$).

Figure 22: Object placement recognition performance in differentially housed and stressed subjects. Bars represent the mean time (± SEM) groups spent exploring the object in the novel location (open bars) versus that in the original location (shaded bars). An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group.

Figure 23: Combined exploration times of objects in sample (T1) and test (T2) trials across groups (± SEM) in the object placement trials. Group designations are the same as figure 20. An asterisk (*) represents a main effect of housing on total object exploration in the 2.5-hour delay trial ($p < .0001$). A main effect of time is evident in the 4-hour delay trial ($p < .0001$).

Figure 24: Mean levels (± SEM) of dopamine and its metabolites (DOPAC and HVA) in prefrontal cortex and amygdala. An asterisk (*) represents a significant housing X stress interaction on prefrontal HVA levels ($p < .01$). A cross (†) represents a marginal effect ($p < .07$) of stress on prefrontal dopamine levels.

Figure 25: Mean norepinephrine levels (± SEM) in prefrontal cortex, amygdala, and hippocampus. An asterisk (*) represents a significant difference between the double-housed stress group and all other groups ($p < .05$).

Figure 26: The growth curve (mean weights ± SEM) of single-housed naïve, double-housed naïve, single-housed stress, and double-housed stress groups through the duration of the experiment. An asterisk (*) reflects a significant difference in the mean weights of the single and double-housed stress groups from the naïve other groups ($p < .05$). A cross (†) reflects a significant difference in the mean weights between double-housed naïve and all other groups ($p < .05$).

Figure 27: Mean number (± SEM) of forced open-field visits in the peripheral sectors (above) and inner sector visits (below) in the first and second 3 minutes of the trial. Group designations are as follows: S-naïve, single-housed naïve; D-naïve, double-housed naïve; S-stress, single-housed stress; D-stress, double-housed stress. An asterisk (*) represents significant group differences in inner sector visits from single-naïve subjects ($p < .05$). A cross (†) represents a significant group difference in inner sector visits from double-housed naïve subjects ($p < .05$). A double-cross (‡) represents a significant difference in inner sector visits between double-housed stress subjects and all other groups ($p < .05$). Peripheral sector visits generally decreased over time (main effect time, $p < .0001$).

Figure 28: Mean number (± SEM) of forced open-field rears and wall climbs recorded in either the first or second 3 minutes of the trial. Group designations are the same as figure 26. An increase in the number of rears occurred in all groups over time (main effect of time, $p < .001$).

Figure 29: Mean latency to enter a free open-field, mean latency to first approach one of two novel objects in the field, and mean total time spent in the field in the first 3 and second 3 minutes of the trial. Error bars reflect (±) SEM. Group identification is the same as figure 26. A single asterisk (*) represents a main effect of housing on field entrance latency ($p < .05$).

Figure 30: Object recognition performance in differentially housed and stressed subjects. Bars represent the mean time (± SEM) groups spent exploring the novel object (open bars) and the sample object (shaded bars). Group identification is the same as figure 26. An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group. Across represents a marginally significant difference ($p = .08$).

Figure 31: Combined exploration times of objects in sample (T1) and test (T2) trials across groups (± SEM) in the object recognition trials. Group designations are as follows: S-N, single-housed naïve; D-N, double-housed naïve; S-ST, single-housed stress; D-ST, double-housed stress. An asterisk (*) represents the D-N group differing in test trial time from all other groups in the 2.5-hour delay trial ($p < .05$). A cross (†) represents the S-ST group differing from all other groups in the test trial time ($p < .05$). A double cross (‡) reflects the D-N sample trial time differing from those of S-N and D-ST subjects ($p < .05$) in the 4-hour delay trial.

Figure 32: Object placement recognition performance in differentially housed and stressed subjects. Bars represent the mean time (± SEM) groups spent exploring the object in the novel location (open bars) versus that in the original location (shaded bars). Group designations are the

same as figure 26. An asterisk (*) indicates a significant difference ($p < .05$, correlated t-test) within each group.

Figure 33: Combined exploration times of objects in sample (T1) and test (T2) trials across groups (± SEM) in the object placement trials. Group designations are the same as figure 26. An asterisk (*) represents a main effect of time on total object exploration in both the 2.5 and 4-hour delay trials ($p < .0001$).

Figure 34: Mean monoamine (5-HT, 5HIAA, and NE) levels (± SEM) in hippocampus CA1, CA3, and prefrontal cortex. Group designations are the same as in figure 26. An asterisk (*) represents a main effect of housing on monoamine levels in CA1 (5-HT, $p < .01$; 5HIAA, $p < .07$). A cross (†) represents a main effect of stress on CA3 monoamine levels (5-HT, $p < .03$; NE, $p < .01$). A double cross (‡) represents a stress X housing interaction in 5HIAA levels in prefrontal cortex ($p < .05$).

Figure 35: Mean amino acid levels (± SEM) in prefrontal cortex and hippocampus CA1. Group designations are the same as figure 26. An asterisk (*) represents a main effect of housing on CA1 levels of glycine ($p < .04$) and GABA ($p < .03$). A cross (†) represents a main effect of stress on prefrontal histidine levels ($p < .03$).

Figure 36: A review of the neurochemical changes (monoamines and amino acids) found in the four experiments. The results are presented by limbic region (medial prefrontal cortex, PFC; hippocampus, CA1, CA3; and amygdala, AMYG) and sex. Stress effects only under conditions of single (S) or double (D) housing are also identified.

General Introduction

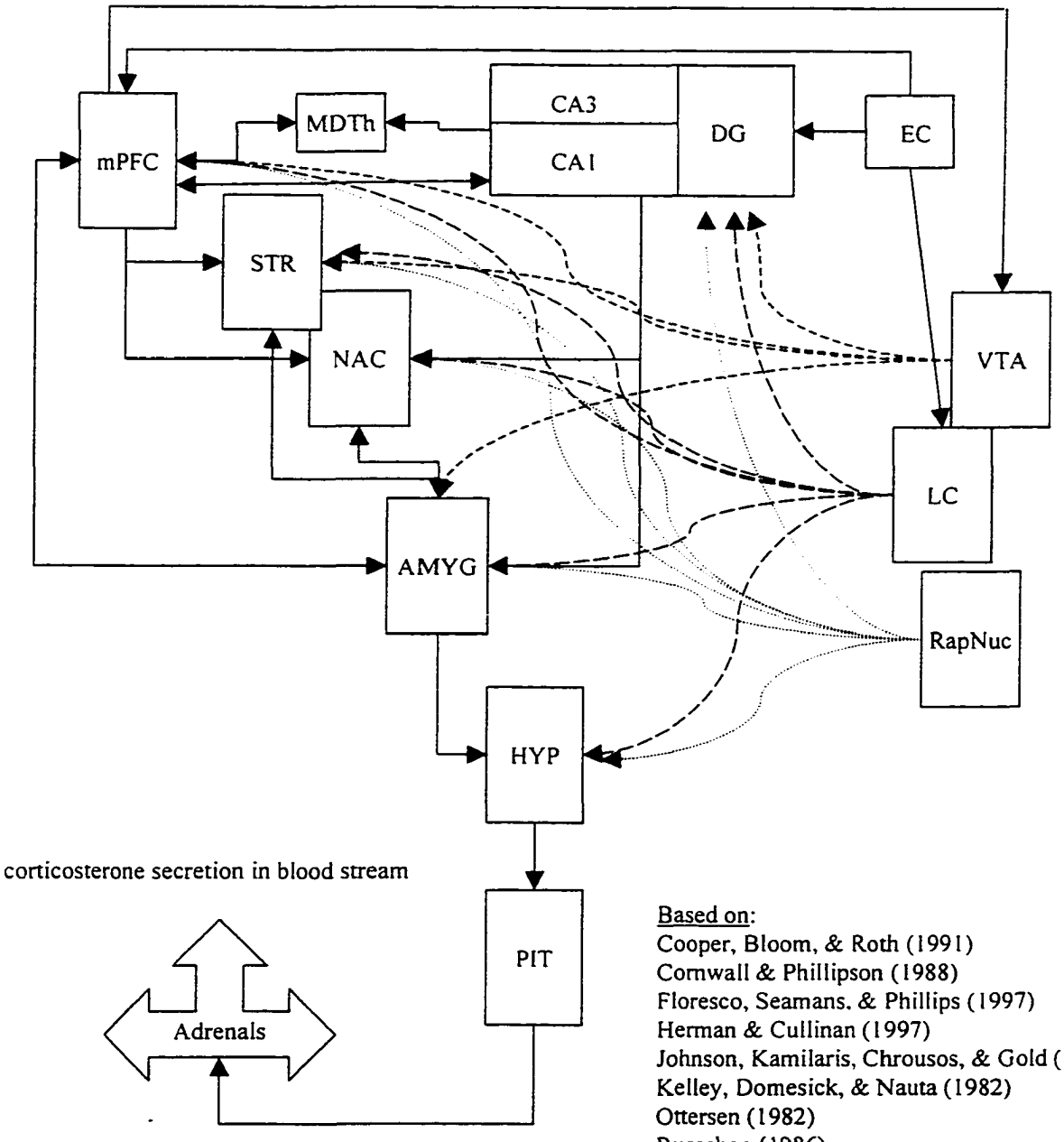
Stress is a topic of intense study under many areas of research interests and methodologies. Many models (i.e. Luine, 1997; 1994; McEwen, 1998; Saplosky, 1997; 1996) have characterized the physiological effects of chronic stress on an individual's cognitive abilities and social interactions. Conditions ranging from shock treatments, housing in a cold environment, immersion in cold water, prolonged and uncontrolled physical exertion (such as on a treadmill), immobilization (pining down the limbs), restraint, noise, flashing light, and observational stress have all been used in acute and chronic stress paradigms. The present body of research follows such a perspective and seeks to clarify some of the current theory surrounding the effects of chronic stress upon an individual's ability to react, interact, and remember elements of its environment. In addition, these studies show neurochemical changes in critical areas of the limbic system that may underlie the behavioral changes, and these changes appear to differ based on the sex of the animal. Thus, these studies illustrate the importance of sex-specific modeling of brain-behavior processes related to stress.

Herman and Cullinan (1997), in their theory of neurological stress mechanisms, attempt to organize the resultant biological events that occur in response to "stressful" stimuli. They describe a dichotomous relationship between an individual's physiological response to and perception of a "stressful" stimulus. Under this distinction, stress is defined as either *systemic* or *processive*. A systemic stressor creates an arousal response denoting an immediate physiological threat that corresponds to heightened activity in the brain's paraventricular nucleus of the hypothalamus (PVN). This activity leads to a cascade of events which up-regulate activity in the hypothalamic-pituitary-adrenal (HPA) axis, causing increases in plasma adrenal hormone levels (corticosterone in the rat, cortisol in the human). In contrast, a processive stress initiates an arousal response in the brain without necessarily involving an increase in the release of adrenal hormones by increasing neural activity in limbic and/or forebrain regions (believed to modulate

the HPA-axis via the PVN). Specifically, the hippocampus, prefrontal cortex, amygdala, bed nucleus of the stria terminalis, preoptic area of the hypothalamus, and brainstem nuclei (monoamine cell body groups) have all been implicated in the neural assessment of environmental stimuli and conditions. Thus, an HPA response could be lesser, greater, or not at all depending on the processing occurring in those limbic regions.

The interconnected areas of the medial prefrontal cortex, hippocampus, and amygdala are the focus in the current studies because of their implied roles in both stress-regulation (Herman & Cullinan, 1997) and memory processing (Kesner, 1998; Squire, 1992; Cahill & McGaugh, 1998). As shown in figure 1, the connectivity between these regions is direct and indirect, as well as, reciprocal. For instance, the basolateral amygdala and the medial prefrontal cortex have direct connections (as illustrated in the figure), however, there are also connection from both of these areas to thalamus, albeit in different nuclei (Russchen, 1986; Su & Bentivoglio, 1990). Yet, local processing in the thalamus could yield a second, more integrated pathway. Similar paths occur between the hippocampus and the prefrontal cortex (Floresco, Seamans, & Phillips, 1997). Direct connections exist both from the entorhinal cortex (the "gateway" to the hippocampus) and the subliculum (a major "output" pathway) to the medial prefrontal cortex. Furthermore, there is the also the tract between the hippocampus and the prefrontal cortex via the dorsomedial thalamus (a less direct route). Thus, as is the case for much of the telencephalon, these structures are heavily inner-twinned in their communication and processing pathways.

These varied anatomical processing pathways, and the behavioral effects of lesioning these structures, have lead many to posit that each is, at least in part, important in mnemonic activity. The amygdala has been studied in terms of the emotional component in memory (Cahill & McGaugh, 1998), and the hippocampus in terms of long-term memory formation, especially for spatial (Whishaw & Maaswinkel, 1998) and declarative information (Squire, 1992; 1986). The prefrontal cortex has been implicated in both working and time-relevant aspects to memory processing (Floresco et al., 1997; Kesner, 1998). The fact that these regions are also involved in



Based on:
Cooper, Bloom, & Roth (1991)
Cornwall & Phillipson (1988)
Floresco, Seamans, & Phillips (1997)
Herman & Cullinan (1997)
Johnson, Kamilaris, Chrousos, & Gold (1992)
Kelley, Domesick, & Nauta (1982)
Ottersen (1982)
Russchen (1986)
Sarter & Markowitsch (1983; 1984)
Segal (1977)

fig. 1

the regulation of the stress response is interesting, although not improbable. It would follow evolutionary theory that species that could process and store information regarding instances of a flight-or-flight reaction would, generally, survive more than those species that could not (Saplosky, 1997). For instance, it would be beneficial for an antelope to learn not to consistently drink from a particular pond where a pride of lions range nearby. This same response has been modeled in the laboratory (i.e. avoidance-learning) most diligently in testing the learned helplessness phenomena (as reviewed by Peterson, Maier, & Seligman, 1993). Rodents learn to avoid aversive stimuli rather quickly and extinguish those responses rather slowly. Therefore, it should not be surprising that stress-related neural activity occurs within structures that have been described as components of the learning and memory system of the brain. However, when stress is chronic and / or not decreased by the individual's neurobehavioral response, activity in those same brain areas may become maladaptive; thus, causing neural changes that may disrupt learning and memory processing in both during stress and post-stress (as reviewed by Luine, 1997).

Previous chronic stress research has focused on the hippocampus in particular because of its sensitivity to chronic restraint stress or corticosterone (CORT) administration, leading to neuron degeneration in the CA3 sub-region (Magarinos & McEwen, 1995; Magarinos, Verdugo, & McEwen, 1997; Saplosky, Krey, & McEwen, 1986) and spatial memory impairments (Luine, Villegas, Martinez, & McEwen, 1994a; 1994b; Conrad, Galea, Kuroda, & McEwen, 1996). Duration of the stress, however, is of key importance. Behavioral impairments are apparent following 21 days of stress (Luine et al., 1994a, 1994b) but not following 7 or 14 days (Luine, Martinez, Villegas, & McEwen, 1995). Prolonged, high levels of CORT have been hypothesized to be the principle initiator of these behavioral changes (Luine, 1997; McEwen, 1998; Saplosky, 1996), and, in fact, hippocampus degeneration (thought to be due to such high CORT levels) has been shown to correlate with post-traumatic stress disorder (Saplosky, 1996). Still, CORT appears to have more general role in the shaping of hippocampus morphology. Adrenalectomized

rats, thereby CORT deficient, also exhibit neuron loss in the hippocampus, but it is predominately in an adjacent region to CA3, the dentate gyrus (Sloviter, Valiquette, Abrams, Ronk, Sollas, Paul, & Neubort, 1989), leading to both spatial (Vaher, Luine, Gould, & McEwen, 1994) and nonspatial (McCormick, McNamara, Mukhopadhyay, & Kelsey, 1997) memory impairments. Following the theory of Luine (1997; 1994) that the recession of dendrites in any sub-region of the hippocampus will lead to memory impairments; then similar nonspatial memory impairments should be evident in chronic stress rats as was found in adrenalectomized rats, since, in both cases, there is recession of dendrites in the hippocampus (CA3 region due to chronic stress, dentate gyrus due to adrenalectomy). Testing this hypothesis is the first objective of these studies.

It is possible that an interaction between the prefrontal cortex and the HPA-axis also occurs during stressful events, although it may differ from that seen between the HPA-axis and the hippocampus. In the hippocampus, prolonged stress is not only correlated with dendrite atrophy, but also with significant corticotrophin-releasing factor (CRF) receptor loss (Saplosky et al., 1986). Herman and Cullinan (1997) proposed that the CRF receptors in the hippocampus serve as part of one likely feedback loop upon the PVN. On the other hand, hypothalamic ACTH has been shown to increase dopamine synthesis (Delanoy, Kramarcy, & Dunn, 1982) and CRF antagonists decrease norepinephrine release (Shimizu, Nakane, Hori, & Hayashi, 1994) in the prefrontal cortex. Others have also reported increases in prefrontal dopamine (Finaly, Zigmond & Abercrombie, 1995; Gresch, Sved, Zigmond, & Finlay, 1994; Carlson, Fitzgerald, Keller, & Glick, 1991), norepinephrine (Flugge, 1996; Gresch et al., 1994; Finlay et al., 1995), serotonin (Adell, Garcia-Marquez, Armario, & Gelpi, 1989), and glutamate (Moghaddam, 1993) in response to stressful stimuli. However, Dunn (1988) showed that these increases in the prefrontal cortex are not dependent on adrenal hormone increases. Still, if chronic stress also affects prefrontal processing, then the possibility arises that changes in either or both the hippocampus and the prefrontal cortex could be mediating the stress effects upon behavior (particularly

memory functioning). Generally, rodent models of neural memory functioning (such as Floresco, et al., 1997; Kesner, 1998; Long & Kesner, 1998; Whishaw & Maaswinkel, 1998; Wan, Aggleton, & Brown, 1999) propose that different limbic structures provide rather specialized components (such as strategy shift, timing, individual spatial-relations) that together work to create memories for events, places, things, rules, etc. The contributions of the hippocampus and prefrontal cortex to cognition have been studied extensively (i.e. Kesner, 1998; Shaw & Aggleton, 1993; Winocur, 1991; Eichenbaum, 1997; Jarrard, 1993), but, determining what each structure provides to memory processing is not the principle concern here. Instead the focus is observing whether chronic stress leads to changes in transmitter activity in either region. Unlike many of the past studies on memory, these studies do not use a lesion approach; thus a change in activity in either area could lead to memory impairment, whether it is encoding, maintenance, or recall. Tissue samples from the hippocampus and prefrontal cortex, for possible monoamine and amino acid changes (following stress), will provide additional information to the known morphological data, thus leading to a more complete physiological profile of chronic stress effects.

Differential stress effects on either prefrontal or hippocampus processing could lead to an array of behavior changes, both memory dependent and not. To date, behavior following 21 days of restraint stress has been assessed using spatial memory tasks for novelty (Conrad et al., 1996) and food placement (Luine et al., 1994a; 1994b). As stated above, nonspatial memory assessment is a primary focus in the studies described herein, but other field tasks, not assessing memory, are also used to better characterize the nature of the stress-induced behavior change. Two versions of the basic open field test, forced and free, in addition to a spatial object memory task, are used to determine whether chronic stress affects memory dependent behaviors in particular, or general exploration and motor activity. Consequently, by using several behavior tests, a more descriptive and broader profile of the behavioral changes induced by chronic restraint can be established.

Another objective is to determine if the same chronic stress regimen causes similar effects (both behaviorally and neurochemically) in males and females. Using the 21-day restraint paradigm, Galea, McEwen, Tanapat, Deak, Spencer, and Dhabhar (1997) have already shown that both baseline and stress-induced CORT levels are different in males and females. Others (i.e. Kennett, Chaouloff, Marcou, & Curzon, 1986; Aloisi, Steenbergen, Van de Poll, & Farabollini, 1994; Haleem, Kennett, & Curzon, 1988) have similarly shown using other paradigms that a sex-difference is evident when measuring the CORT response. If plasma CORT levels are indeed a reliable measure of an animal's stress response (Hennessy, Heybach, Vernikos, & Levine, 1979), then females should exhibit more robust changes in behavior, as compared to males. However, Galea et al. (1997) have also found that hippocampal morphological changes are not as extensive in stress females, as compared to stress males. This sex-difference in morphological response to CORT could be due to gonadal hormone influences upon the distribution (Patchev & Almeida, 1996) and responsiveness (Turner, 1992) of glucocorticoid and mineralcorticoid receptors in the hippocampus in males and females. Such a discrepant pattern of effect across sex, on the morphological level, could also lead to a different pattern of effect in memory processing. Female memory then may not be impaired after chronic stress since CORT has a lesser influence upon female hippocampal morphology. This hypothesis will be tested for both spatial and nonspatial object memories, as well as, open field activities.

A final objective is to determine the extent to which housing conditions may moderate the effects of chronic stress on behavior and neurochemistry, and whether these conditions differentially influence males and females. There are indications of a possible interaction between sex, corticosterone release and the environment (such as type of housing, number of cagemates, etc.) For instance, females exhibit basal corticosterone levels three times that of males when isolated, but their corticosterone is reduced (to male group levels) when more females are added to the tub (Brown & Grunberg, 1995; 1996). Males housed with other stress males exhibit more activity in an open field than differently housed controls, but do not show

altered amounts of plasma corticosterone (Ottenweller, Servatius, Tapp, Drastal, Bergen, & Natelson, 1992). Moreover, individually housed males do not exhibit the as high an increase in prefrontal dopamine activity as group housed (Holson, Ali, & Scallet, 1988). Shors and Dryver (1996) found combinating stress and another environmental condition, food-deprivation, leads to radial-arm maze perseverations (redundant arm visits). The interaction of stress and food-deprivation on memory functioning could be explained by research suggesting prefrontal dopamine utilization increases when subjects are placed under dietary restraints (Carlson, Herrick, Baird, & Glick, 1987). Hence, if HPA-axis activity is regulated by the prefrontal cortex (or any other limbic region that is sensitive to such environmental conditions), then known environmental influences upon either limbic neurochemistry or behavior need to be understood within the context of a particular stress model. Here, the common range of food restriction used for radial-arm training (85-90% post-stress baseline weight) and individual versus paired housing are manipulated within the stress experiments to assess whether these factors enhance or buffer the stress-induced changes in behavior and neurochemistry.

The following studies, then, investigate how environmental conditions influence chronic restraint-induced behavior change in males versus females using several behavioral indexes. The order of studies is as follows. In the first pair of studies, the variable of induced-motivation (i.e. food deprivation) is tested using tasks that typically do not require the extra "motivational" component of food-deprivation. The tasks utilized, open-field and object recognition, do not require a reward contingency. Thus, if food depriving previously stressed subjects influences behavior, then these tasks give us an initial assessment by which to assess this interaction. We can also determine if food restriction influences other non-cognitive aspects (i.e. amount of object exploration) on which these tasks depend (such as object memory), and if these factors are constant across sex. In the second pair of studies, the housing environment is manipulated in order to assess its possible role it in the 21-day chronic stress paradigm. In these studies, subjects are housed either individually or in pairs (similar to Wade & Maier, 1986). This manipulation

provides several comparisons. First, we can assess whether a particular housing status is necessary for stress to be effective in the 21-day paradigm. Second, we can assess whether housing influences the behaviors observed in these field-based tasks. And, thirdly, we can determine whether the patterns seen in the first two points are similar or different in males versus females. The first study in each pair uses male subjects and the second uses female subjects.

Food Deprivation Modulates Chronic Stress Effects on Object Recognition in Male Rats: Role of Monoamines and Amino Acids

Regimen of stimuli that create arousal in an animal, via the sympathetic nervous system or the hypothalamic-pituitary-adrenal (HPA)-axis, lead to transitory changes in behaviors, specifically those that require an animal to learn or remember specific, adaptive responses to environmental stimuli. Luine (Luine 1997; 1994) has shown how the duration (days) of 6-hour restraint leads to either performance enhancements, after 13 days (Luine, Martinez, Villegas, Magarinos, & McEwen, 1995), or performance decrements, after 21 days (Luine, Martinez, Villegas, & McEwen, 1994a), using the radial-arm maze, a spatial memory task. Conrad (Conrad, Galea, Kuroda, & McEwen, 1996) similarly showed spatial memory deficits after 21 days of restraint using the Y-maze. Others have shown that stress influences performance of a number of other behavioral tasks (Diamond, Fleshnet, Ingersoll, & Rose, 1996; Flint, Metzger, Beson, & Ricco, 1997; Marby, McCarthy, Gold, & Foster, 1996; Servatius & Shors, 1994; Shors & Servatius, 1997) that has led to intensive research into the underlying mechanisms of these changes.

In general, stress-dependent behavioral change has been ascribed to heightened release of adrenal corticosterone (CORT) that is known to alter both neuroanatomy and neurochemistry (Saplosky, 1996). Luine, Spencer, and McEwen (1993) found chronic high CORT plasma levels (via ingestion) increased serotonin (5-HT) tissue levels in dentate gyrus, but decreased 5-HT and norepinephrine (NE) levels in frontal cortex. Magarinos and McEwen (Magarinos & McEwen, 1995; Magarinos, Verdugo, & McEwen, 1997) and Saplosky (1996; 1986) have continually demonstrated that the chronic release of CORT from the adrenal glands, in response to stress, causes remodeling of the CA3 dendrites in the hippocampus, through excitatory amino acids (EEA) via NMDA receptors (Moghaddam, Bolinao, Stein-Behrens, & Sapolsky, 1994; Stein-Behrens, Lin, & Sapolsky, 1994).

The temporal relationship between the stress-dependent physiological effects and behavioral change is critical in these animal models. For instance, chronic high CORT and / or stress causes hippocampal CA3 apical dendrites to recede, but the effect appears to reverse following one week without stress (Conrad, Magarinos, LeDoux, & McEwen, submitted manuscript). Interestingly, a similar relationship exists between stress and radial-arm maze performance, in that, ten to fourteen days post-stress, the impairing effect of stress on spatial memory diminishes (Luine, et al., 1994). Dachir et al. (Dachir, Kadar, Robinzon & Levy, 1993) also found that chronic, high CORT levels lead to transitory spatial memory deficits on the radial arm maze, but that this effect dissipates with continual training beyond the second week post-treatment. The diminishment of the stress effect over time creates a problem when extensive behavioral training is needed for certain tasks (such as radial-arm maze). Conrad et al. (1996) circumvented this procedural difficulty by using a non-reward-contingent spatial task – the Y maze. All trials could then be conducted within the known "window" of detriment, and they found stressed subjects did not exhibit a significant preference for the novel "location/space" (the novel arm) following a four hour delay.

These previous studies showed that chronic stress could impair spatial memory performance. In this study, we investigated whether similar deficits are also observable in a non-spatial memory task. Object recognition (Ennaceur & Aggleton, 1994), like the Y-maze, uses novelty as an indirect measure of previously experienced stimuli, thus reward-contingent training is not necessary. Therefore, it meets the criteria of a behavioral task, involving little training, which can be used within the apparent "window" of detriment (as the Y-maze). However, in contrast to the Y-maze, object recognition does not appear to be hippocampal dependent because fornix and hippocampal lesions do not significantly affect object recognition performance (Ennaceur & Aggleton, 1994; Rothblat & Kromer, 1991).

An additional issue surrounding these behavioral models of stress is the role of task-requirements. For instance, habituation to swim-stress aids performance in the Morris water-

maze, such that typical deficits were not observed in aged, chronic-swim subjects, as compared to aged, handled controls (Marby et al., 1996). Similarly, food deprivation is used in many behavior tasks as a "motivating" or "facilitating" stimulus, including the radial-arm maze. However, food deprivation has been also used as a stressor (Katz, Roth, & Carroll, 1981; Soblosky & Thurmond, 1986). Food deprivation increases serum CORT levels, decreases the basal dopamine (DA) level in the nucleus accumbens, and decreases the DA release after eating food (Pothos, Creese, & Hoebel, 1995). In addition, Carlson (Carlson, Glick, Hinds, Baird, 1988; Carlson, Herrick, Baird, Glick, 1987) found that DA utilization increases in the prefrontal cortex at least during the first 48 hours of food deprivation. These neurochemical changes could influence the behaviors associated with learning and memory as exemplified by Miller and Dess's finding (1996) of attenuated exploration of the radial-arm maze.

In this study we used object recognition to test whether chronic stress impairs non-spatial memory performance. We additionally determined whether food deprivation modulates those observed stress effects. Since critical areas involved in working-memory dependent tasks, such as medial prefrontal cortex, hippocampus, nucleus accumbens and amygdala (Floresco, Seamans, Phillips, 1997; Quirarte, Roozendaal, McGaugh, 1997), are also affected by stress (Herman & Cullinan, 1997), we examined monoamine and amino acid levels to determine how chronic restraint and subsequent food deprivation influence neural processing in these four areas.

Experiment 1

Methods

Subjects

Thirty-three male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), 40-60 days old upon arrival were used. All subjects (but one control) were double housed in plastic tubs during the initial acclimation week and throughout the three-week stress period on a 12/12 reverse light-dark cycle (lights on at 8:00 p.m.). In the two days prior to stressing, all subjects were weighed. Based on these baseline measures, each double-housed tub of subjects was matched with another tub of similar average combined weight subjects and designated as stress subjects. Throughout the twenty-one days of stress, half of the paired subjects (n=16) were placed in Plexiglas restrainer tubes for 6 hours (in the their home cage in the animal room) during the dark phase (always ranging between 9a.m and 5:30 p.m.) Restrainers allow ample air, but limit head and limb movement. On stress days 7-8, 14-15, and 20-21, all subjects were weighed in the a.m.

Following the last stress regimen, subjects were immediately changed to single housing (in identical size tubs), and food was taken away from the assigned food-deprived subjects (see figure 1). Half of the naïve and stressed subjects were randomly selected (one from each housing pair) for food deprivation. Thus, there were four groups of single housed subjects for the subsequent week of behavioral testing: naïve-controls (NC, n=9), naïve-food deprived (FD, n=8), stress (ST, n=8), and stress-food deprived (STFD, n=8). All subjects had free access to water and were weighed each day. Food deprived subjects' weights were lowered to 85-90% of their average weight taken in the morning on stress day 21 (prior to restraint) and post-stress day 1. Based on the increasing weights of the NC subjects, five grams were added to each food deprived subject's goal deprivation weight level on post-stress day 4. Under single housed conditions,

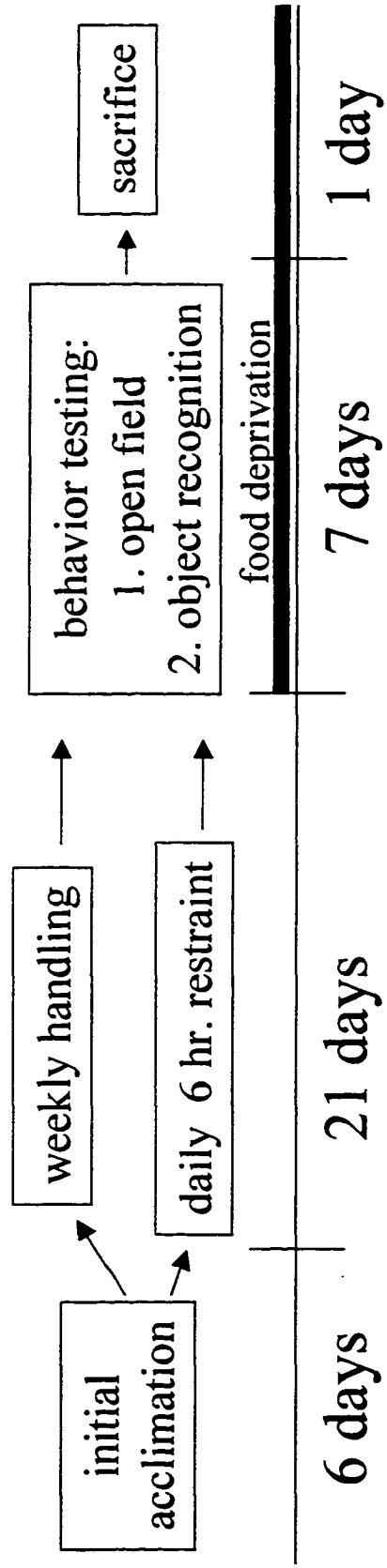


fig. 2

free-feed subjects had full-access to their food at all times (ad-lib). Deprived subjects were given a food ration of 2-4 rat chow pellets depending on their weight loss no earlier than 1 hour after testing. All subjects were sacrificed 8 days post-stress.

General Procedure and Apparatus

Behavioral testing was conducted on an open field (102.5 cm X 61.5 cm). A Formica table top with temporary opaque walls, approximately 40-50 cm high, created the enclosed field. For the initial open field trial, the floor was marked off into 15 equal (20.5 cm) squares (5 X 3). For subsequent trials, that had objects in the field, the area was shortened to 9 squares (3 X 3). Behavior testing occurred over 7 days, post-stress. Day one included an open field trial and an object recognition habituation trial. Over the next 6 days, 3 more object recognition trials were conducted.

Open Field

Day one post-stress, subjects were tested on the open field. They were placed in the center of the open field (in a matched predetermined order) and observed for 6 minutes. Behaviors recorded include sector visits, rears, grooms, and defecations. A visit was defined as the subject bringing at least half of its torso into a sector (no subject can be recorded having visited two adjacent sectors at the same time). Rearing was defined as a subject raising its upper torso, so that its forelimbs are at least at the position of its head during ambulation (3-4 cm of the surface). Behaviors were tabulated for the first 3 minutes and the second 3 minutes of the 6-minute trial (Katz, et al., 1981).

Object Recognition

Two objects were placed at the south end of the field, opposite the experimenter. The objects were placed one sector from the south, east, and west wall. Thus, the objects were equidistant from each other by one sector. Following the basic procedures of Ennaceur and

Aggleton (1994) and Willig, Van de Velde, Laurent, M'Harzi, and Delacour (1992), we used the first object recognition test with no delay to acclimate the subjects to the presence of unfamiliar objects within the shortened field. It was conducted exactly as the subsequent trials, but the data collected was not included in the analysis nor were any of the objects reused. Trials that were included in the analysis began post-stress day two.

All object recognition sessions consisted of two 3-minute trials: a sample trial (T1) and a recognition trial (T2). The two sessions were separated by an inter-trial interval of 1 minute, 10 minutes, 1 hour, or 4 hours. In T1, two similar (practically identical) objects were located in the two positions in the south end of the field. For T2, one object was replaced by a distinctly different, novel object. Placement positions and determination of which objects were used as sample versus novel were fully counterbalanced within each separate delay session across groups. All items and the field were washed with water after each subject trial.

In both trials, the subject was placed in the opposite north-center sector facing the north wall. Time spent exploring the objects was recorded using two stopwatches (one for each object). An exploration behavior was defined as the subject facing the object, with its nose within 2 cm of the object. If the subject manipulated the object with his paws while facing it, also was counted as an exploration; however, if the subject touched the object, or crawled over it, without actively facing it, it was not counted. Object exploration time was recorded during both T1 and T2. It was determined a priori that any subject that did not spend at least 10 combined seconds exploring the two objects was to be excluded from that session's behavioral analysis.

The day following the last behavior test, subjects were sacrificed and their brains removed (as previously described Luine, Bowling, & Hearns, 1990; Luine, Grattan, & Selmanoff, 1997). Briefly, each subject was singularly brought into a separate room and decapitated, without anesthesia, and the brain was quickly removed and placed in dry ice. The brains were stored at -70 degrees until chemical analyses.

Neurochemical Analyses

Brains were sectioned at 300 μm at – 6-8 °C in a microtome cryostat. Tissue punches were taken from the sections on a microscope stage maintained at -11.5 °C and placed in 1ml Ependorf tubes. A sodium acetate buffer containing α-methyl-dopamine and homoserine was added prior to centrifugation as internal standards for monoamine and amino acid analysis, respectively. The pellet was resuspended for protein analysis by the Bradford method (Bradford, 1976). High performance liquid chromatography (HPLC) with electrochemical analysis (EC) analysis was used to quantify supernatant levels of monoamines (5-HT, DA, and NE) and metabolites (5HIAA, DOPAC, and HVA). The mobile phase, described elsewhere [36], contained 3 percent acetonitrile and was pumped through a Waters Alliance module connected to an ESA Coulochem II detector (+ 0.48 - +0.50 V potential) via a C-18 reverse phase column (Brownlee Velosep RP-18, 3um). An additional 100% methanol gradient was introduced into the flow (99.5% mobile phase: 0.5% methanol) to increase peak sharpness. Single sample runs averaged between 12-20 minutes.

In order to quantify amino acids [using methods described elsewhere (Grattan & Selmanoff, 1993; Luine et al., 1997)], an aliquot of the remaining supernatant was derivatized with o-phthalaldehyde and ß-mercaptoethanol and injected into a Waters 717 automated refrigerated injected system using a 510 pump. The mobile phase (12% methanol and 5% acetonitrile) was pumped through a C-18 reverse-phase column (Waters Nova-Pak) then an ESA 5011 analytical cell. An ESA 5200 Coulochem II detector was set at +0.20 V to oxidize and remove derivatization contaminants and + 0.40 V to oxidize and detect amino acids. Sample runs averaged between 90 and 120 minutes in order to separate aspartate, glutamate, asparagine, serine, histidine, homoserine, glycine, threonine, and GABA.

Data Analysis

For open field measures, a mixed 2 X 2 X 2 ANOVA was used to test differences between stress condition, food deprivation, and time (first 3 minutes vs. second 3 minutes in the field). In the object recognition task, recognition of a sample object is defined as spending a significantly greater amount of time with the novel object. Correlated measures t-tests determined significant object discriminations within groups during T2. Inter-group comparisons for object preference during T2 were conducted by using a between subjects 2 X 2 (stress X food) ANOVA for each of the following: an index of habituation (T1/2 - T2 novel), novel preference (T2 novel – T2 sample), and an index of discrimination [(T2 novel - T2 sample) / T2]. In addition, a mixed 2 X 2 X 2 (stress X food X time) ANOVA tested whether object exploration times differed across T1 and T2 between groups (see 16 for further details behind these analyses). Subjects that did not explore the objects a minimum 10 of seconds (combined) were not included in the object recognition analysis.

Neurochemical tissue levels for monoamines are expressed as pg / ug protein and amino acids as ng / ug protein. Monoamine and amino acids levels that fell beyond 2 standard deviations beyond the group mean were considered outliers and were subsequently removed from the analysis. A between subjects 2 X 2 (stress X food) ANOVA tested group differences for all neurochemicals in reference to each group's stress and food deprivation status. Fisher's LSD was used for all post-hoc analyses. All statistical analyses were conducted using GB-STAT (Dynamic Microsystems Inc).

Results

Weight Gain

Subjects' weights were monitored throughout the stress and behavioral testing period. The restrained subjects weighed less than naïve subjects from stress day-7 (naïve = 295.38 ± 2.71g, stress = 285.94 ± 2.91g) through stress day 21 (naïve = 359.44 ± 4.15g, stress = 341.25 ±

4.13g). The weight differences were significant for stress ($F_{1,31}$= 5.86, p = .02), time ($F_{3,93}$= 1904.56, p < .001), and the stress X time interaction ($F_{3,93}$= 17.35, p < .0001).

Behavioral Measures

Open field measures were tallied in the first versus second three minutes of the trial. The number of outer sector visits decreased over time across all groups ($F_{1,30}$= 20.77, p < .001) with no other significant interactions or main effects of stress or food deprivation (NC = 64.67 ± 3.54, 56.56 ± 3.87; FD = 66.25 ± 4.25, 52.00 ± 5.82; ST = 60.50 ± 4.67, 56.00 ± 2.04; STFD = 55.63 ± 4.07, 49.13 ± 3.72). Inside sector visits showed no significant differences between or within groups (NC = 6.22 ± 1.47, 7.00 ± 1.50; FD = 6.25 ± 1.73, 5.38 ± 1.00; ST = 6.13 ± 1.44, 5.88 ± 1.11; STFD = 6.00 ± 1.04, 6.25 ± 1.19). Due to the rare occurrence of inside sector rears, outside and inside rears were collapsed within the two 3 minute trial halves. After combining data, the same main effect, decrease over time ($F_{1,30}$= 5.59, p < .02), was observed in total rears (NC = 16.11 ± 1.85, 12.67 ± 2.11; FD = 17.38 ± 1.92, 15.13 ± 1.64; ST = 14.38 ± 1.86, 11.13 ± 1.20; STFD = 15.25 ± 2.19, 14.88 ± 1.54).

As shown in figure 2, all groups: NC (naïve-control), FD (naïve-food-deprived), ST (stressed), and STFD (stressed-food-deprived) generally attended to the novel object more than the sample object. Notable exceptions occurred in the 1 minute and 4 hour delay conditions. In the 1 minute delay, the FD group exhibited a preference to the novel object, although statistically marginal (t_7 = 2.03, p = .08). This was due to one subject spending 13 seconds more with the sample over the novel object; otherwise, the group showed an overall preference to all novel objects. The other exception was the performance of the ST group following the 4-hour delay. As a group, they did not exhibit a significant preference to the novel object (t_7 = 1.07, p = .32). In fact, only 2 stressed subjects exhibited any preference toward the novel object. This is in stark contrast to the STFD group that did prefer the novel object. Thus, the ST subjects did not discriminate between a sample versus novel object at four-hour delays (i.e. the ST are unable to

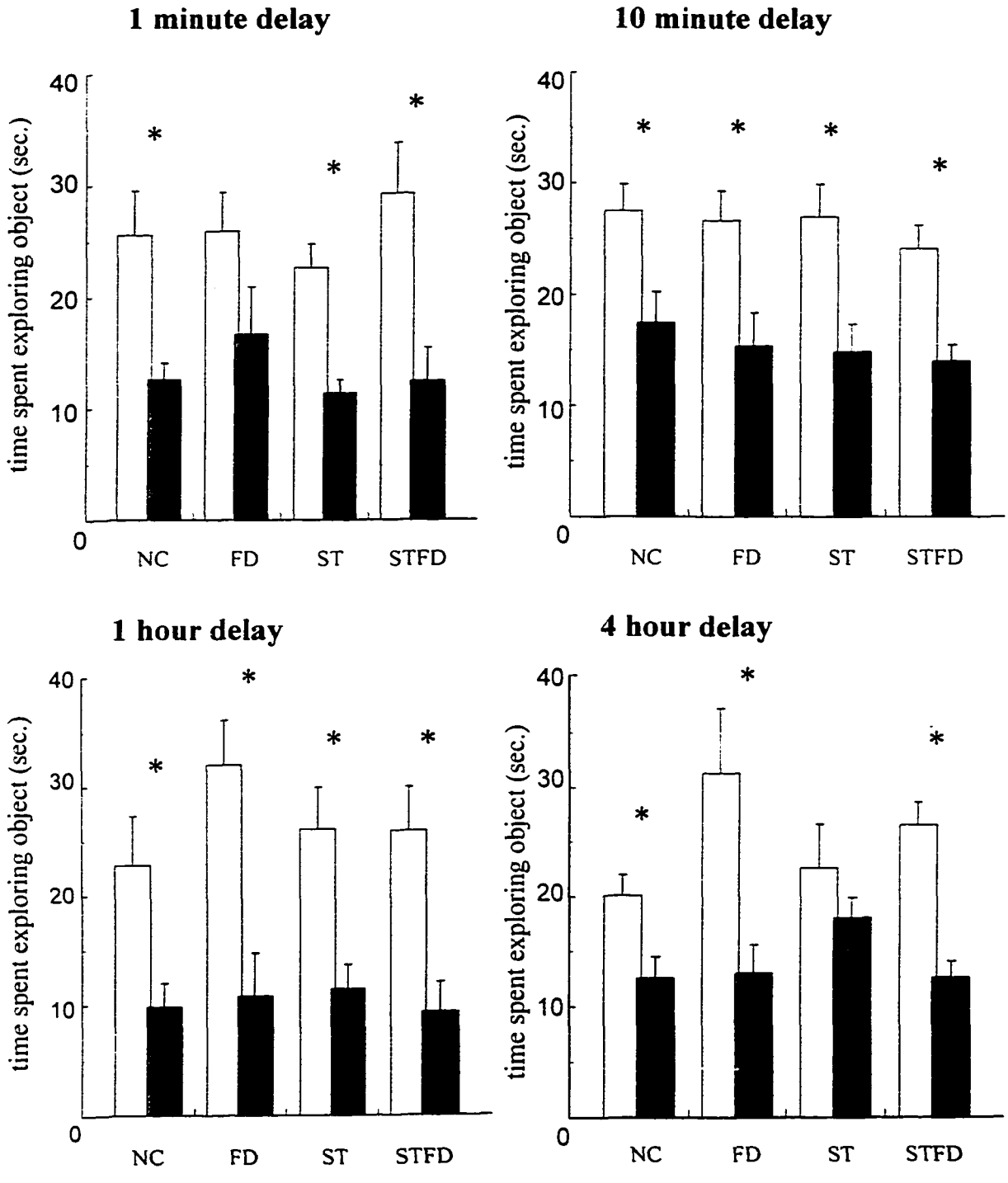


fig. 3

remember sample objects following a 4-hour delay). All other groups (NC, FD, and STFD) exhibited a clear preference to the novel object at 1 minute, 10 minute, 1 hour, and 4 hour delays at the $p = .01$ level or better.

Additional differences between groups on the measures of habituation, novel versus sample time and discrimination ratio also occurred across the delay trials. Food deprived subjects, regardless of stress condition, exhibited a significantly greater amount of habituation to the sample objects after a 1-hour delay [$F(1, 26) = 7.72$, $p < .01$]. Similarly, food deprivation was associated subjects spending a greater time with novel versus sample objects [$F(1, 29) = 9.44$, $p < .005$] and exhibiting a higher discrimination ratio [$F(1, 29) = 14.45$, $p < .0008$] after a 4-hour delay. At four hours, the ST group had the lowest discrimination ratio (NC = 0.25 ± 0.05, FD = 0.41 ± 0.06, ST = 0.08 ± 0.08, STFD = 0.36 ± 0.03), however, the main effect of stress was only marginally significant [$F(1, 29) = 3.54$, $p < .07$].

General object exploration differences across all groups were evident in both the 10 minute and 1 hour trial (see figure 3). In the 10-minute delay, all groups spent more time exploring the objects in the test trial. However, in the 1 hour delay, both FD and STFD groups generally explored more in both trials, while NC and ST subjects generally explored objects less, although they did increase their exploration in the test trial.

Neurochemical Analyses

Food deprivation, but not stress, affected monoamine levels. 5HIAA levels were higher in the prefrontal cortex and CA1 in both food-deprived groups. Using a 5HIAA/5HT ratio to assess serotonergic activity, prefrontal cortex ($F_{1, 26} = 9.84$, $p < .005$) and CA1 ($F_{1, 26} = 6.21$, $p < .02$) showed greater activity when the subject was food deprived (see figure 4).

Amino acid levels changed in both stressed and food-deprived groups in CA3 and nucleus accumbens (see figure 5). Stress increased CA3 histidine and glycine, and food deprivation decreased nucleus accumbens histidine levels. Prefrontal aspartate and GABA, CA3

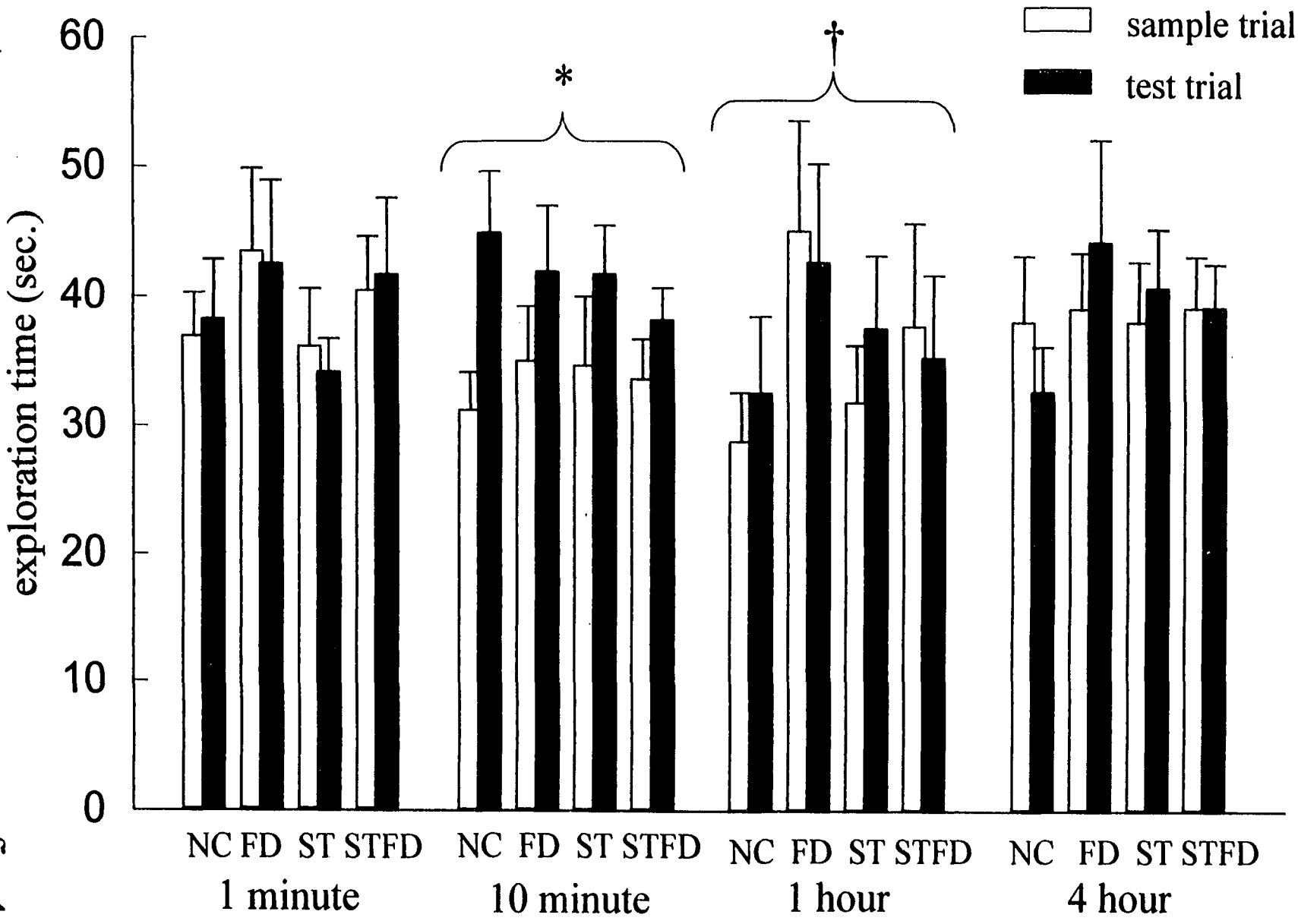


fig. 4

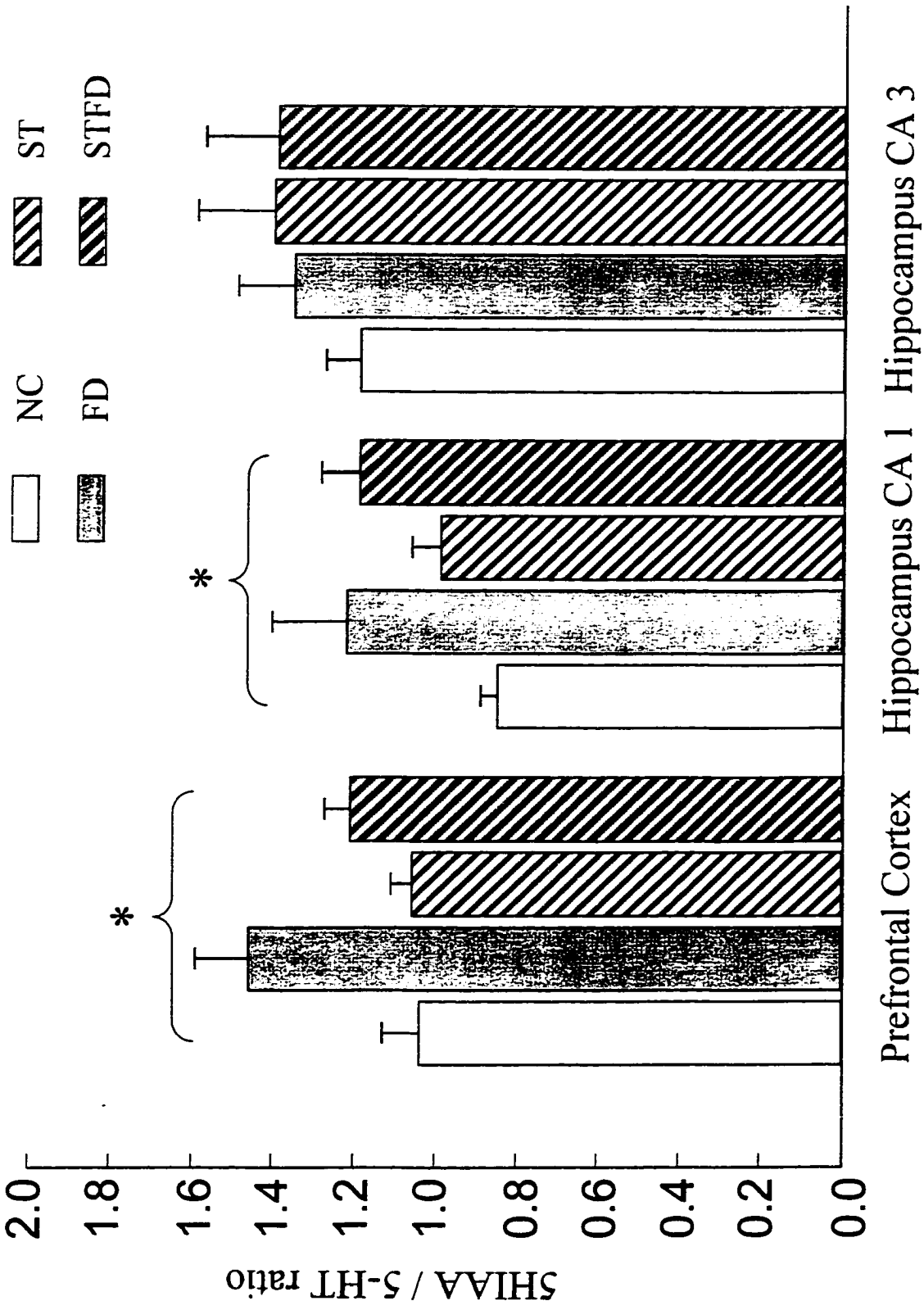


fig. 5

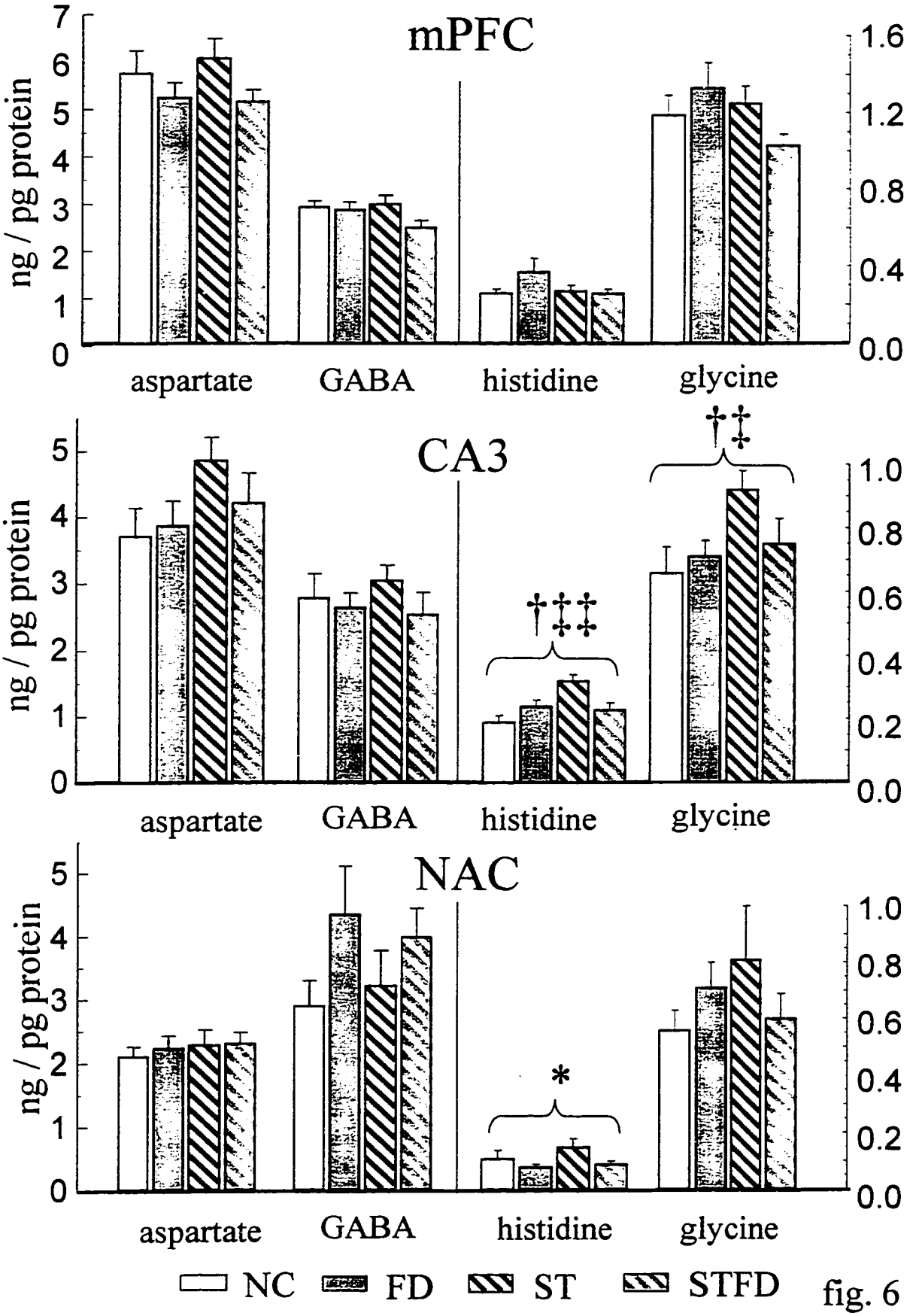


fig. 6

aspartate, and nucleus accumbens GABA were changed due to either stress or food deprivation, but only reached a marginal level of significance ($.05 < p < .10$). This was also the case for asparagine in prefrontal cortex, serine in CA3, and threoine in nucleus accumbens (data not shown).

In summary, stress and food deprivation, alone and in combination, did not affect general activity, but these variables did affect performance of an object recognition task. As the delays between sample and test lengthened, discrimination between the sample object and the new object was impaired by stress. One week of food deprivation in stressed subjects mitigated the stress effect. Therefore, results suggest that impairments in non-spatial memory, induced by chronic restraint, can be reversed by sub-chronic (several days) food deprivation. Food deprived subjects exhibited greater 5-HT activity in the frontal cortex and hippocampus and decreased histidine in the nucleus accumbens, regardless of stress condition. Chronic stress, regardless of food deprivation status, increased histidine and glycine levels in CA3.

Discussion

Behavioral Effects of Restraint Stress and Food Deprivation

These results show that chronic stress impairs object recognition memory at a 4-hour delay. Moreover, the fact that STFD subjects significantly differentiated between novel and sample objects, suggests that sub-chronic food deprivation can modulate chronic stress-induced behavioral changes in this non-reward contingent task. The effects of stress on 4-hour delay object recognition, are consistent with those reported by Conrad et al. (1996), but additionally suggest that stress-induced behavioral changes extend beyond those associated with spatial memory.

We must also consider the differences observed in exploration of the objects in the sample and test trials. Most notably, the groups did exhibit differences in the amount of time exploring the objects, regardless sample or test trial, in the 1-hour delay trial. The FD subjects

generally spent more time than the other groups exploring the objects. This trend continued in the 4-hour trial although not to statistical significance. Renner previously observed that the time spent exploring non-manipulable objects (as well as how they are explored) is dependent upon the age and sex of the rat (Renner, Bennett, & White, 1992) as well as the adult housing conditions during testing (isolated versus enriched) (Renner, 1987). Thus, we must consider the possibility that food deprivation may increase the tendency for these rats to approach field stimuli. However, it appears that this effect occurs regardless of object familiarity (Renner, 1987).

In contrast to results here, previous studies have shown a decrease in ambulation by stressed subjects in the open field (Conrad et al., submitted; Katz et al., 1981; Soblosky & Thurmond, 1986). However, it should be noted that such studies utilized multiple stressful stimuli (including shock, tail pinch, and cold) over the chronic stress period, or immediately following, that cause a greater HPA-axis reaction (Herman & Cullinan, 1997) with greater CORT release (Armario, Hidalgo, & Giralt, 1988; Hennessy, Heybach, Vernikos, & Levine, 1979) than chronic restraint alone.

Neurochemical Effects of Stress Restraint and Food Deprivation

Food deprivation had a more influential effect then stress on monoamine transmission in critical memory areas (i.e. increased serotonergic activity in both hippocampal CA1 and prefrontal cortex), and decreased nucleus accumbens histidine synthesis. Chronic stress selectively increased histidine and glycine in CA3. In both cases, the ST subjects had higher levels than all other groups, including the STFD group. Both of these amino acids are involved in inhibitory neurotransmission: glycine and histidine (the precursor for histamine). Since these methods cannot determine the precise tissue distribution of these amino acids, these levels could reflect either an increase in glycine storage and histamine synthesis or an increase in glycine usage and a decrease in histamine synthesis. Still, both glycine and histamine are known to modulate hippocampal electrical activity and affect memory-dependent processing (Bekkers,

1993; Goldstein, Rasmusson, Bunney, & Roth, 1994), and we only observed changes in the ST group. The increased activity in prefrontal and CA1 5-HT pathways may have contributed to the reversal in behavior and / or the activity in CA3 histamine and glycine, but the nature of these interactions needs exploration.

Therefore, altered neuronal activity, predominantly 5-HT, was only observed in food deprived groups, while stress effects were observed in inhibitory amino acids. The lack of monoaminergic stress effects, which was surprising based on previous studies, could be related to the timing of sacrifice (one day following the last behavior test). Thus, the animals might not have been in an "active" or "aroused" state. In the next experiment, we explored whether increasing the difficulty of the task and sacrificing immediately after a sample trial (during the onset of the delay) would change the behavioral and neurochemical profile of these subjects.

We followed the same general behavioral procedures as in experiment one, but we increased the size of the open field used for all tasks (open field and object recognition). McCormick's (McCormick, McNamara, Mukhopadhyay, & Kelsey, 1997) object exploration times, using a much larger field (125 X 125 cm), averaged only 6-13 seconds per object. Since we observed differences in total exploration time, across groups, in two of the four recognition trials, a larger field may lend to greater group distinction in the time spent exploring objects. If chronic restraint or food deprivation is causing the subjects to react to the objects differently, then, in a larger arena, it should be easier to discern differences in object approach-avoidance. Furthermore, if stressed subjects explore the objects to the same degree and still fail to exhibit preference for the novel object during the test trail, then we can feel more confident in accepting the hypothesis of a memory deficit. Delays of 1 hour, 2.5 hours, and 4 hours were chosen to determine if the minimal delay needed to observe impairment was actually less than that observed in experiment one.

The time of tissue sampling was also changed from experiment one. Unlike subjects in experiment one, the subjects in this experiment were sacrificed after placement on the field for a

sample trial. Therefore, assayed monoamine levels better represent trial arousal levels (in contrast to homecage, baseline activity in experiment one). We believed that monoamine activity differences due to previous stress exposure might become more evident following task-induced arousal. Furthermore, post-mortem CORT was also assessed to determine if serum CORT levels are differentially elevated in naïve, previously stressed, and food deprived subjects during the trial delay (one week post-stress).

Experiement 2

Methods

Subjects

Twenty-five male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) aged 40-60 days were used. Subjects were assigned to groups, weighed periodically, and behaviorally assessed as in experiment one. Post-mortem blood samples were collected at sacrifice, post-stress day 8.

General Procedure and Apparatus

For both open field measures and object recognition, we used a larger field than previously in experiment 1 (88 X 88cm square field with 60cm high walls). In these trials, we differentiated between rears that involved leaning against the field walls (wall climbs), versus those away from the walls (rears). For object recognition, objects were placed at the far end of the field from where the subject was initially located, equidistant from the walls. Experimenter was seated approximately 1 m from the field, and observed subjects using a monitor connected to a Panasonic video camera that faced the field from above the north wall. All trials were coded at a later date from the tape. In addition, subjects were sacrificed, in a separate room, following an object recognition sample trial. Neurochemical analyses followed the same procedures as in

experiment one and serum CORT levels were assessed using RIA methods described elsewhere (Luine et al., 1995).

Results

Stress Effects on Weight Gain and CORT Levels

Stressed subjects weighed less from day 7 of stress through termination of the stress period ($F_{4, 92}$= 13.36, $p < .0001$) (data not shown). Serum CORT levels (μg / 100 ml) at sacrifice showed significant group differences (NC = 4.11 ± 1.14, FD = 13.45 ± 1.60, ST = 4.48 ± 4.48, STFD = 10.49 ± 2.16), but these were only due to food deprivation not stress ($F_{1, 21}$= 25.54, $p < .0001$). Food deprivation increased serum CORT levels 2-3 fold.

Behavioral Measures

There were no effects of stress or food deprivation on ambulation, defections or grooms (data not shown). Wall climbs did differ across time ($F_{1, 22}$= 6.44, $p < .02$) and rears occurred more often in STFD subjects [stress X food deprivation $F_{1, 22}$= 5.50, $p < .03$] over the six-minute trial (see figure 6).

All groups showed a general preference for the novel object following a one-hour delay (see figure 7), and in addition, the food deprived groups spent more time exploring the objects during test trials. These effects are also evident in significant differences for exploration time, object discrimination, and discrimination ratio. One NC subject was dropped because of failing to meet the minimum exploration criteria. As early as a 2.5-hour delay, the differences between groups became evident. The NC and STFD groups exhibit clear discrimination, and the FD subjects show a marginal discrimination. The ST subjects did not show discrimination below the .10 level of significance and exhibited the least amount of habituation [$F(1, 21) = 4.06$, $p = .05$] to the sample objects (NC = 6.29 ± 2.21, FD = 2.87 ± 3.73, ST = -0.75 ± 2.81, STFD = 7.04 ± 2.24). Such results may have been due to those subjects spending the least amount of time with

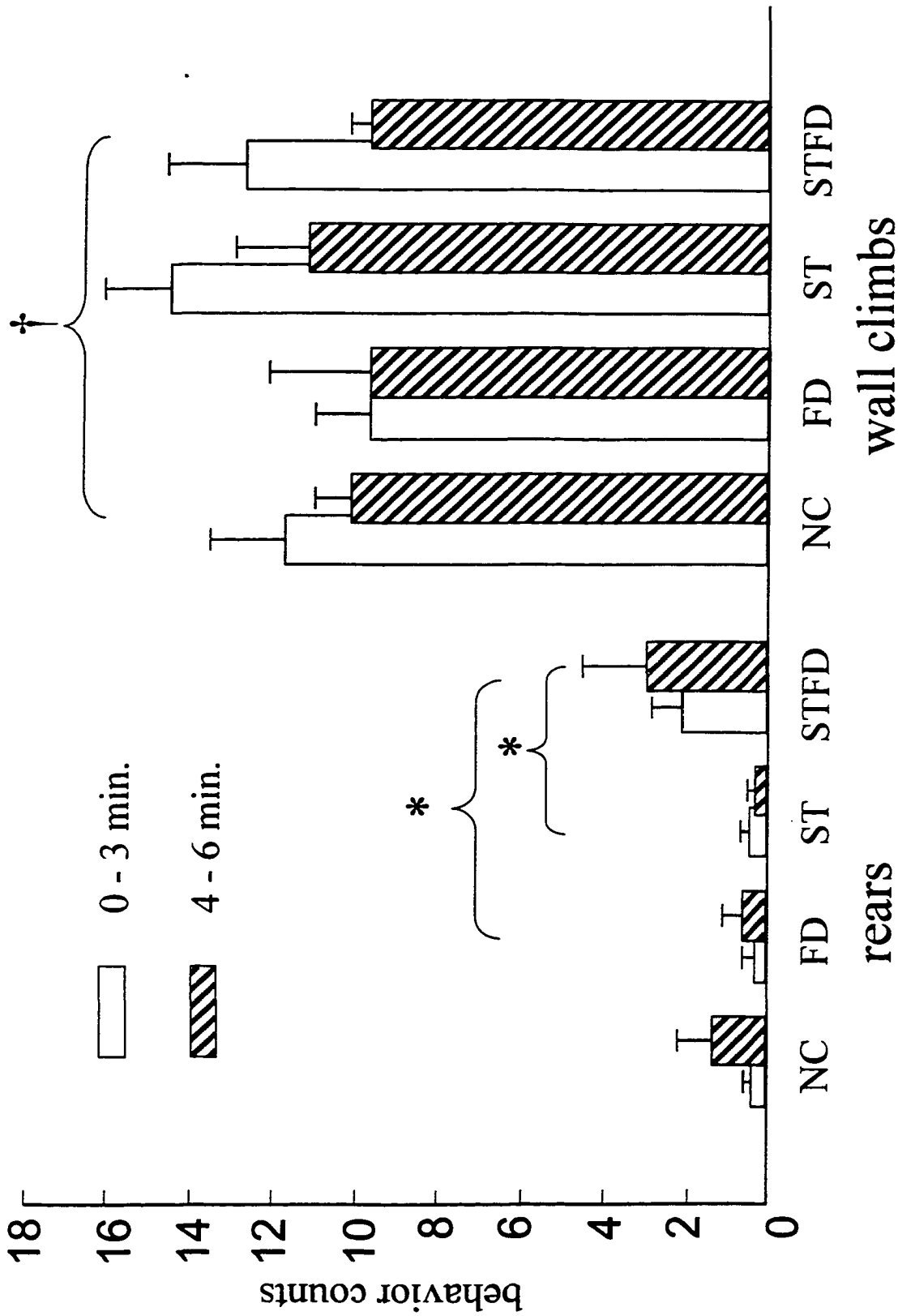


fig. 7

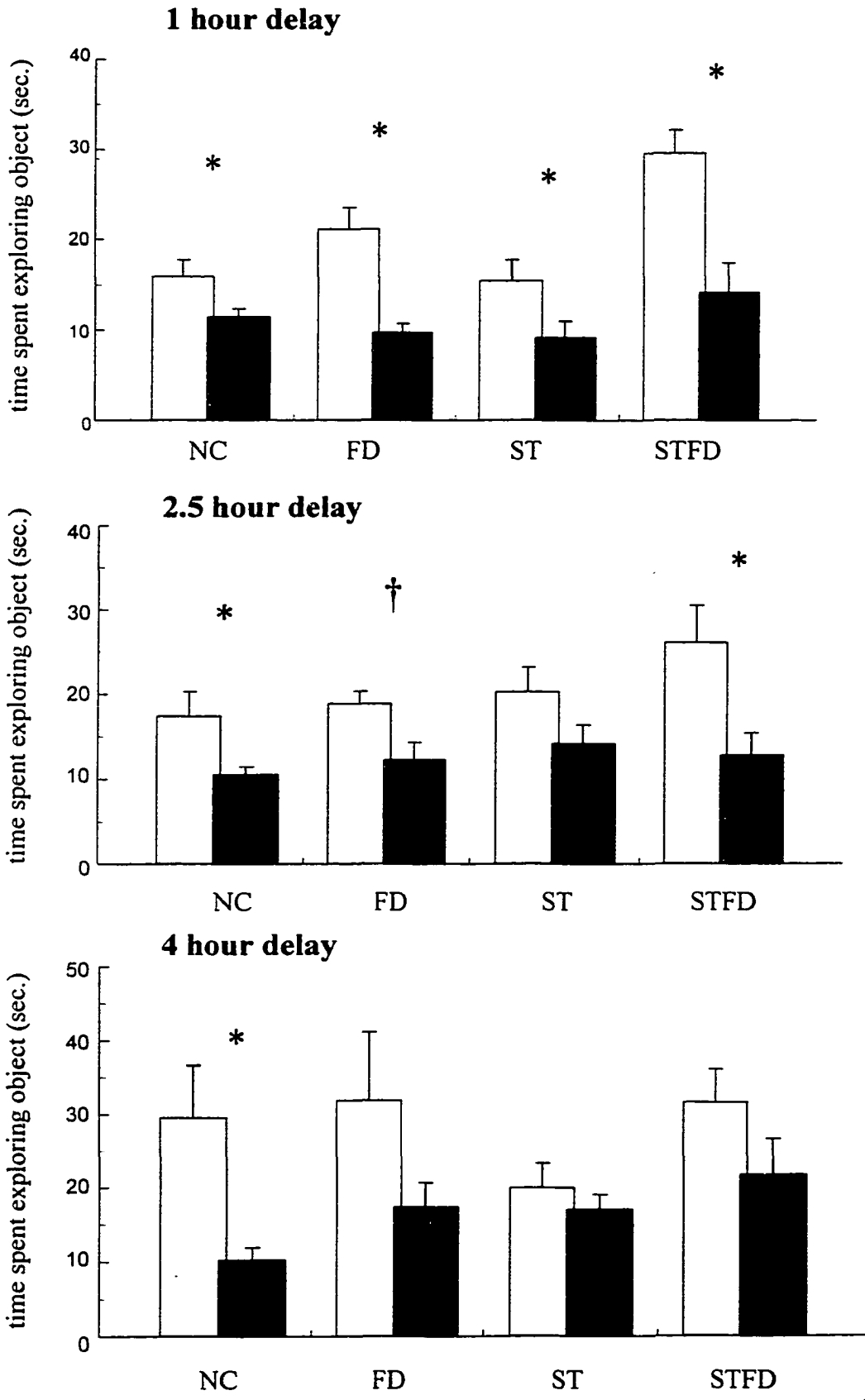


fig. 8

the sample objects in T1 (see figure 8). The pattern of performance was altered in the 4-hour delay trial where only the NC group exhibited a significant exploration preference for the novel object. While food deprived groups spent more time exploring objects in the sample trial and in the test trial, they still did not show a preference for the novel over sample object. However, the ST group also showed the least habituation to the sample object (NC = 4.27 ± 1.13, FD = 3.25 ± 2.70, ST = -3.04 ± 1.96, STFD = 5.09 ± 3.32) as evidenced by a marginal interaction [$F(1, 21) = 3.82$, $p = .06$].

Thus, the ST group, in contrast to the STFD group, was the first to exhibit a clear lack of novel object preference, and additionally showed no habituation to the sample objects beyond a 1-hour delay. Even though only the NC subjects continued to exhibit a preference for the novel objects following a 4-hour delay, both food-deprived groups still habituated to an equal degree to the sample objects as the NC subjects.

Neurochemical Analyses

Monoamines and metabolites measured in brains of subjects when they were sacrificed immediately upon removal from the field (sample trial), were altered by both stress and food deprivation. As evidenced in figure 9, the ST group had significantly higher NE levels in basolateral amygdala and in CA1 ($.05 < p < .10$). Prefrontal DA, HVA, and DOPAC levels did not differ, however DA utilization, as indexed by HVA/DA ($F_{1,21} = 3.45$, $p = .07$) and DOPAC/DA ($F_{1,21} = 3.21$, $p = .08$), was marginally significant with the ST group exhibiting the greatest amount of DA activity (ratios not shown). Food deprivation was associated with a significant decrease in CA3 NE levels. Lower prefrontal cortex 5-HT levels were also found in both FD and ST subjects.

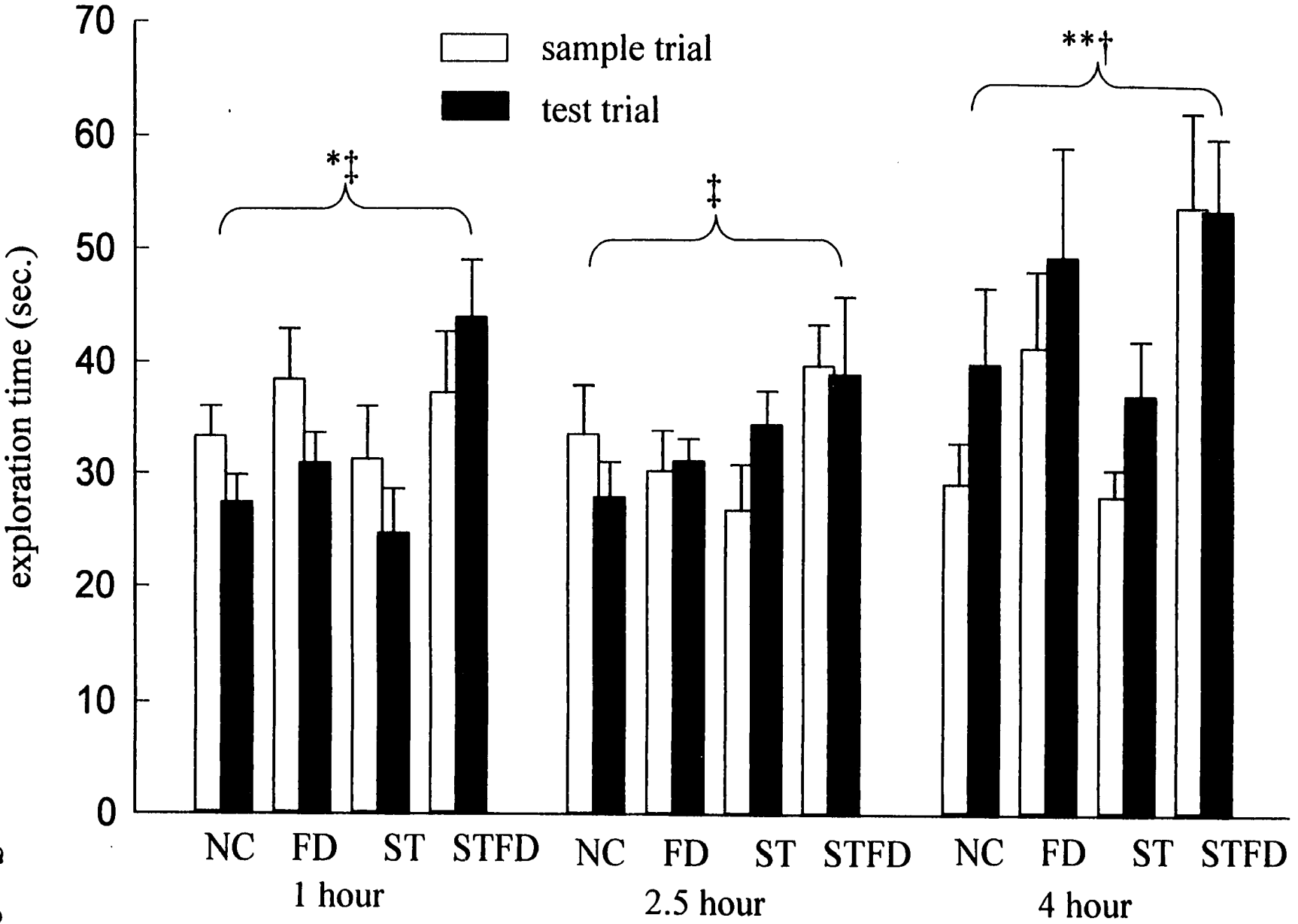


fig. 9

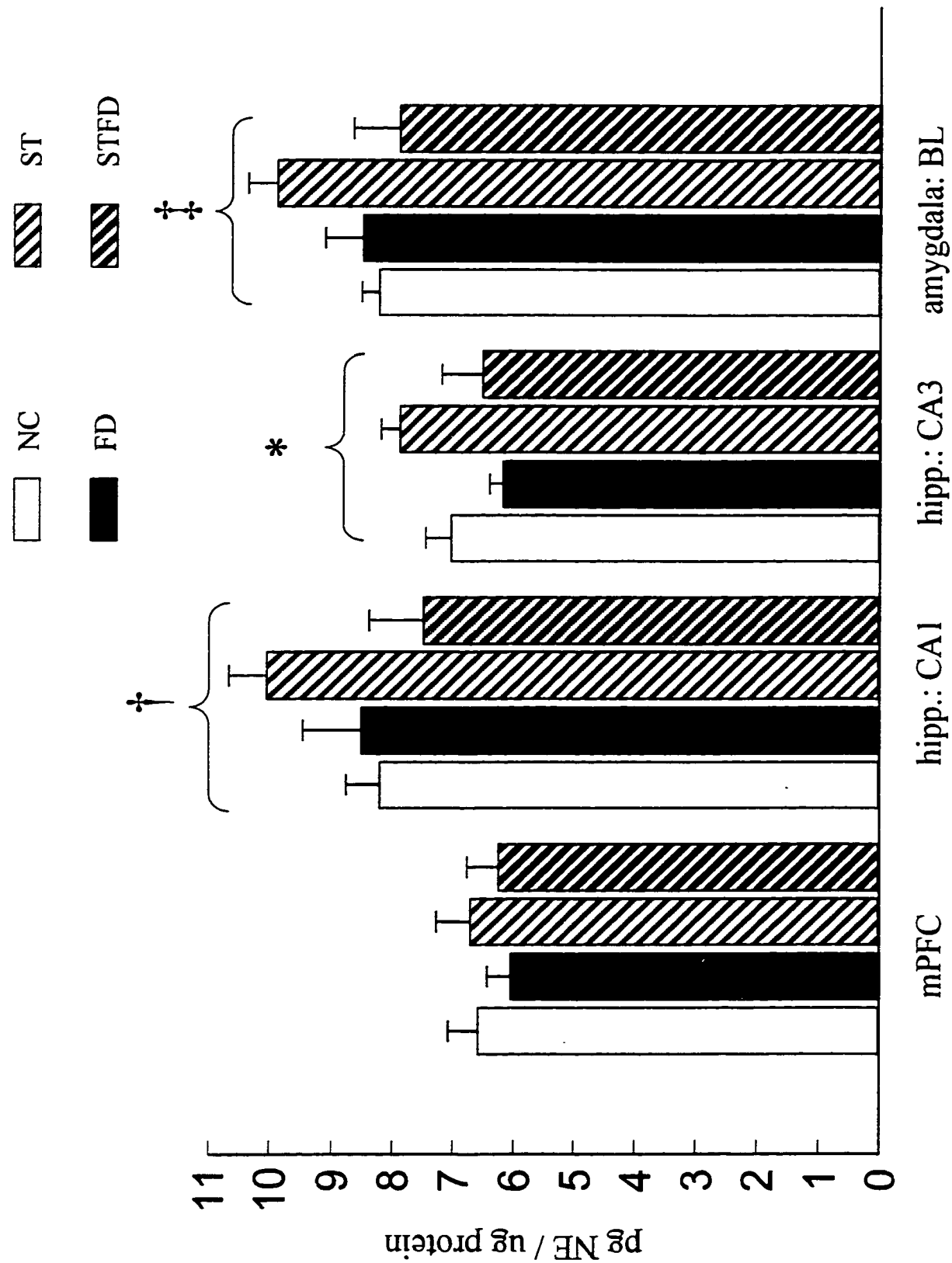


fig. 10

Discussion

Behavioral Effects of Restraint and Food Deprivation – Large Field

As expected, the usage of a larger field altered the extent to which subjects recognized the novel objects (especially at delays greater than 1 hour). It is noteworthy that, as in experiment one, the ST subjects showed the least amount of discrimination once the delay extended beyond 1 hour, and their habituation to the sample objects was also less than the other three groups. This impairment continued in ST subjects through the 2.5 and 4-hour delays. As the delay extended, the food-deprived groups also exhibited impairments in recognition (unlike experiment 1), but still showed comparable degrees of habituation as the NC group. Generally, the larger field seemed to make object recognition more difficult for all treatment subjects (food deprived and/or stressed). Therefore, when testing under similar conditions as used by McCormick (McCormick et al., 1997), where task difficulty seems to increase with a more extensive environmental setting, ST subjects showed the greatest impairment. After a four-hour delay, FD and STFD groups also exhibit less recognition, even though, they significantly explore the objects more than their naïve control counterparts. Naïve controls showed no impairment at any delay. It appears that increasing object exploration via manipulation of internal states (such as through food deprivation) influences behavior in this type of task to a degree that may suggest a lack of impairment under some conditions (experiment 1) but not under others (experiment 2). Obviously, this shows the importance of task demand in modeling the interaction of multiple internal states through separate manipulations as done here.

Serum CORT Effects of Restraint and Food Deprivation

The post-testing serum CORT levels suggest that food-deprivation increases plasma CORT levels in food deprived subjects and previous exposure to chronic restraint does not change that effect. This being the case, studies using task-required reward (Luine et al., 1995; Luine et al., 1994a) are then comparing groups with similar CORT levels during behavioral

testing, even if those levels are elevated by food deprivation. Moreover, in studies not utilizing food deprivation or noxious stimulation (Conrad et al., 1996) behavioral impairments cannot be readily ascribed to heightened CORT levels in stressed subjects.

Neurochemical Effects of Restraint and Food Deprivation

The neurochemical results suggest that differences due to stress are more evident following conditions of task-induced arousal. In this regard, several regions implicated in memory processing show a different degree of monoaminergic activity immediately after a sample object trial. The ST group had higher NE levels in basolateral amygdala, CA1, and CA3, as well as possible increases in prefrontal DA activity. Our observed increase in NE levels may reflect a decrease in release particularly among the ST subjects, while food deprivation appeared to increase hippocampal release of NE (as evidenced by lower NE levels). However, it should be noted that changes in NE levels could also reflect changes in synthesis.

A decrease in NE release could explain the greater recognition impairment by the ST group. NE release in the amygdala facilitates memory-processing (Quirarte et al., 1997) and hippocampal NE release normally increases in response to environmental stimuli (Britton, Segal, Kuczenski, & Hauger, 1992). Thus, the higher levels in our ST subjects could reflect a lack of arousal that is important for normal memory processing. Interestingly, STFD NE levels were lower compared to ST subjects in both the amygdala and hippocampus, and might explain the difference between the recognition impairments between these groups. In CA3, FD and STFD subjects had lower NE than those of NC subjects. Whether this increase in NE release reflects an over-arousal that leads to deficits under more difficult conditions, still needs to be determined, but those subjects did spend more time with the objects, in comparison to NC subjects, without necessarily discriminating as well.

In regards to the possible increase in DA activity in the prefrontal cortex, we have observed a similar DA release sensitization, to an acute dose of nomifensine, after 21-days of

restraint (Beck, Sterbank, & Luine, 1996) and others (Gresh et al., 1994) have observed DA sensitization to acute stress after 3-4 weeks of chronic cold. Others have reported similar DA sensitization in relation to chronic psychostimulant usage (Piazza & Le Moal, 1998). The prefrontal cortex is implicated in both working memory processing (Floresco et al., 1997; Goldman-Rakic, 1992; Kesner, 1990; Winocur, 1991; Zahrt, Taylor, Mathew, & Arnsten, 1997) and novelty preference (Kolb & Nonneman, 1975). Thus although the dopamine activity ratios were only marginally significant, both the chemical and behavioral effects mirror previous reports that would implicate a possible DA role in the observed impairment.

General Discussion

These experiments demonstrate that 21 days of chronic restraint affects the performance of male rats in object recognition, a non-spatial, non-reward associated, memory task. As similarly reported by Conrad et al. (1996), using a Y-maze, a lack of recognition when comparing novel versus sample stimuli occurs when delays, between sample and novel trials, are extended beyond one hour. Moreover, these experiments illustrate stress subjects, that are subsequently food deprived, do not exhibit the same level of impairment. These shifts in performance occurred under both versions of the task (small and large environment). Neurotransmitters and metabolites were measured both under basal conditions (sacrificed the day after training from the home cage) and under trial-induced arousal conditions (sacrificed after a sample trial). Those groups that were food deprived showed heightened basal 5-HT activity in both the prefrontal cortex and CA1. These animals also had higher serum CORT and CA3 NE release after behavior testing. Stressed subjects showed basal differences in CA3 histidine and glycine and a general decrease in NE release and increase in prefrontal DA activity. Thus, these results illustrate that stress-induced changes in monoamine activity are observable only after task-induced arousal, whereas, amino acid changes are observable even at basal (non-arousal) levels.

Chronic stress, as modeled by 21-day restraint, has been hypothesized to compromise memory processing in rats by creating a prolonged high-CORT level state. Thus, the known detrimental effects of chronic high CORT on hippocampal CA3 morphology would explain the probable miscommunication (i.e. interference) that leads to memory deficits. Following this theory, the current results, showing CORT levels are not elevated at 1 week post-restraint, would suggest any stress induced impairments due to heightened CORT activity must be long lasting (i.e. changes in morphology, chemistry, or physiology). However, this explanation does not fully explain the observed behavioral and neurochemical differences between the ST and STFD groups. Assuming that the ST and STFD subjects had increased plasma CORT during the 21 days of restraint, the STFD group, which exhibited an elevated level for 8 additional days (due to food restriction), should have shown the greatest decrements in memory. This leads us to seek other alternatives in explaining the underlying neurochemical actions that may be causing the memory impairment.

Two changes in monoamines appear to dissociate the ST group from the other three groups: prefrontal DA and hippocampal and amygdala NE. Stress-induced changes in NE are influenced by corticotrophin releasing factor (Shimizu, Nakane, Hori, & Hayashi, 1994), but the release of prefrontal DA appears independent of corticosterone activity (Imperato, Puglisi-Allegra, Casolini, & Angelucci, 1991). Comparing the ST and STFD groups, increased CORT levels, due to food deprivation, were associated with lower hippocampal and amygdala NE levels (suggesting greater release) than in NC subjects. Thus, the ST subjects had the highest levels of NE (suggesting lesser release) which may reflect restraint-induced desensitization to task-generated NE arousal (Dalley & Stanford, 1995). Chronic regimens of restraint have been shown to decrease NE release over time (Shimizu, Tanaka, Yokoo, Gondoh, Mizoguchi, & Matsuguchi, 1994), but since the HPA-axis does not show cross-desensitization to different stressors (Armario et al., 1988), it appears that food deprivation may have reinstated NE arousal-induced release. The observed increase in prefrontal DA activity by stress matches past studies (Carlson,

Fitzgerald, Keller, & Glick, 1991; Finlay, Zigmond, & Abercrombie, 1995; Gresh et al., 1994). Furthermore, heightened DA turnover is known to cause memory impairments (Murphy, Arnsten, Goldman-Rakic, & Roth, 1996; Zahrt et al., 1997), as does decreases in amygdala NE release (Quirarte et al., 1997). The increase in NE release in CA3 may also underlie the greater amount of time spent exploring objects in food deprived groups. The heightened basal levels of serotonin activity also indicate a stress-response in both food-deprived groups (Adell, Garcia-Marqueq, Armario, & Gelpi, 1989; Kennett, Dickinson, & Curzon, 1985). This would suggest that monoaminergic activity changes evident following chronic stress could underlie observed memory deficits but are observable only after trial-induced arousal.

The fact that the CA3 region had higher amino acid levels, in ST subjects, suggests enduring effects from chronic restraint for up to 8-9 days post-stress. The elevated histidine and glycine levels in ST subjects could reflect changes in transmission and may explain the apparent lesser release of NE. Both glycine and histamine are known to modulate NMDA receptor activity (Bekkers, 1993; Goldstein, Rasmusson, Bunney, & Roth, 1994), thus, these changes may underlie the lasting effects of stress on hippocampal processing and morphology. Giaume, Grange, Baubet, Gay, Sermet, Sarda, and Bobillier (1995) found protein synthesis, as assessed by methionine incorporation, remains high in CA3 after chronic stress, so, our results could also reflect changes in intracellular protein synthesis. Previous studies also show stress-dependent increases in the release of EEA (Moghaddam et al., 1994), but we did not detect any large change in aspartate levels. Unfortunately, glutamate was off-scale in most of the areas (in amounts 10 times greater than the other amino acids). Previously, Luine, Grattan, and Selmanoff (1997) found glutamate levels as much as eight times higher than those of GABA in other neural areas, thus, the results here are not unexpected. These results then suggest glutamate, not aspartate, may be the principle amino acid involved in the CA3 dendrite recession due to heightened excitatory input. Interestingly, histidine levels were decreased in nucleus accumbens in both the FD and STFD groups. These changes may suggest a shift in histamine utilization in nucleus accumbens

synaptic processing, although, it is unclear whether the histidine decrease is due to type of stress (food deprivation) or the temporal aspect of it acting as a novel sub-chronic stress (8 days).

When comparing the behavioral results from both experiments, task demand appears to distinguish the four groups from each other. When the task was less demanding (shorter delays, smaller testing arena), FD and STFD groups performed similarly to NC subjects. However, when the task demands are increased (longer delays and / or larger testing arena), the effects of food deprivation and restraint upon subjects' behaviors in the object recognition task are dissociated from that of NC subjects. The three treatment groups showed varying degrees of behavioral decrements in object recognition beyond the 1-hour delay in experiment 2. In contrast, those currently experiencing the acute phase of sub-chronic stress (food deprivation) were not readily distinguishable from naïve subjects in experiment 1, even following a 4-hour delay, until we analyzed the total exploration time. The instatement of food deprivation increased exploration time in both naïve and stressed subjects, which may have aided in object discrimination. However, this effect is limited. Food-deprived subjects under low-demand conditions (small arena) perform as well as naïve controls, but under high demand (long-delays in a large arena), they do not show the same level of discrimination in spite of exploring the objects more than naïve controls.

These results show that task demands, both task difficulty and the use of reward contingencies, can influence the behavioral and neurochemical consequences of stress. The use of food deprivation has been widely debated in the past in relation to a subject's drive-state and how that might influence learning and memory. In addition to the current results, Adlerstein and Fehrer (1955), Kamback (1966), Hoyenga and Hoyenga (1974), Richards and Leslie (1962), and Timberlake and White [64] have found that food deprivation increases exploratory drive in a non-reward environment that includes novel cues and objects. Food deprived rats are especially prone to exploratory behaviors when environmental cues are continually changed (Adlerstein & Fehrer, 1995; Zimbardo & Miller, 1958). Here we found food deprivation increased the general

reactivity towards the objects, but not necessarily in a manner that always aided them in discriminating the novel from sample object. Food deprivation also selectively increased open field rearings in previously stressed subjects. Thus, the attenuation of the recognition impairment in STFD subjects may be related to increases in exploratory behaviors. This finding contrasts those found by Miller and Dess (1996) using a radial-arm maze, but it should be noted that they used electric shocks both before and on the day of testing as the stressor. Furthermore, since their food-deprived subjects showed increased activity as compared to their stressed subjects (with the combination group exhibiting the least), it suggests that different "stressful" conditions placed upon the animal, both alone and in combination, can yield diverse reactions. Under certain conditions, these conditions may appear to enhance performance, as observed here and by Servatius and Shors (1994), but as we also observed, these effects appear to have limits. Exploration appears to be generally increased by food deprivation, but when combined concurrently with electric shock it is decreased. Here, when chronic restraint preceded food restriction (which then became a novel stress), exploration of the field was not decreased, thus suggesting an interactive role of timing and the use of multiple stressors leading to these contrasting results.

From this discussion, two hypotheses for the apparent buffering of the stress-induced recognition impairment are possible. The buffering is simply an artifact of increased activity caused by food deprivation (as a novel stress), or food deprivation is acting upon the subject's internal state as a motivational stimulus. As stated above, the increased exploration that was most evident in experiment 2 could appear to support the artifact argument. However, Maren and Fanselow (1998) recently theorized that food and water deprivation could either enhance the strength of contextual encoding or the amount of context that is encoded. Thus, an increase in exploration may actually reflect a state in the animal that is able to hold more information about the environment than its non-deprived counterparts. Still, the current results further suggest that this issue is more complex. First, both FD and STFD groups explored more in the 4 hour delay in

the large field (experiment 2), yet neither showed the degree of preference to the novel object as the NC group. This was not the case in the smaller field where both food-deprived groups exhibited an overall greater preference to explore the novel objects, more than either the NC or the impaired ST group. Yet, in the degree of habituation to the sample objects, it is obvious that NC, FD, and STFD groups generally do not differ across trials, but they differ in prefrontal and hippocampal (CA1) serotonin activity because of food deprivation. Clearly, this is an issue that still needs further investigation to determine: a) the limits of food deprivation in influencing contextual encoding because of task demands, b) the extent to which task requirements, such as food deprivation, can hinder the observation of performance deficits in behavioral tests, and c) the role serotonin has in neural processing in these situations.

Results from these two experiments illustrate that chronic stress affects memory processing for non-spatial information. Thus, both spatial and non-spatial memory is impaired by 21days of chronic restraint, but impairments can be modulated by sub-chronic food deprivation. Food deprivation appears to buffer the detrimental affect of chronic restraint upon object recognition, and also attenuates the monoamine changes (NE and DA) in chronically stressed subjects. The attenuation of these changes could be due to the heightened basal 5-HT activity observed in food deprived subjects, suggesting food deprivation is acting as a novel stress. Amino acid level changes due to chronic restraint were only observed in CA3, and involved glycine and histidine, suggesting increased inhibitory transmission. Therefore, three areas implicated in memory processing (hippocampus, amygdala, and prefrontal cortex) all exhibited differential changes among the four groups. Current work is determining how other factors, such as housing, differentially modulate chronic stress induced behavioral impairments and neurochemical activity.

Chronic Restraint Stress and Food Deprivation Affect Female Monoamine Levels without Causing Behavioral Deficits in Object Recognition

The 21-day restraint-stress paradigm (6 hrs. restraint over 21 consecutive days) clearly illustrates how brain morphology (Watanabe, Gould, & McEwen, 1992; Sunanda, Rao, & Raju, 1995), chemistry (Luine, Villegas, Martinez, & McEwen, 1994a; Beck & Luine, in press), and behavior (Luine et al., 1994a; Luine, Villegas, Martinez, & McEwen, 1994b; Luine, Martinez, Villegas, Magarinos, & McEwen, 1995; Conrad, Galea, Kuroda, & McEwen, 1996; Beck & Luine, in press) can be changed when male rats are subjected to a rather mild, but prolonged, stressful condition. The prevailing theory for explaining this effect is that chronic restraint creates a prolonged, heightened level of corticosterone (CORT) release from the adrenal cortex which leads to a cascade of neural changes, most notably in the hippocampal CA3 region (as reviewed in McEwen, 1998; Saplosky, Krey, & McEwen, 1986) but also in other related limbic areas (Herman & Cullinan, 1997). These changes are thought to cause disruption in neural processing which are evident in tasks of spatial memory (as reviewed in Luine, 1997, 1994). However, Beck and Luine (in press) found deficits in non-spatial memory as well. Since all these studies modeled the stress effect in males, our objective here was to determine if similar behavior change in a non-spatial memory task is observable in stressed female rats.

Questions have recently surfaced when attempting to model the hippocampal morphological changes in female subjects. Galea, McEwen, Tanapat, Deak, Spencer, and Dhabhar (1997) reported chronic restraint did not affect hippocampal CA3 apical dendrite length or the number of branch points in females. In fact, restrained females only exhibited a decrease in basal dendrite branch points, but not to the degree observed in males. These reduced effects occurred despite consistently higher plasma CORT and corticosterone-binding globulin (CBG) levels in the females, in comparison to males, over the 21 days. Furthermore, Turner (1992) found that the CORT binding affinity for mineralcorticoid (type I) versus glucocorticoid (type II)

receptors was less specific compared to males, and McCormick, Smythe and Beers (1994) showed that differences in the type I binding become more evident after birth (although some exist prenatally). This pattern of increased female responsiveness in both adrenocorticotropin (ACTH) and CORT in the acute phase of the stress response appears to be due to estrogen (Burgess & Handa, 1992; Handa, Burgess, Kerr, & O'Keefe, 1994). OVX females do not exhibit as high or as prolonged CORT release as their estrogen treated counterparts (Burgess & Handa, 1992), and when intact, proestrous females exhibit the greatest increase (followed by estrous) in both ACTH and CORT release to acute restraint (Viau & Meaney, 1991). These results lead both Handa et al. and Galea et al. to conclude that the role of CORT in the general feedback-regulation of the hypothalamic-pituitary-adrenal (HPA) axis may be different in males and females.

Since the HPA-axis appears to respond differently in males and females (in both basal conditions and under arousal), we may expect to see a different affect of chronic restraint upon female learning and memory. Past studies (Davis, Porter, Burton, & Levine, 1976; Kirk & Blampied, 1985; Steenbergen, Heinsbrock, Van Hest, & Van de Poll, 1990; Steenbergen, Heinsbrock, Van Hest, & Van de Poll, 1989; Shors, Lewczyk, Pacynski, Mathew, & Pickett, 1998; Wood & Shors, 1998) suggest that males and females exhibit different learning deficits following exposure to restraint and/or shock. Generally, stressed females exhibit less (if any) impairment, compared to males, in acquiring avoidance learning (Kirk & Blampied, 1985; Steenbergen et al., 1989; Steenbergen et al., 1990) or escape conditioning (Davis et al., 1976). Conversely, eye-blink conditioning is enhanced in stressed males (Wood & Shors, 1998), unaffected in OVX-stress females (Wood & Shors, 1998), and deteriorated in stressed females in proestrous (Shors et al., 1998). These studies suggest, in addition to the physiological changes, stress influences learning and memory differently in males and females.

In conjunction with this idea, we attempted to replicate our previous study where stressed male rats were found to show a lack of object recognition after a 4-hour delay, in comparison to naïve controls. After 21 days of restraint, an initial open field test was followed by several days

of object recognition trials (delays ranged from 1 minute to 4 hours). Moreover, as in the previous study, half the controls and stressed subjects were additionally food deprived during the behavior-testing week since food deprivation buffered the stress effect in males. This aspect of the previous work was also repeated to establish whether females respond differently than males to the onset of a secondary stress-condition during testing. Monoamine and amino acid level changes were measured in the hippocampus, amygdala and medial prefrontal cortex to determine if those changes followed the male pattern observed previously (Beck & Luine, in press).

Methods

Subjects

Forty-nine female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), 40-60 days old upon arrival were used. Subjects were double-housed (one tripled) in plastic tubs during the initial acclimation week and throughout the three-week stress period on a 12/12 reversed light/dark cycle (lights on at 8:00 p.m.). Baseline weight measures were used to match tub pairs to naïve or stress condition (both tub subjects were in the same condition). Due to normal fluctuations in female weight, each time-point in the study is reflects the mean weight for each subject over two consecutive days. For 21 consecutive days (following acclimation and initial baseline weight measures), 8 tubs (n=16) of subjects were placed in Plexiglas restrainer tubes for 6 hours (during the dark phase, approximately (9:30am – 3:30pm). During the 6 hours of restraint, all subjects were in their home cages in the lab's animal room; those restrained were idle in the plastic tubes while controls were freely active. All subject weights were measured (in the a.m.) on stress days 1, 7-8, 14-15, and 20-21.

Subjects were immediately changed to single housing (in identical size tubs) immediately following the last restraint session (day 21). Based on matched weights within each group (naïve and stress), subjects were assigned as food-deprived or control conditions. Matching by weight

was used to equate the distribution of stressed subjects in the two subgroups to control for level of stress (as defined by weight loss) before proceeding with the second stress (food deprivation). All food deprived subjects (naïve and stress) were weighed and rationed 2-3 rat chow pellets daily in order to maintain 85-90% of their baseline body weights, as recorded on days 20-21 of the restraint period. All subjects were weighed after testing and food deprived subjects were fed no earlier than 1 hour later. Food deprived subjects remained on their restricted diets until sacrifice (post-stress day 8).

Behavioral and Neurochemical Apparatus

A 102.5cm X 61.5-cm open field constructed of a Formica table with temporary opaque, wood walls, approximately 40-50 cm high, was used for the initial open-field activity trial. The floor was marked off into 15 equal (20.5 cm) squares (5 X 3). In the subsequent object recognition trials, the arena was shortened to 9 squares (3 X 3). Neurochemical analyzes were conducted using the same equipment as described in experiment 1.

Behavioral Measures

The procedures used here are the same as those used in experiment 1a. Briefly, subjects' activities (sector visits and rears) were observed in open field (6-min. trial) on post-stress day one. Object recognition trials were conducted over the next 6 days. Two objects were placed at the south end of the field, opposite the recording experimenter (approx. 20cm from the walls) equidistant from each other. Object recognition sessions consisted of two 3-minute trials: a sample trial (T1) and a recognition trial (T2), each separated by an inter-trial intervals of 1 minute, 10 minutes, 1 hour, or 4 hours. All object placements (sample and novel / left versus right) were fully counterbalanced within each separate delay session across groups. Time spent exploring the objects was recorded using two stopwatches (one for each object). Definitions of exploration were identical to experiment 1, as was the a priori determination that any subject that

did not spend at least 10 combined seconds exploring the two objects (on either trial) was to be excluded from that session's behavioral analysis.

Seventeen of the forty-nine subjects were used in another study after begin tested for the 4- hour delay, thus, their results are only reflected in the open field, 10 min., 1 hour, and 4 hour trials. Similarly, those subjects were not included in the neurochemical analysis. Otherwise, as in experiment 1a, the day following the last behavior test (post-stress day 8), all other subjects were sacrificed, their brains were removed and placed immediately in dry ice. Each subject was singularly brought into a separate room (in their home cage) and decapitated without anesthesia. The brains were subsequently stored at -70 °C until HPLC analysis.

Neurochemical Analyses

Thirty-two of the forty-nine subjects used in behavioral analysis were used in the neurochemical analysis for monoamines and amino acids. All procedures were identical to those used in experiment 1.

Data Analysis

Identical analyzes were used as in experiment 1. For open field measures, a mixed 2 X 2 X 2 (stress X food X time) ANOVA was used to determine differences in sector visits and rears. Correlated t-tests were used to determine significant object exploration differences (novel vs. sample) during the T2 recognition trial. Inter-group comparisons for object preference (during T2) were conducted by using a between subjects 2 X 2 (stress X food) ANOVA over the indexes of habituation (T1/2 - T2 novel), novel preference (T2 novel – T2 sample), and discrimination ratio [(T2 novel-T2 sample)/ T2]. General exploration differences due to condition and trial were determined by a mixed 2 X 2 X 2 (stress X food X trial) ANOVA.

Neurochemical tissue levels for monoamines are expressed as pg / ug protein and amino acids as ng / ug protein. A between subjects 2 X 2 ANOVA tested group differences for all

neurochemicals. Fisher's LSD was used for all post-hoc analyses. All statistical analyses were conducted using GB-STAT (Dynamic Microsystems Inc).

Results

Weight Gain Analysis

All subjects gained weight between pre-stress day 1 and stress day 21. However, stress subjects gained weight at a much slower rate than did naïve subjects [$F(4, 184) = 4.84$, $p < .001$]. During behavior testing (as assessed by protected t-tests), NC and ST subjects did not differ in weight, but food deprivation decreased weights significantly in FD subjects (below NC and ST groups, $p < .01$) and STFD subjects (below NC and ST subjects, $p < .01$; and FD subjects $p < .05$).

Behavioral Measures

The number of open field sectors visited was similar across all groups (see figure 10). No significant differences were found in either the number of outer or inner sector visits due to condition (stress or deprivation). The number of outer sector visits did decrease between the first 3 minutes and the second 3 minutes in all groups [$F(1, 46) = 16.92$, $p < .001$], but no such change occurred in the number of inner sector visits. The number of rears observed also did not differ between groups or over time (data not shown).

In the object recognition trials, all groups showed a general preference for the novel object during the discrimination trial (T2). This occurred consistently over all four delay times (see figure 11). Indexes of habituation, exploration differences, and discrimination ratio did not differ between groups for any of the delays. All groups spent less time exploring the previously examined sample objects, as compared to the novel. The total time exploring objects

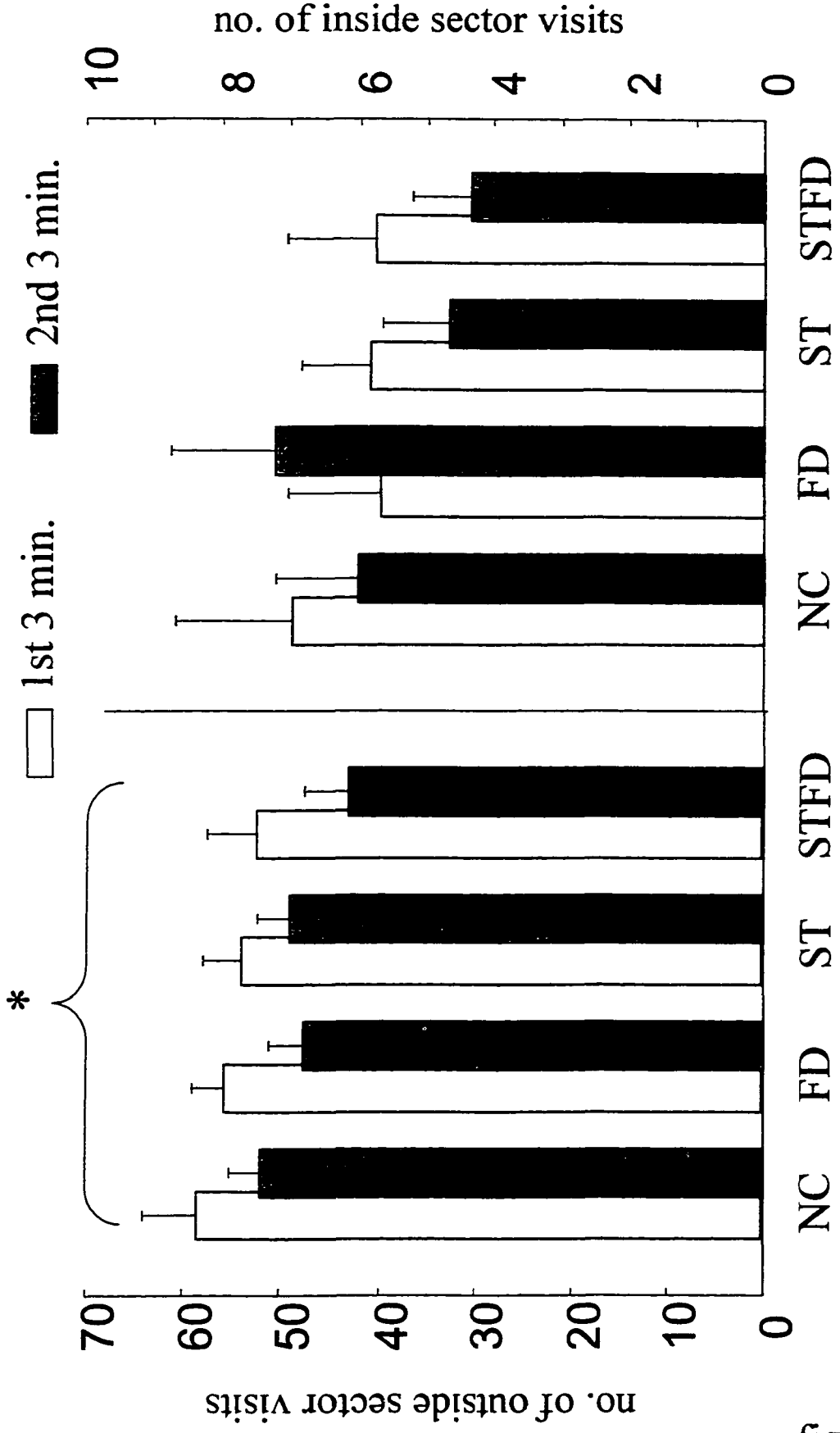


fig.11

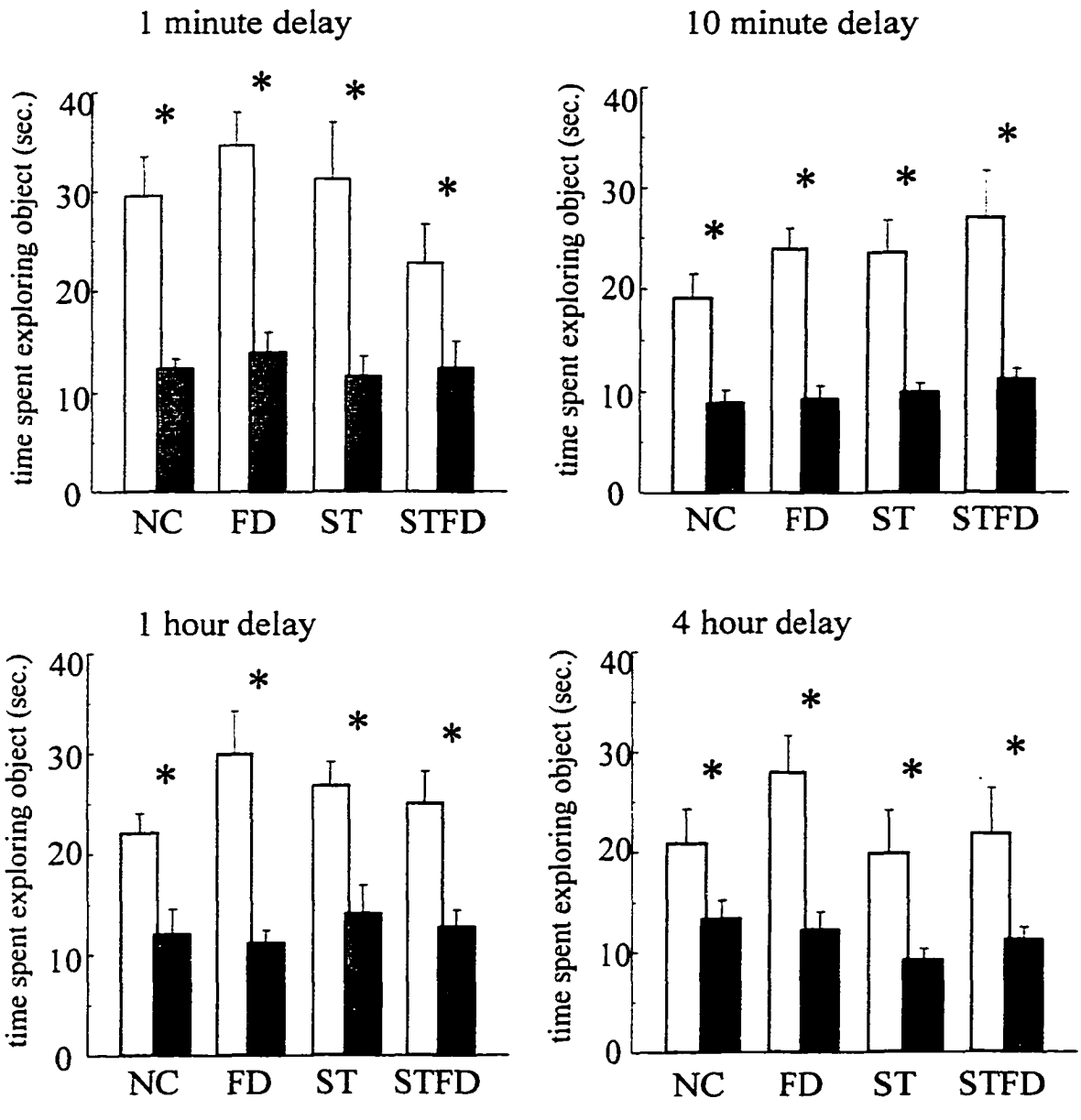


fig. 12

at each of the delays did not differ from sample to discrimination trial, nor did it between groups (see figure 12). Thus, chronic stress and food deprivation did not influence behavior in the open field or object recognition trials.

Neurochemical Measures

Tissue levels of monoamines and amino acids in prefrontal cortex and CA1 appeared to be most affected by the experimental manipulations. Stress increased NE turnover in CA1, as measured by MHPG levels [stress $F(1, 27) = 3.88$, $p = .05$], and decreased prefrontal dopamine activity in conjunction with food deprivation [stress $F(1, 26) = 5.87$, $p < .03$; food $F(1, 26) = 5.16$, $p < .04$]. As seen in figure 13, only the HVA/DA ratio was changed to a significant degree in the prefrontal cortex, not DOPAC/DA or 5HIAA/5-HTratios. The four amino acids affected by stress and food deprivation in these areas were aspartate, asparagine, glycine, and histidine (see figure 14). Stress was also associated with a decrease in measured aspartate levels in prefrontal cortex [$F(1, 26) = 5.98$, $P < .02$]. Interactions occurred in prefrontal glycine and histidine levels with FD and ST subjects showing both increases in glycine [stress X food $F(1, 26) = 4.33$, $p < .05$] and decreases in histidine [stress X food $F(1, 26) = 4.52$, $p < .05$]. These two groups also showed increases in CA1 asparagine [stress X food $F(1, 26) = 4.35$, $p < .05$] and glycine [stress X food $F(1, 26) = 4.75$, $p < .04$].

The amygdala and hippocampus CA3 did not exhibit the degree of changes described above. However, serotonin levels in the amygdala were decreased in stressed subjects [stress $F(1, 26) = 3.94$, $p = .05$]; although, the ratio of 5HIAA / 5-HT was not significantly changed. Amino acids were not changed. No significant changes were observed in CA3 for either amino acids or monoamines.

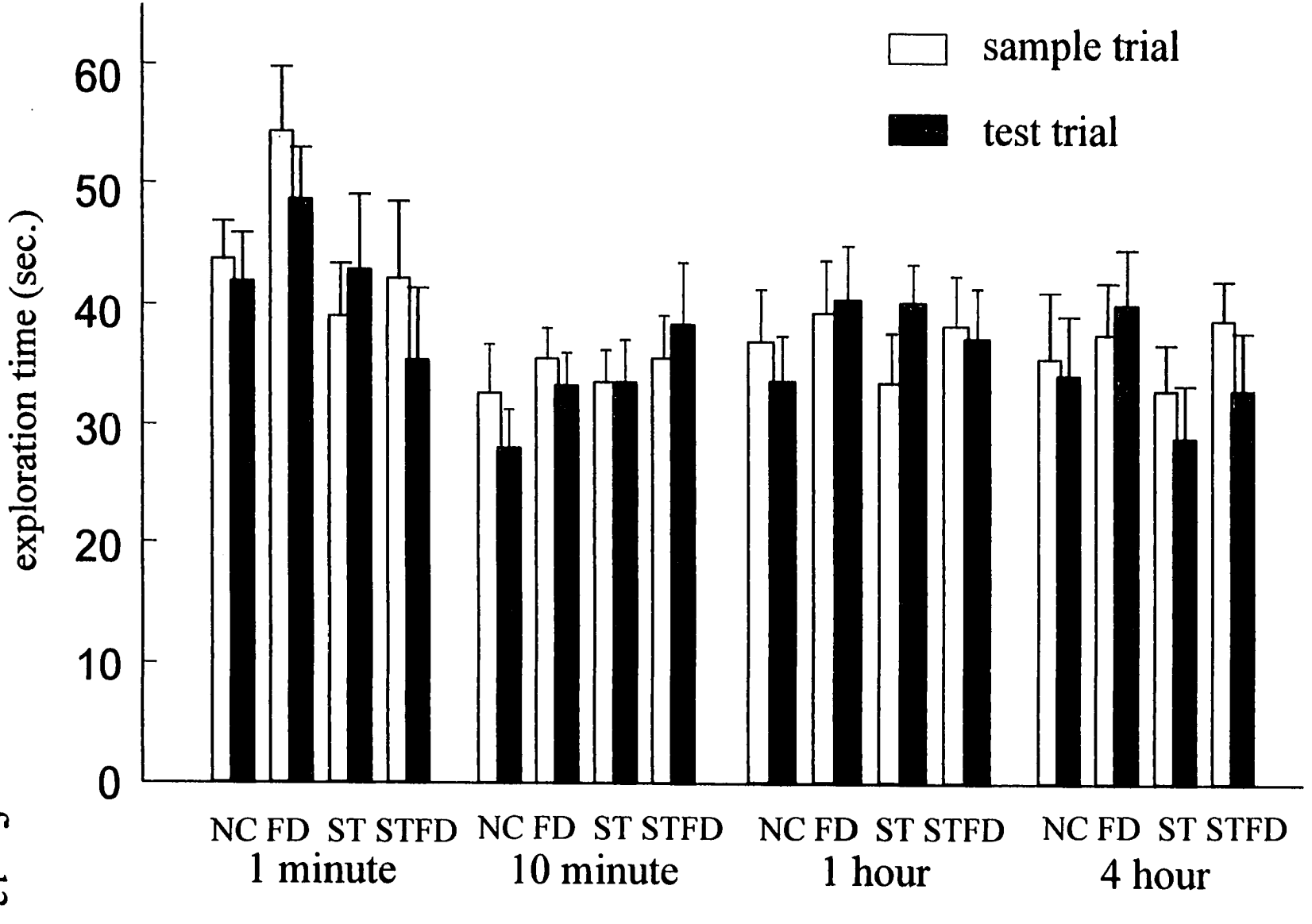


fig. 13

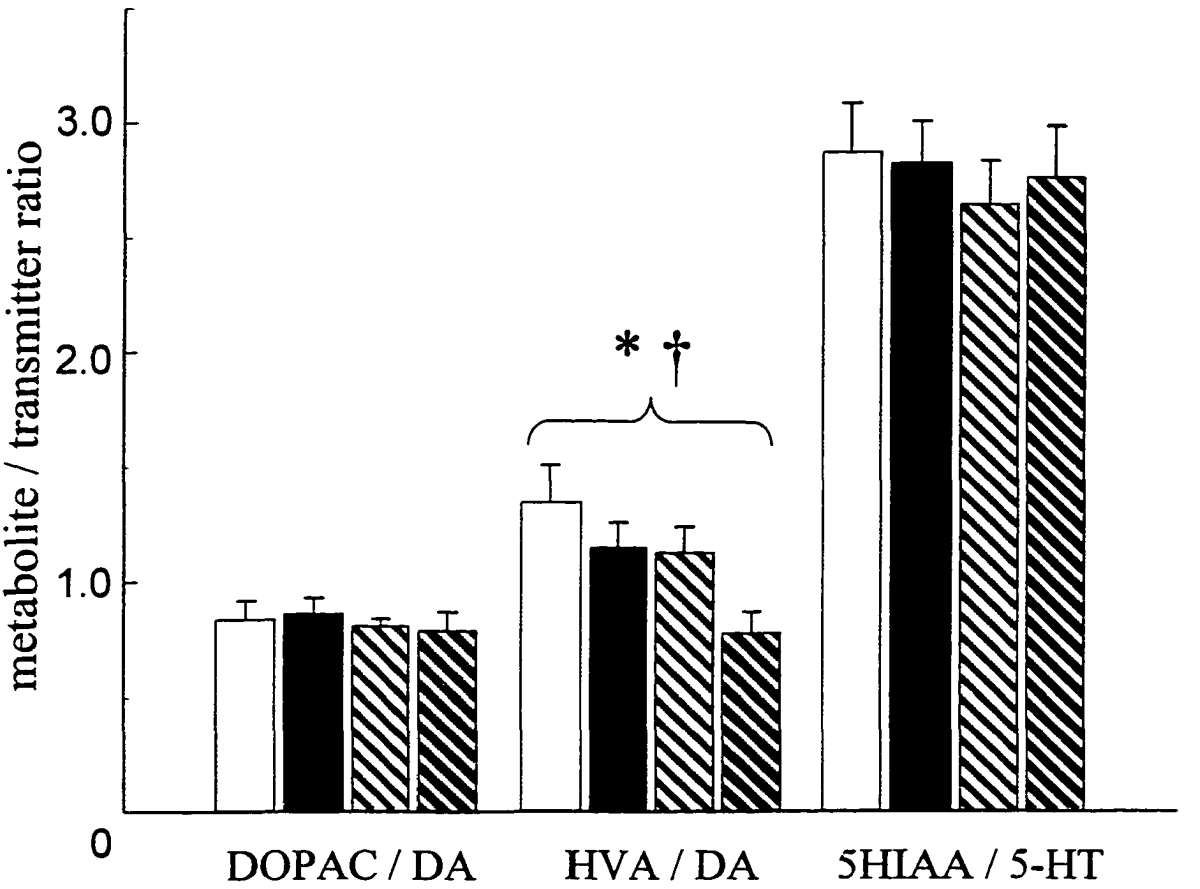


fig. 14

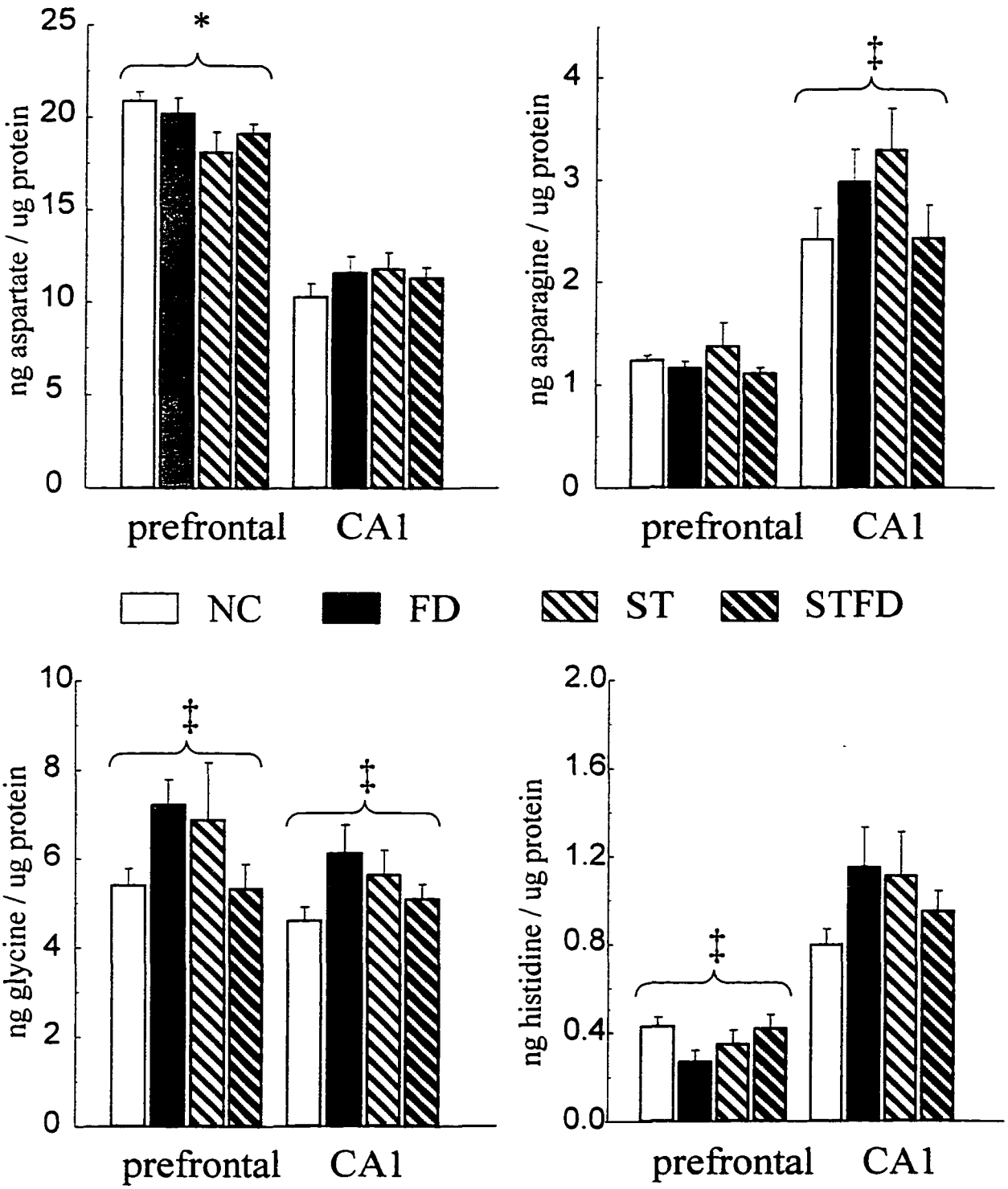


fig. 15

Discussion

The results of this study suggest that 21 days of restraint does not alter object recognition performance (i.e. increased exploration of a novel object over a previous sample object) in female rats. All groups in this study consistently exhibited more exploration toward the novel objects. These findings also show that food restriction does not influence object recognition either, even in subjects that were previously restrained over three weeks. No differences were evident in open field exploration either. These results are in dramatic contrast to males who are impaired by stress, and influenced by food deprivation in object recognition performance (Beck & Luine, in press).

These two manipulations did however appear to change region-specific neurochemistry. The greatest amount of change was observed in the prefrontal cortex and hippocampus CA1, involving both monoamine turnover and amino acid levels. Most notably, prefrontal dopamine activity was decreased in all the manipulated groups, with the combination group (STFD) showing the least activity, and while prefrontal aspartate, glycine, and histidine were all differentially affected by the manipulations. Aspartate was similarly decreased in both restraint groups, but glycine and histidine levels show an opposing pattern of affect. For each of these (glycine and histidine), the single manipulation groups (FD and ST) showed similar up-regulation (glycine) or down-regulation (histidine); which contrasts the little change measured in the combination group (STFD) in reference to the naïve-controls (NC). This pattern of up-regulation was mirrored in CA1 with respect to asparagine and glycine in the single manipulation groups, along with a greater amount of NE metabolism (as assessed by MHPG) in the two restrained groups (ST and STFD). Hence, chemical changes in two key regions implicated in mnemonic-associated neural processing occurred without affecting either open field exploration or object recognition.

These finding contrast the effects we previously observed in males (Beck & Luine, in press) in terms of both behavioral effects and regional change in neurochemistry. The females here did

not exhibit any discernable deficits in object recognition or increases in exploration time due to food restriction; whereas in males, food deprivation appeared to buffer stress induced object recognition performance deficits (possibly through increased exploratory drive). This distinction is also evident in the amino acid changes in hippocampus and prefrontal cortex. Males exhibited the greatest changes in hippocampal CA3, but females exhibited no changes in CA3. Females showed the most changes in CA1 and prefrontal cortex. Interestingly, both glycine and histidine were changed in males and females. Both were increased for ST males in CA3, but in females, glycine was increased (in PFC and CA1) and histidine was decreased (in PFC) in the ST and FD groups. Similar contrasts were also evident for serotonin activity. Males exhibited an increase in activity with food deprivation, but the females here did not. Since Mendelson and McEwen (1991) showed sex difference in CA1 serotonin receptor binding under control and stress conditions, this may reflect a condition of enhanced release without receptor up-regulation. Clearly, these results further show that males and females respond differently to the conditions of chronic stress, whether it is restraint or food deprivation.

The lack of a stress effect upon female behavior is not necessarily unexpected. Many previous works (as reviewed by Archer, 1973; 1975), attempted to model anxiety in rats but found that male responsiveness to behavior change differed from females. Typically, since males were studied first, "anxiety" was defined as the deviation in behavior between treated (shocked, restrained, etc.) males and non-treated control males. However, females exhibit different baseline measures (when compared to males) in many of the tests used to model anxiety. Thus, measures like open field activity (Meng & Drugan, 1993; Kiemiesky, Sick, & Kruppenbacher, 1977), escape conditioning (Davis et al., 1976), and the Porsolt swim test for immobility (Alonso et al., 1991) are seen as reasonable tests for male responsiveness to anxiety or stress, but not so for females. This we replicated here with our open field results.

Still, this relationship between stress and behavior does not always yield detriments in males and no change in females. As Shors and her colleges (Servatius & Shors, 1994; Shors &

Servatius, 1997; Beylin & Shors, 1998; Shors et al., 1998; Wood & Shors, 1998) have shown, eye-blink conditioning performance is enhanced in stressed males, but not in stressed females. This impairment appears to be estrous cycle related; in fact, baseline performance is cycle dependent as well (Shors et al., 1998). Hence, even in a paradigm where the sex-difference pattern of detriment is reversed, there is still a problem with baseline equivalency.

The results of Renner, Bennett, and White (1992) led us to believe that there would be little difference between baseline performance in males and females in a task of object recognition. Since Renner et al. found that sex differences were, overall, insignificant for object exploration in an open field, we thought that a paradigm involving object exploration could provide a common baseline from which we could observe stress-induced determents in each sex. Indeed, in terms of naïve-control performance, both males (Beck & Luine, in press) and females (shown here) explore the novel objects up through a 4-hour delay. However, unlike in the females observed in this study, males are affected by both chronic restraint and food deprivation to varying degrees (Beck & Luine, in press). Taken together, these results, appear to mimic those of the past anxiety studies, except that baseline performance at the outset do not differ.

Still, even with observing similar baseline performance in females (as we did males), we cannot rule out the possibility that females could be encoding the stimuli, the field and all its component parts (including objects), differently. Einon (1980) illustrated such a strategy sex difference in exploring a radial-arm maze. Males tend to sequence (using adjacent arms) more over trials, whereas females do not. This strategy-difference would not be evident if only a tally of errors committed was recorded. Since we only measured time with the objects (in contrast to a behavioral profile, i.e. Rosellini & Widman, 1989; Renner & Seltzer, 1994), there could be a difference in *how* objects are explored. Rosellini and Widman (1989) did find, after a day of stress (shock), male rats exhibit less diversity in object exploration (as defined as the amount of different behaviors recorded per exploration time). If this was the case, our findings may suggest that female rats do not change their exploratory behaviors after chronic restraint. Moreover,

based on the results of Shors et al. (1998), we cannot rule out the possibility that the estrous cycle may influence these behaviors as well. Williams, Barnett and Meck (1990) and Roof (1993) showed that cue and strategy usage in mnemonic tasks have a hormonal basis (organizational). Thus, the stable behavior, observed in our stress females, suggests that the female reaction to the stress regimens may be the underlying factor in this apparent sex difference.

Food deprivation, as a mild stress, had no affect on behavior, but in females, the level we used (85-90%) may not have been drastic enough to create behavior change. Wong (1979) found the most robust changes in females when holding weights at 70% baseline, however, such a drastic restriction was not appropriate here since we were trying to mirror the conditions used for radial-arm maze training. As seen in our previous study using males, food restriction can bring an added confound into a stress-testing behavior paradigm. It does not appear that normal weight loss for training is an issue in 21-day restrained females. At this point, we are left with accepting the hypothesis that the female lack of behavioral responsiveness to stress generalizes to a) the 21-day restraint paradigm (as the chronic stress), b) 85% food deprivation (as an added stress), and object recognition (as a test of memory assessment).

The changes we observed in underlying biology are quite intriguing since they predominately occurred in prefrontal cortex and CA1. Estrogen is implicated in modulating CA1 drendritic spine density (Gould, Woolley, Frankfurt, & McEwen, 1990) and prefrontal NE, DA, and5-HT (Luine, Richards, Wu, & Beck, 1998). The changes reported here might suggest an interaction between estrogen levels and apparent changes due to the stress of restraint and / or food deprivation. Restraint and food deprivation (alone and in combination) decreased prefrontal dopamine activity. This is also the case for 28-day OVX females (Luine et al., 1998) who also fail to exhibit differences from their E_2-treated counterparts in 1-hour radial-arm maze delay trials (Luine & Rodriguez, 1994). Interestingly, GDX males do exhibit significant decrements in comparison to those given a 28-day E_2 supplement on the radial-arm maze (Luine & Rodriguez, 1994). Therefore, the gonadal hormone response to these stressful conditions could distinguish

the male and female patterns of behavioral and neurochemical responsiveness to restraint and food deprivation.

Both organizational and activational effects have been suggested to explain gonadal hormone participation in the regulation of the physiological stress response. For instance, Patchev, Hayahi, Orikasa, and Almeida (1995) showed neonatally estrogenized females have decreased dark-cycle CORT levels (but not quite to male level) and hippocampal glucocorticoid receptor mRNA levels equal to males. Similarly, McCormick and Mahoney (1999) showed that temporary prenatal inhibition of androgen receptivity causes male rats to exhibit higher levels of CORT (towards the normal female range). Still, the activational effects of the gonadal hormones have received the most attention (as reviewed by Handa, Burgess, Kerr, and O'Keefe, 1994). The female response to stress, as measured by HPA activity, is modulated by the current state of estrogen. This has been shown both pharmacologically (Burgess & Handa, 1992) and across the estrous cycle (Viau & Meaney, 1991). Furthermore, the potency of the anxiolytic diazepam, inhibiting open field activity, is decreased in OVX females (Bitran, Hilvers, & Kellogg, 1991). The pattern in NE receptor regulation in prefrontal cortex shows an opposite pattern; K_2 receptor mRNA is increased in OVX females and subsequently decreased by estrogen (Karkanias, Li, & Etgen, 1997). Meanwhile, chronic stress (14 days of intermittent shock) does not appear to suppress the estrous cycle (as shown by Anderson, Saviolakis, Bauman, Chu, Ghosh, & Kant, 1996), although a recent report suggests estrogen levels may be generally higher (Shors, Pickett, & Paczynski, 1998). The sex-differences seen in CORT reactivity (Viau & Meaney, 1991) or in CORT receptor binding (McCormick et al., 1994; Turner, 1992; Turner & Weaver, 1985), thus appear to be modulated, in part, by gonadal hormone regulation of glucocorticoid and mineralcorticoid receptor synthesis (Patchev & Almeida, 1996). However, it should be noted that even under baseline conditions (prior to stress) males and females show different 2-DG activation levels in several limbic substructures, including CA1 (Brown, Siegel, & Etgen, 1996) and different hippocampal c-Fos labeling (Aloisi, Zimmermann, & Herdegen, 1997). In both cases, females

showed either higher baseline and / or treatment reaction to Formalin. Therefore, both organization and activational effects of estrogens and androgens must be considered in attempting to delineate the basis for the physiological reactions seen in males and females.

In conclusion, our study has shown that, unlike males, 21-day restrained females do not exhibit non-spatial memory deficits as assessed by object recognition. We also found no significant deviation in object recognition or open field behavior when stress was followed by 85% food deprivation. The failure of chronic stress to elicit behavioral deficits occurred while several physiological changes were observed. Restraint caused females to lose weight (as typically observed in males). Moreover, dopamine, norepinephrine, aspartate, histidine and glycine were all effected by stress or food deprivation in prefrontal cortex and hippocampus (CA1). These changes, from NC subjects, also differed than what was previous observed in males. The neurochemical changes observed in the female prefrontal cortex and CA1, apparently, are either not necessary for object recognition or not great enough to cause interference. Still, as shown recently by Shors (Shors et al., 1998; Wood & Shors, 1998), the current level of estrogen is a critical factor in female performance following being stressed. Clearly, further investigations into the underlying mechanism of both object recognition (as a task) and stress/coping in females need to be conducted. At this time, we believe that females react differently to chronic restraint and food deprivation (in terms of neurological processes) which buffer them from being affected in tests of object recognition and open field.

The Influence of Housing Status and Chronic Restraint on Male Object Memory and Neurochemistry

Our previous two studies illustrate how a secondary environmental condition, food deprivation, can moderate stress-induced changes in behavior and neurochemistry (Beck & Luine, in press; previous chapter). Male Sprague-Dawley rats showed a buffering in their behavior changes, after being chronically restrained, when they were subsequently food deprived during the behavior testing. Yet, in this procedure, there was a second manipulation. Subjects had been restrained (or left alone) under paired housing conditions, but for the subsequent manipulation, each pair was separated (one was then placed on food restriction, the other was not). In this study, our goal was to determine if the change in housing condition, from double-housed to single-housed, could also impact the behavior and neurochemical changes we previously observed in the restrained males.

Research into the effect differential housing conditions have upon the neural structure, physiology, and behavior profile of animals is rather extensive (as reviewed by Brain & Benton, 1979). In fact, Brain and Benton (1979) criticized the first decade of housing-condition studies, in rats and mice, for proposing a multitude of theories suggesting that both social isolation and social crowding were detrimental to animal behavior and physiology (i.e. via stress, over-arousal, under-arousal, disinhibition, maladjusted social behavior, etc.). Still, dopamine and norepinephrine neurochemical levels (Stolk, Conner, & Barchas, 1974; Thoa, Tizabi, & Jacobowitz, 1977; Weinstock, Speiser, & Ashkenazi, 1978), single-neuron neurochemical sensitivity to dopamine, norepinephrine, serotonin, and acetylcholine (Oehler, Jahkel, & Schmit, 1987), and acute corticosteroid (CORT) response to restraint (Giralt & Armario, 1989) have all been shown to be moderated by individual versus group housing. Thus, since the rat response to differential housing leads to several changes in both behavior and neurochemistry, the issue of the housing status must be addressed.

Here, the question arises to whether there might be an interaction between the behavioral and physiological effects of chronic restraint and those of housing status. For instance, chronic restraint has been shown to affect norepinephrine, dopamine, and serotonin (Luine, Villegas, Martinez, & McEwen, 1994a; Beck & Luine, in press), as has housing conditions (Thao et al., 1977; Oehler et al., 1987). Moreover, both stress and differential housing affect avoidance conditioning (Overmier & Seligman, 1967; Flint, Metzger, Benson, & Ricco, 1997; Kaneto, 1997; Viveros, Hernandez, & Gallego, 1990), general operant behavioral responses (Rosellini & Seligman, 1978; Harmer & Phillips, 1998), open field activity (Katz, Roth, & Carroll, 1981; van Dijken, Mos, van der Heyden, Tilders, 1992; Reboucas & Schmidek, 1997; Dalrymple-Alford & Benton, 1984), and water-maze escape recognition (Bodnoff, Humphreys, Lehman, Diamond, Rose, & Meaney, 1995; Mabry, McCarty, Gold, & Foster, 1996; Wade & Maier, 1986). Wade and Maier (1986) directly illustrated this interaction by finding that isolates' performances in the water maze were enhanced (to group-housed levels) when they were subjected to either noise or restraint stress prior to training for 3 weeks. Also important for our stress-behavioral paradigm, however, is the fact that both Rosellini and Widman (1989) and Renner (1987) each found that stress and housing effect the extent to which rats will explore objects in an open field.

Clearly housing status is a complex issue when viewed in the context that task/paradigm demands force utilization of either group or isolation housing without knowledge of whether housing influences performance of the task. Thus, in this experiment, we manipulated the purported non-stress housing conditions of our male rats (either one or two subjects per tub), but we kept the stress period as a group-condition (i.e. for the six hours they were all restrained in a separate room in a sound-attenuating chamber). Restraining the stress subjects together or separately should help a) determine if housing conditions are critical for the paradigm and b) remove whatever effect having restrained subjects in the same room as control subjects (during the restraining period).

To maintain continuity with the measures used in our previous studies, we used several field-related tasks to assess activity, tendency to explore, and object memory. Object recognition and open-field testing were used to measure of non-spatial memory and general activity levels, respectively. We also added 2 other field-related tasks. First, a "free" open-field, an open-field with a shaving-filled "burrow" attached to it (similar to Welker, 1957; Renner & Seltzer, 1991), was used to assess whether chronic restraint or housing affects the latency to enter the field, latency to approach two novel object in the field, and the total time subjects spend exploring the field. Dalrymple-Alford & Benton (1984) used a similar paradigm to show housing influences field-entrance latency. Second, we used the methods of Ennaceur, Neave, and Aggleton (1997) that change the object recognition task from a non-spatial memory test to a spatial memory test. In this version of the task, instead of replacing one of the sample objects with a novel object, one of the sample objects is moved to a new location on the field for the test trial. In this task of object placement recognition, subjects will preferentially explore the sample object that has been moved to the new location (Ennaceur, et al., 1997). In order to maintain the same total number of field trials as were conducted in our previous experiments, we only conducted two object recognition trials (instead of four), thus, there were 2 object recognition trials and 2 object placement trials (all conducted within 1-week post-restraint). All subjects were sacrificed after a prototypical sample trial on post-stress day 9. Monoamine and amino acid levels were measured in the hippocampus, amygdala and medial prefrontal cortex to determine if housing affected the stress-induced changes we observed previously in male rats (Beck & Luine, in press).

Methods

Subjects

Thirty-four male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), 50-60 days old upon arrival, were used. Upon arrival, subjects were either single or double-housed in

plastic tubs. The housing status of each subject remained constant throughout the experiment, and the room was set on a 12/12 reversed light/dark cycle (lights on at 8:00 p.m.). Baseline weight measures were used to match tubs to either naïve or stress conditions. Double-housed subject pairs were both placed in the same condition. For 21 consecutive days (following acclimation and initial baseline weight measures), 8 single-housed and 8 double housed (4 pairs) were taken daily to an adjacent room where they were placed into Plexiglas restrainers and housed in a sound-attenuating chamber (white noise was supplied by the inflow and outflow fans). Subjects remained restrained in the chamber for 6 hours (during the dark phase, approximately (9:30am – 3:30pm). All subject weights were measured (in the a.m.) on stress days 1, 7-8, 14-15, and 20-21.

Behavioral Apparatus

For all open-field trials (forced open-field, free open-field, object recognition, and object placement), we used an 88 X 88cm square open-field (with 60cm high walls). The experimenter was seated approximately 1 m from the field, and observed subjects using a monitor connected to a Panasonic video camera that faced the field from above the north wall. All trials were coded at a later date from the recorded tape. An additional tub and cardboard tunnel were used in the free open-field trial.

Behavioral Measures

Figure 15 illustrates the time-line of post-stress behavioral assessment. In the first two days post-stress, two open field trials were conducted. For the forced open-field trial (post-stress day 1), subjects' activities (sector visits, rears, wall climbs) were recorded both in reference to time (occurring in either the first or second 3 minutes of the 6 minute trial) and location (occurring in either inside or perimeter sectors). In the free open-field (day 2), instead of being placed at the midpoint of the south-end wall (as in the forced), each subject was placed in the plastic transport tub, and the tub itself was connected to a cardboard tunnel that opened to the

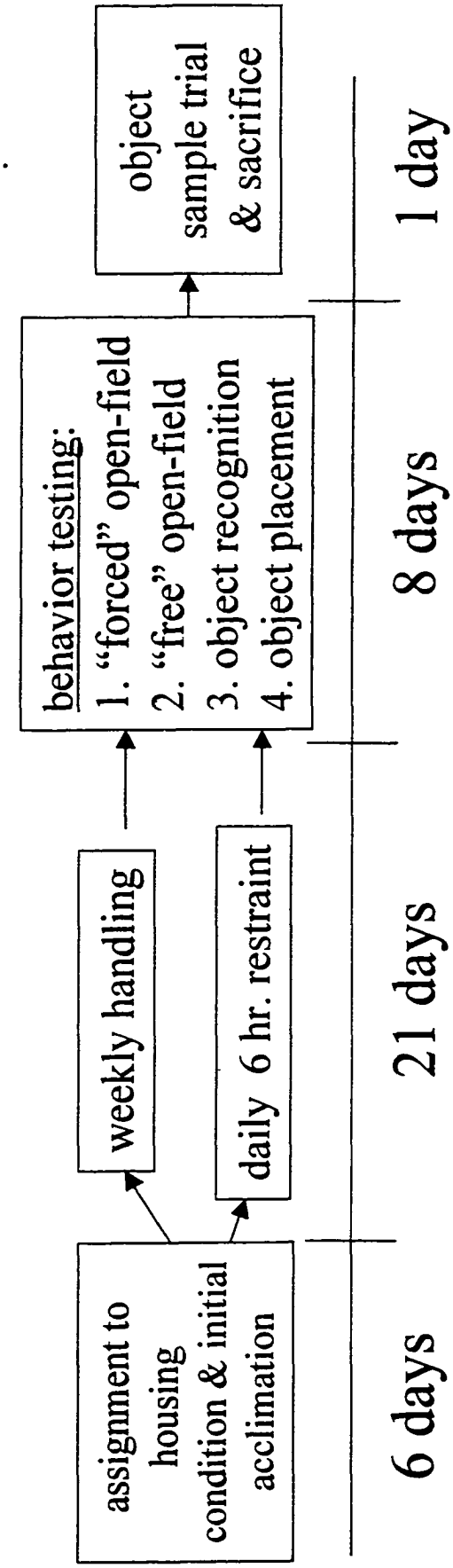


Fig. 16

field (from the midpoint of the south wall). Thus, the location of entry is the same as the starting point of the previous days forced open-field trial. However, two objects were added to the far (north) end of the field (equidistant from the corners). This task served three purposes: 1) as an assessment of the degree of approach/avoidance shown toward the field (total field time and latency to enter the field); 2) as a measure of the degree of approach/avoidance shown toward the objects (object exploration latency); and 3) an initial object-exposure habituation trial (before object recognition / object placement trials).

Over the next 5 days, 4 object-exploration memory trials were conducted. Object recognition sessions consisted of two 3-minute trials (in the forced open-field): a sample trial (T1) and a recognition trial (T2), each separated by an inter-trial interval of either 2.5 hours or 4 hours. The total time each subject interacted with each of the objects was recorded. In these trials, two identical objects were placed equidistant from the north corners during T1, but for T2, one of them is switched with a novel object. The left/right locations of the novel object (and which object was the sample or the novel) were fully counterbalanced within each separate delay session across groups. For object placement trials, the procedures in T1 are identical, but in T2 one of the objects was moved to the SW corner of the field. Because subjects typically begin trials along the south wall, we placed the subjects (for these trials in particular) closer to the SE wall (facing the east wall) so that they would not be biased to the closer proximity of the newly located, sample object. Again, total time exploring each object was recorded. In both tasks, exploration was defined as facing the object (within 2cm of the object), handling the object (while facing it), sniffing the object, or whisking the object.

Neurochemical Analyses

On post-stress day 9, subjects were each placed on the field for a single T1 (sample) trial. Immediately after the 3-minute trial, each was taken to a separate room and sacrificed by

decapitation (without anesthesia). Their brains were quickly removed and placed immediately in dry ice. The brains were subsequently stored at -70 °C until HPLC analysis.

Thirty-two of the forty-nine subjects used in behavioral analyses were used in the neurochemical analysis for monoamines and amino acids. All procedures were identical to those described previously (Beck & Luine, in press).

Data Analysis

For forced open-field measures, a mixed 2 X 2 X 2 (stress X housing X time) ANOVA was used to determine differences in sector visits and rears. The same procedures were used for free open-field measures of total field time and object exploration time. Differences in the latency to enter the field was assessed by a 2 X 2 (stress X housing) ANOVA. Correlated t-tests were used to determine significant object exploration differences (novel vs. sample) during the T2 recognition trial for both object recognition and object placement. Inter-group comparisons (in both tests) for object preference (during T2) were conducted using a between subjects 2 X 2 (stress X housing) ANOVA. These comparisons included indexes of habituation (T1/2 - T2 novel), novel preference (T2 novel – T2 sample), and discrimination ratio [(T2 novel-T2 sample)/ T2]. General exploration differences due to condition and trial were determined by a mixed 2 X 2 X 2 (stress X housing X trial) ANOVA.

Neurochemical tissue levels for monoamines are expressed as pg / ug protein and amino acids as ng / ug protein. A between subjects 2 X 2 ANOVA tested group differences for all neurochemicals. Fisher's LSD was used for all post-hoc analyses. All statistical analyses were conducted using GB-STAT (Dynamic Microsystems Inc).

Results

Weight Gain Analysis

As shown in figure 16, all subjects began with equal baseline weights and then gained weight between stress day 1 and post-stress day 8. However, the double-housed stress subjects had lower weights than other groups [stress X housing $F(1, 32) = 6.97$, $p < .01$; stress X time $F(4, 128) = 3.28$, $p < .01$]. No other group differences were found.

Behavioral Measures

Results from the forced open field trial suggest that general activity is not compromised in the stress subjects. The number of open field sectors visited during the 6-minute trial was not significantly different between any of the groups. The number of visits to the inside sectors, in particular, increased from the first to the second half of the trial in all groups [$F(1,32) = 7.33$, $p < .02$]. Although marginal in significance, stress groups generally did not visit the center as much as naïve groups [stress $F(1, 32) = 3.43$, $p < .07$]. As shown in figure 17, the stress groups also did not rear as much as the naïve groups [stress $F(1, 32) = 6.88$, $p < .01$], despite all groups showing similar increases of wall climbing over the trial [time $F(1, 32) = 5.52$, $p < .02$]. Thus, stress appeared to decrease the exploration behaviors of inside sector transversals and rears without decreasing activity toward the walls (i.e. outer sector visits and wall climbs).

The results in the "free" version of the open field were different, across groups, than we observed in the "forced" open field (see figure 18). In this task, the stress groups showed a quicker latency to enter the field [stress $F(1, 28) = 3.25$, $p < .08$] and approach the objects [stress $F(1, 29) = 4.24$, $p < .05$]. Housing affected the pattern of total field time [housing $F(1, 32) = 4.55$, $p < .04$; time $F(1, 32) = 80.24$, $p < .0001$]. The most obvious effect was upon the single-housed stress group. This group spent much more time exploring the field in the first 3 minutes than the other 3 groups. Moreover, even though all groups increased their time in the field in the

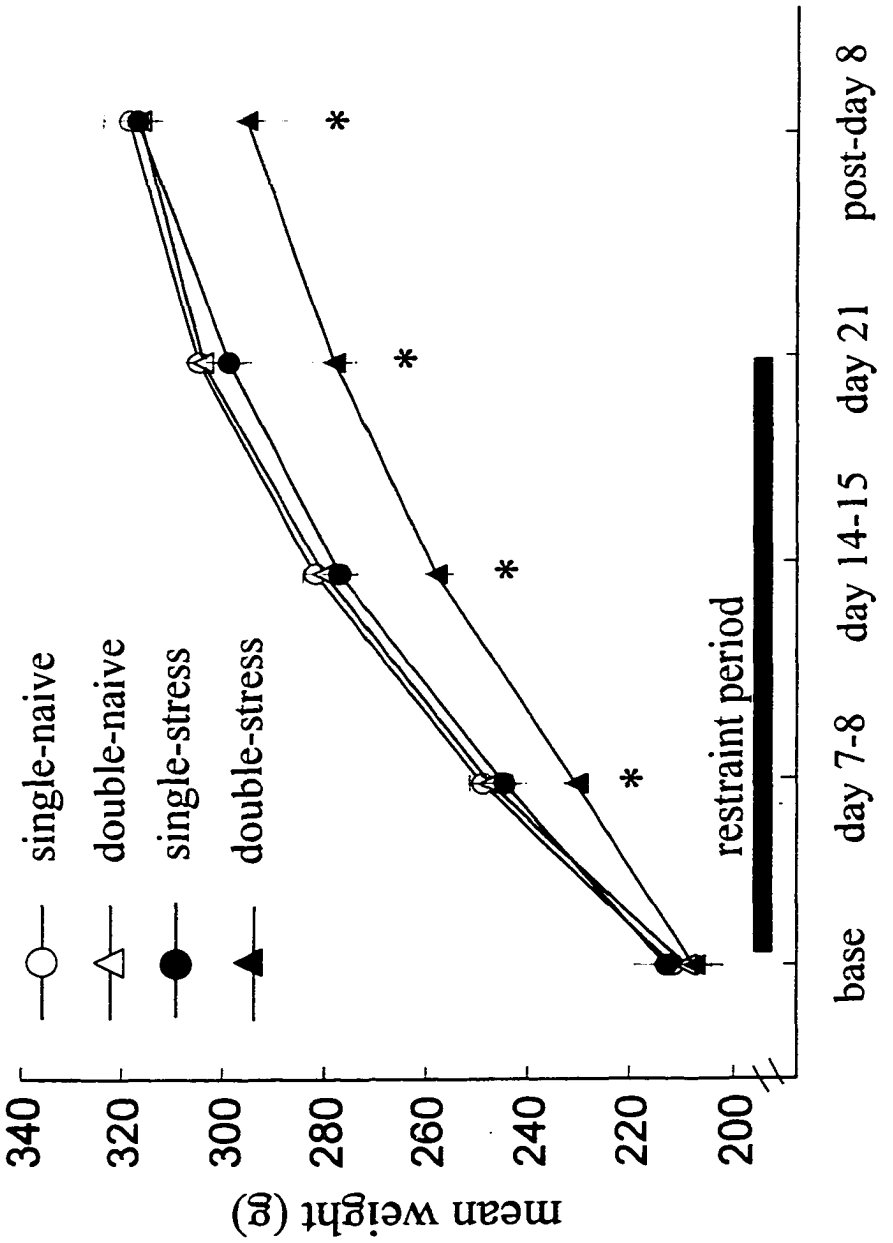


fig. 17

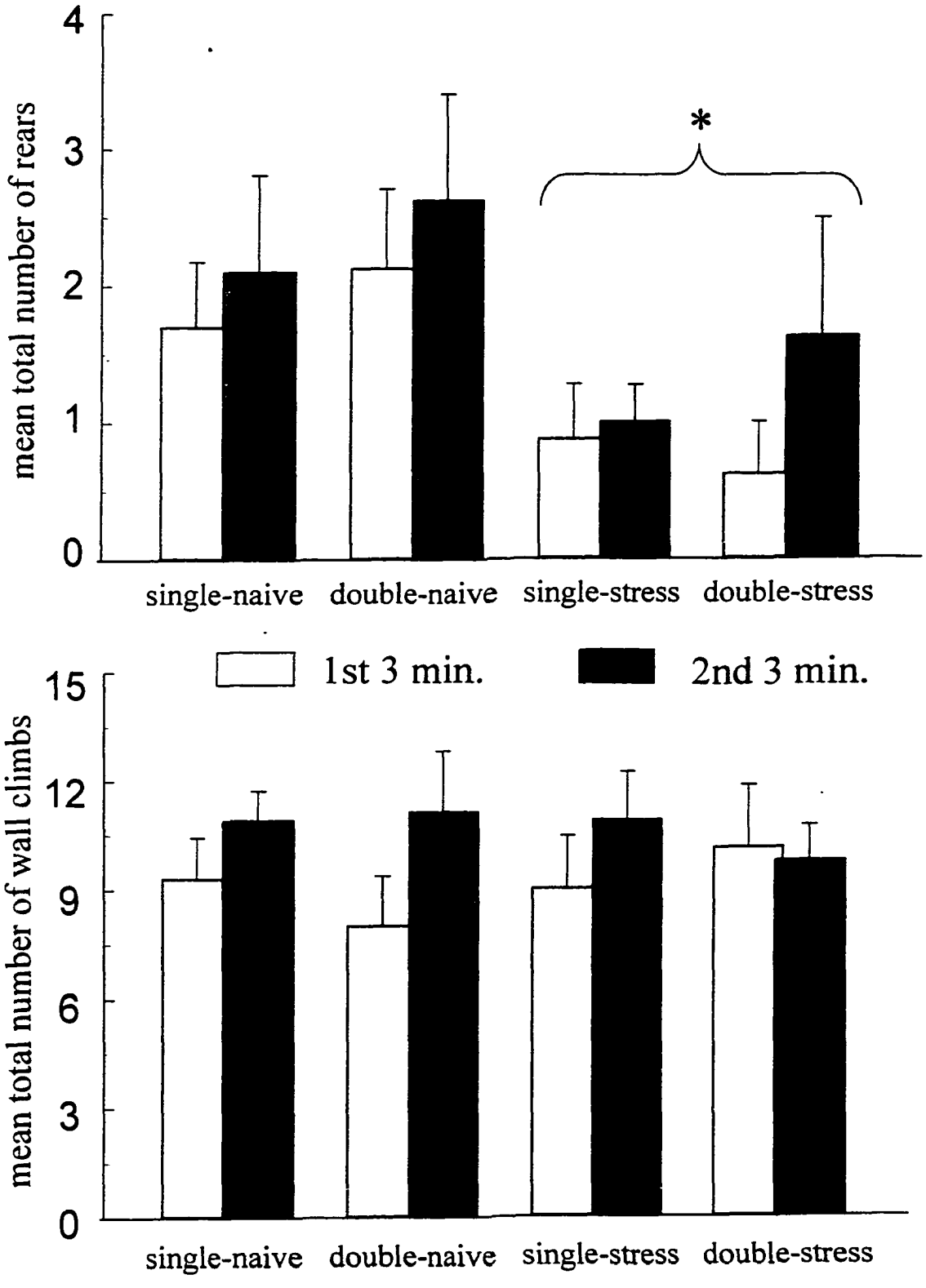


fig. 18

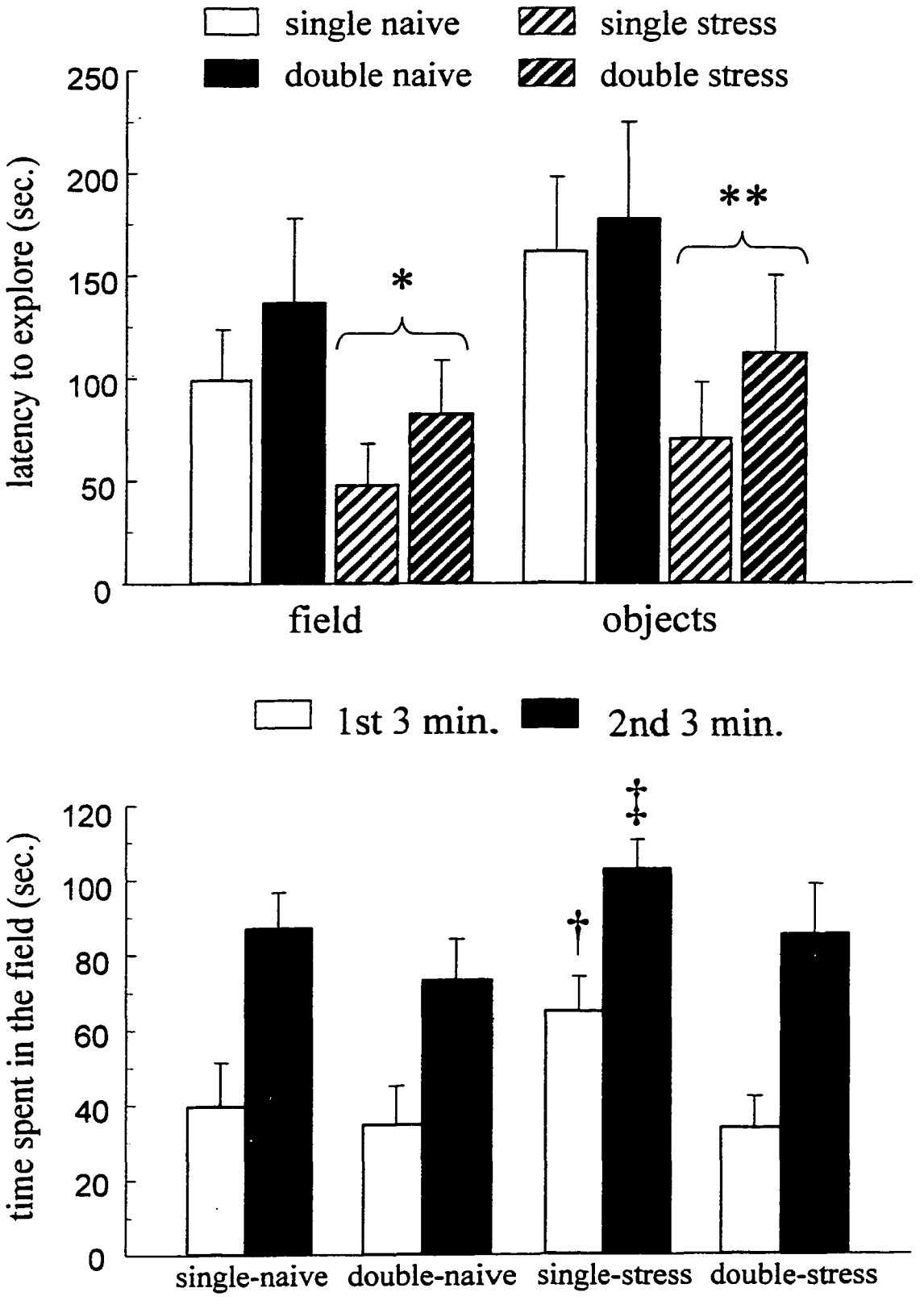


fig. 19

second 3 minutes of the trial, the single-housed stress group still was more active than double-housed controls. In fact, 2 double-housed controls never approached the objects (even though they entered the field). This pattern suggests, when subjects are freely allowed to access a previously explored open-field, stress subjects are quicker to enter the field and quicker to approach the novel stimuli (the objects). Still, it should be noted that the single-housed stress group spent the most time in the field (compared to all other groups), suggesting that stress decreased any inhibition to explore the field and single-housing increased field activity in stressed subjects.

Object recognition was greatly influenced by the housing status of the groups. As illustrated in figure 19, the subjects showed a significant preference for the novel object (in the test trial – T2), if they were singly housed. This was true following both delays, 2.5 and 4 hours, and for both single-housed control [$t(9) = 7.33$, $p < .0001$; $t(9) = 2.44$, $p < .04$] and single-housed stress groups [$t(7) = 4.13$, $p < .005$; $t(7) = 2.92$, $p < .02$]. The indexes of habituation, novel preference, and discrimination ratio were not significant. Both double-housed groups, regardless of stress condition, did not show a significant preference for the novel object. The correlated-t was marginally significant [$t(6) = 2.08$, $p = .08$] for the double-housed stress group at the 2.5-hour delay, but was not at the 4-hour delay. The double-housed controls did not show a significant preference in either delay trial. This lack of preference shown by double-housed subjects could be due to the lesser amount of time they spent exploring the stimuli. As illustrated in figure 20, single-housed groups explored more than double-housed groups in both the 2.5 [$F(1, 30) = 14.80$, $p < .0006$] and 4 hour [$F(1, 32) = 5.64$, $p < .02$] trials. Thus, it appears that double housing is deleterious for object recognition at long delays because subjects do not explore sufficiently.

The object placement trials yielded a different pattern of results compared to object recognition (see figure 21). The correlated t-tests, for significant discrimination, showed significant differences in exploring the novel versus the sample object following a 2.5-hour delay

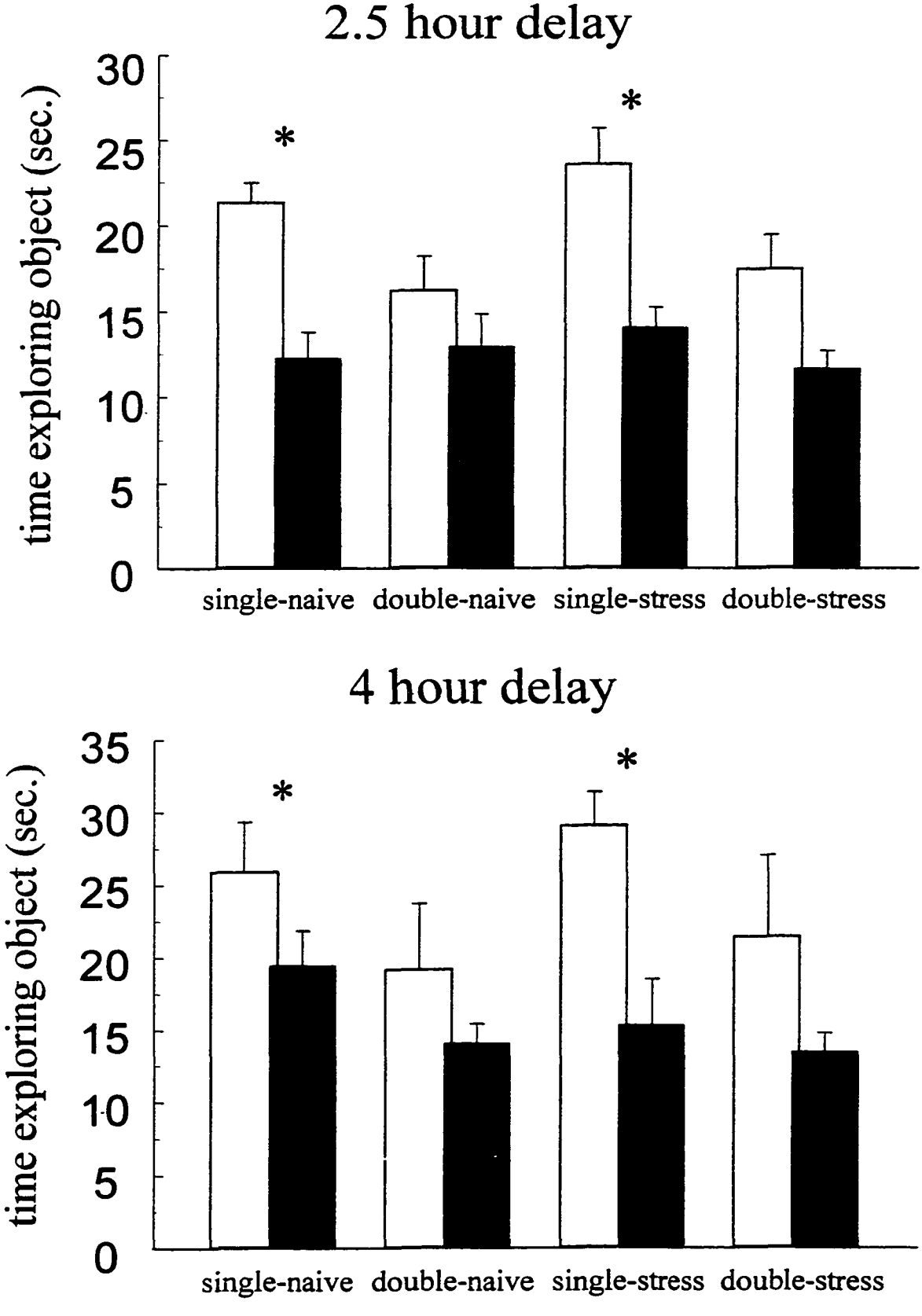


fig. 20

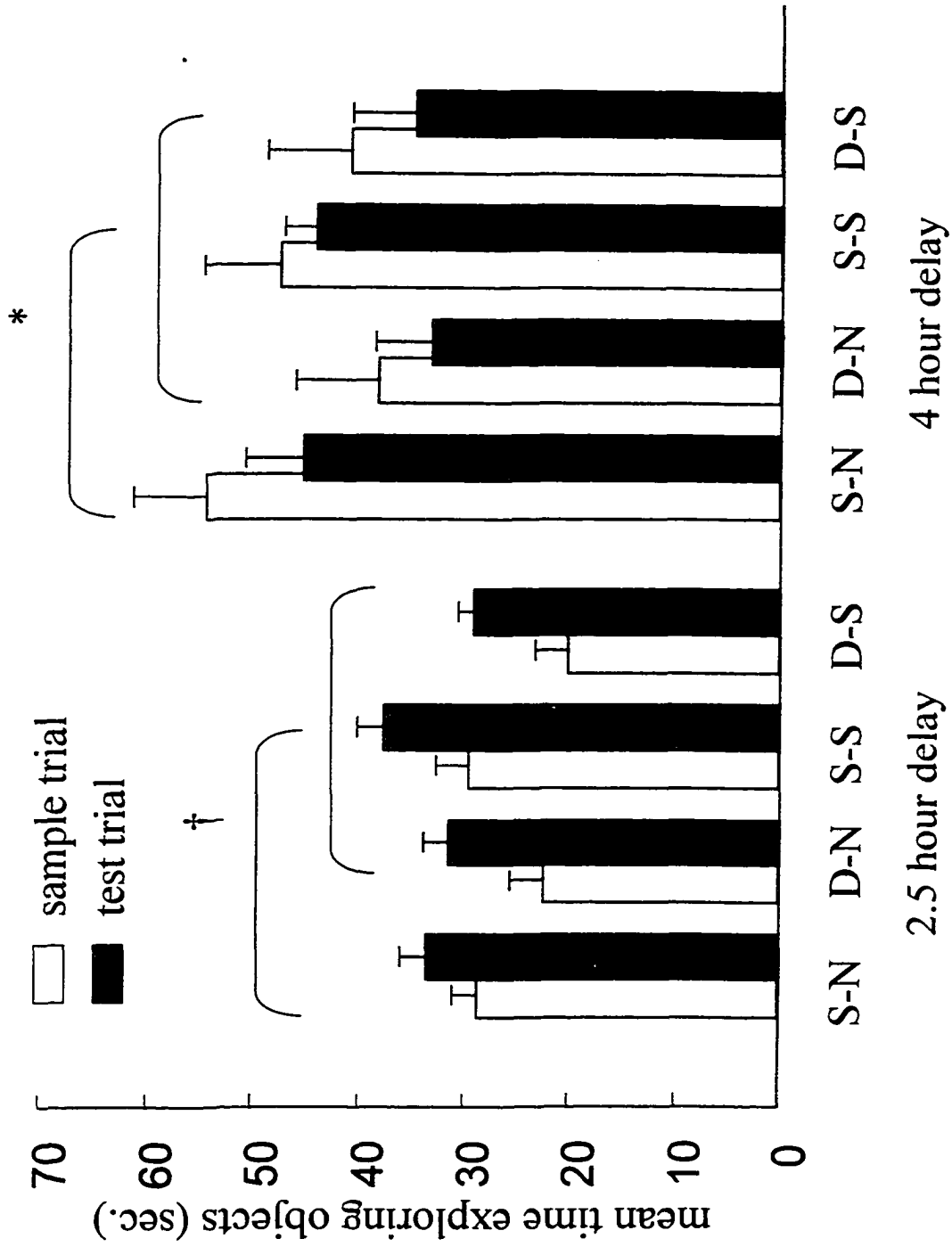


fig. 21

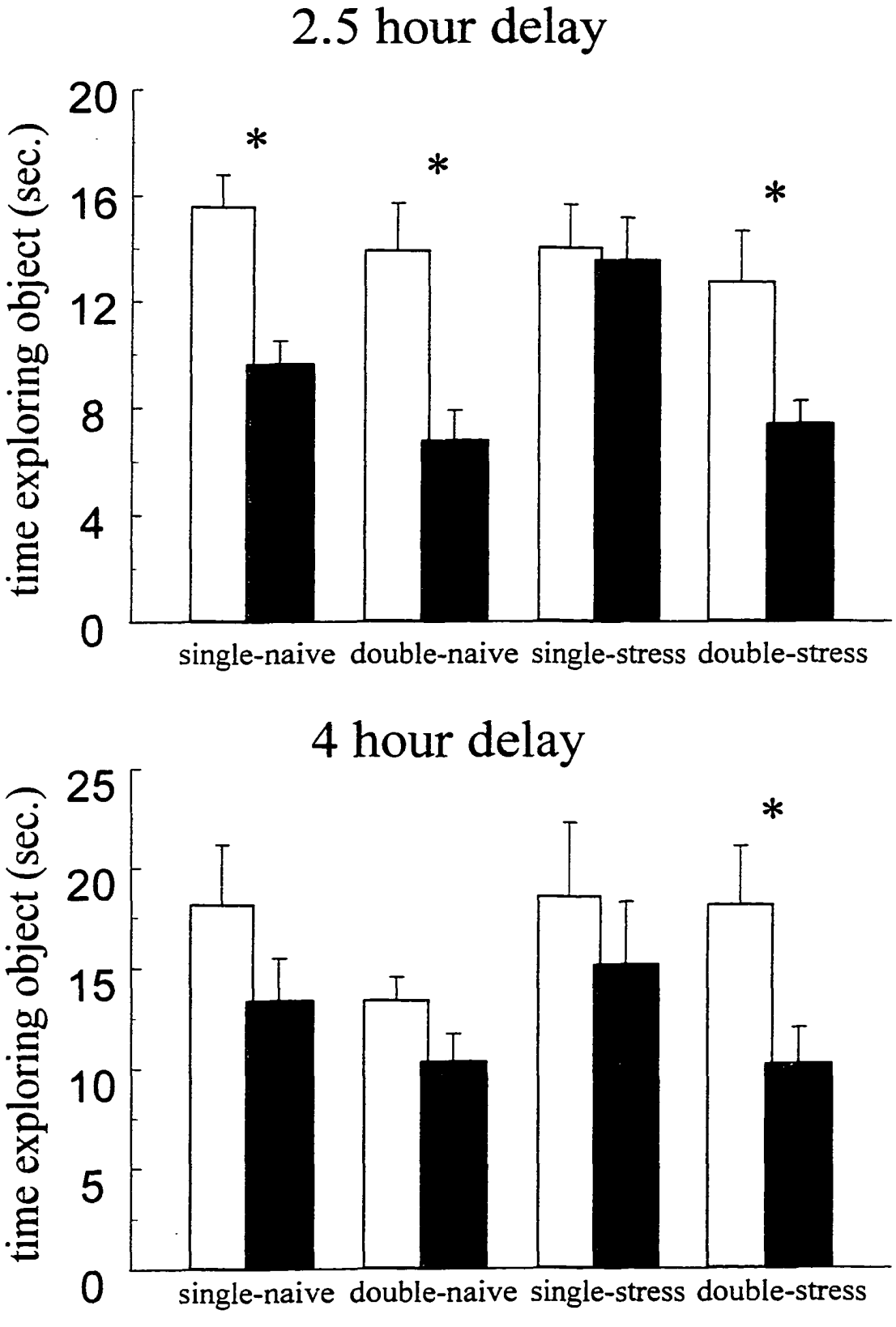


fig. 22

($p < .01$) for all groups except the single-housed stress group. Both the novel preference index and discrimination ratio were also significant for stress [$F(1, 30) = 4.45$, $p < .04$; $F(1, 30) = 7.40$, $p < .02$] and housing [$F(1, 30) = 3.08$, $p < .08$; $F(1, 30) = 5.93$, $p < .02$]. When the delay was extended to 4 hours, both single-housed groups failed to approach significance. The double-housed naïve group showed a preference that was marginally significant [$t(6) = 2.18$, $p < .07$], and the double-housed stress group showed a clear preference [$t(6) = 4.81$, $p < .003$]. Indexes of habituation, novel preference, and discrimination ratio were not significantly different. Again, when the total exploration time was assessed (see figure 22), we found that single-housed subjects explored the objects more than double-housed subjects in the 2.5-hour delay trial [$F(1, 32) = 29.91$, $p < .0001$]. The same pattern is also evident in the 4-hour delay trial, although high variability and the greater effect of time [$F(1, 26) = 77.20$, $p < .0001$] muted the statistical significance of the effect. Still, single-housing subjects generally increased their exploration time of objects; yet, greater exploration in the single-housed stress group did not result in a significant preference for the novel location of the object.

In summary, stress and housing had selectively different patterns of affect on each of the behavioral measures. Generally, stress appeared to decrease exploration latency, and single housing increased total exploration time. These two influences appeared to interact in both the recognition and placement trials to yield opposing results. Increases in exploration were associated with greater discrimination of the novel object over the sample object in recognition trials, but increased exploration did not lead to preference for the sample object in the new location.

Neurochemical Measures

Monoamine levels were affected by both experimental manipulations across the four brain areas. Both the dopaminergic and noradrenergic systems appeared to be affected by either the

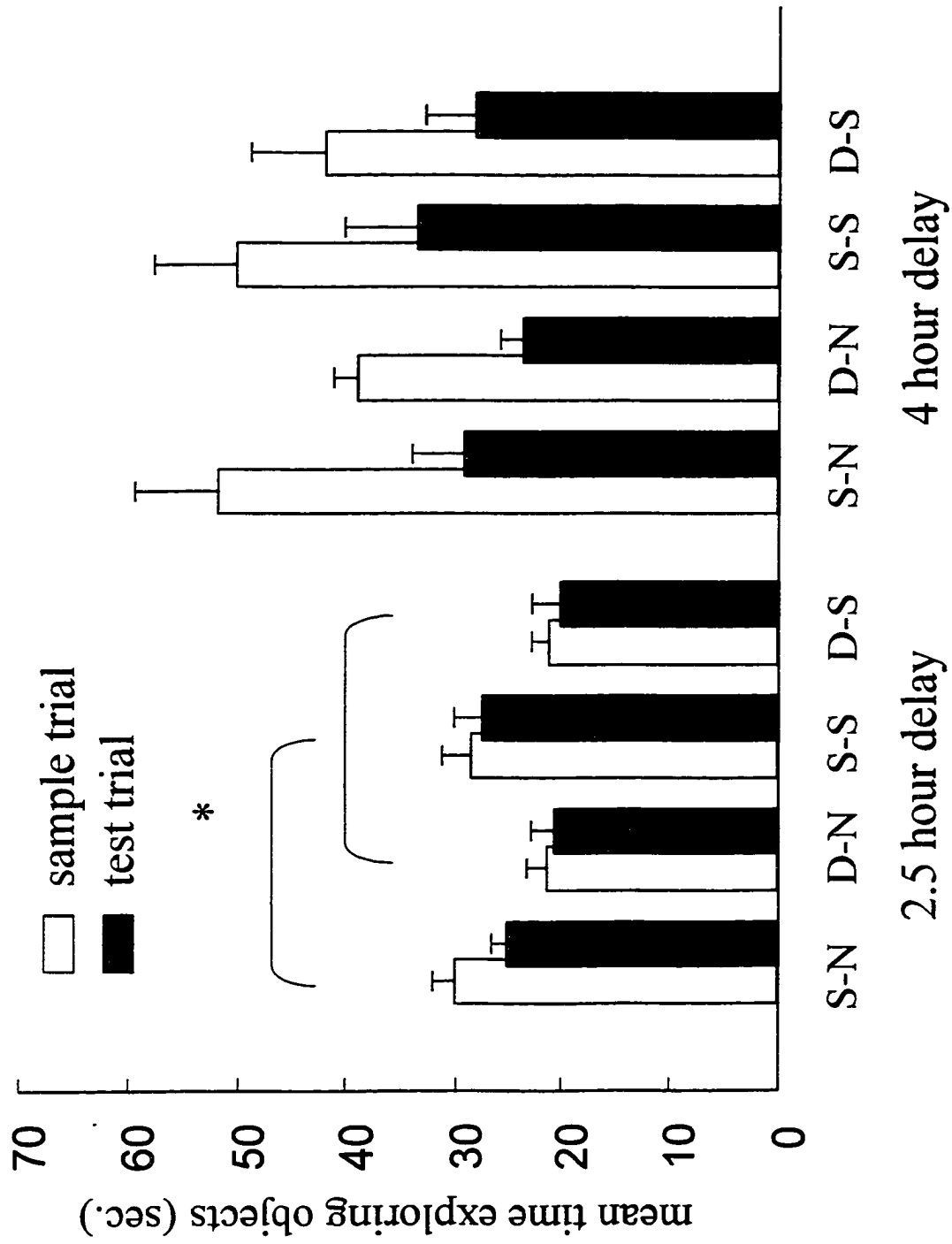


fig. 23

stress or the housing conditions. In the prefrontal cortex, both housing and stress affected dopamine activity (see figure 23). Dopamine levels were lower in stress subjects [$F(1, 30) = 3.48$, $p < .07$], but metabolism (HVA levels) was higher in single-housed naïve and double-housed stress groups [$F(1, 30) = 6.53$, $p < .01$]. Dopaminergic activity (as assessed by DOPAC / DA ratio) was also affected in the amygdala [$F(1, 30) = 4.75$, $p < .03$], with single-housed groups (naïve: 0.38 ± .04, stress: 0.48 ± .05 pg / ug protein) exhibiting less activity than double-housed groups (naive: 0.56 ± 0.07, stress: 0.76 ± 0.19 pg / ug protein). As shown in figure 24, norepinephrine levels were selectively higher in CA3 in double-housed stress subjects [housing $F(1, 30) = 4.40$, $p < .04$; stress $F(1, 30) = 3.49$, $p = .07$; housing X stress $F(1, 30) = 3.36$, $p < .07$]. There was also a marginal effect for norepinephrine in CA1 in both stress groups [$F(1, 30) = 3.04$, $p < .09$].

Amino acid analyses in each of the four brain regions showed the most consistent changes were in histidine levels. In the prefrontal cortex, histidine was significantly lower [stress $F(1, 29) = 3.49$, $p < .07$; stress X housing $F(1, 29) = 4.44$, $p < .04$] in the double-housed stress group (0.11 ± .01ng / ug protein) in comparison to double-housed controls (0.18 ± .03 ng / ug protein) while single housed groups exhibited similar levels to each other (naïve: 0.13 ± .02, stress: 0.14 ± .01 ng / ug protein). Histidine also exhibited changes in CA3 [interaction $F(1, 28) = 3.64$, $p < .06$) with the single-stress group having higher amounts (0.80 ± .15 ng / ug protein) than the single-naïve group (0.50 ± .05 ng / ug protein). The levels for double-housed subjects were intermediate (naïve: 0.65 ± .11, stress: 0.56 ± .07 ng / ug protein). A similar interaction pattern appears evident for amygdala glutamine levels, although, statistically it was only a trend ($p < .10$). No changes were found in CA1.

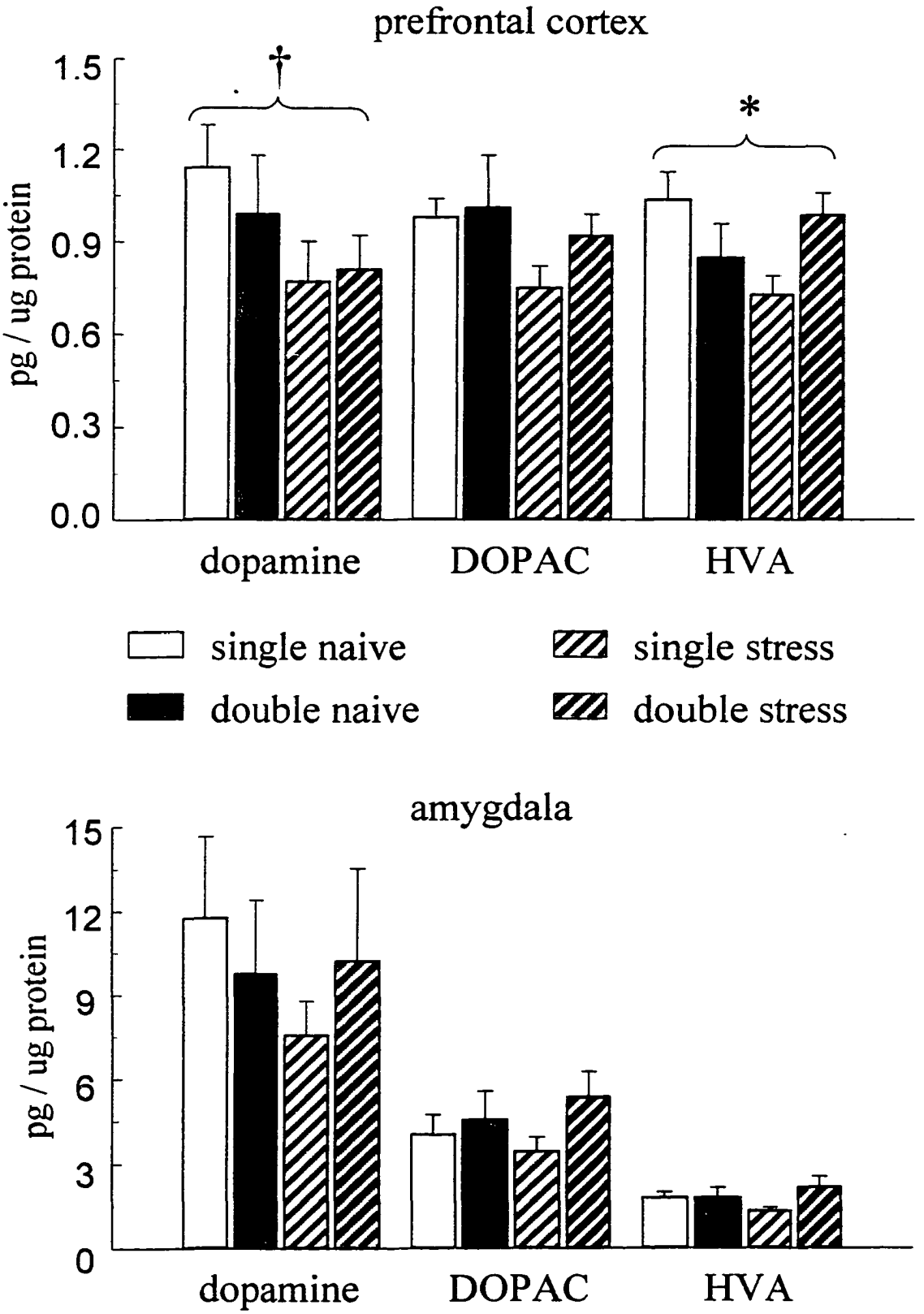


fig. 24

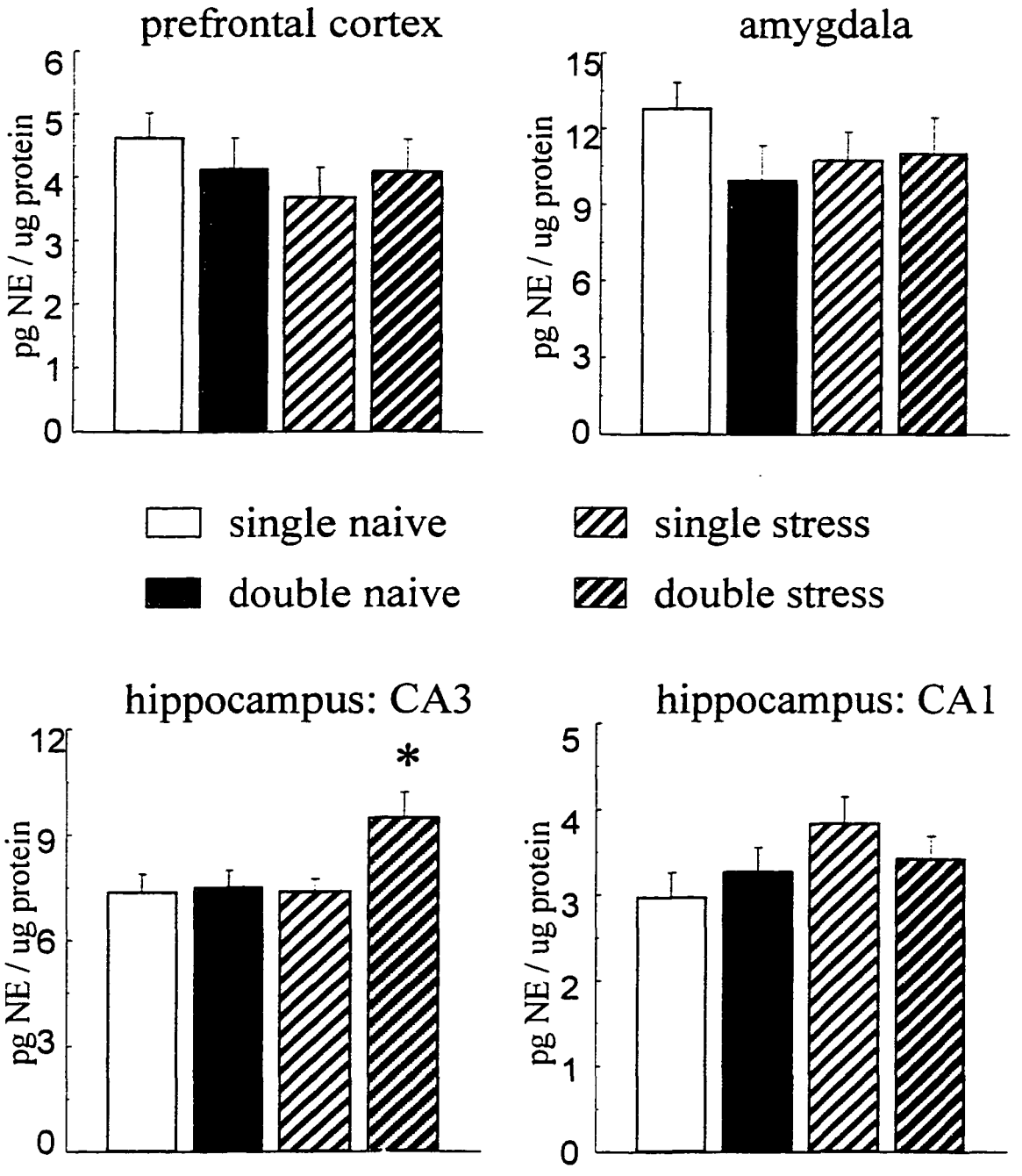


fig. 25

Discussion

The current findings illustrate the importance of the housing environment on behavioral and neurochemical changes in male rats following chronic restraint stress. Stress appeared to have different effects on object exploration depending on housing status. Stress subjects that were single-housed showed the typical preference for a novel object after either a 2.5 or 4-hour delay, but did not show a preference for exploring an object that was moved to a new location in the field. The opposite pattern was observed in double-housed stress subjects. They did not exhibit a significant preference for a novel object, but did show a clear preference for exploring the sample object in a new location. We also found that object recognition is also influenced by the housing status of the subjects. The double-housed naïve subjects did not show a novel preference, as single-housed subjects had. Yet, we did find that both single and double-housed naïve subjects (as well as double-housed stress subjects) do show a preference for the novel placement of an object (after a 2.5-hour delay). These findings should also be viewed in conjunction with the "free" open-field results which suggest that stress subjects tend to approach objects quicker than naïve controls when placed in a choice situation. In addition, we also found underlying neurochemical changes that were related to the stress and housing status of each group.

Differences in neurochemistry across groups included dopamine, norepinephrine, and histidine levels, all of which were found before (Beck & Luine, in press) to be affected by 21-days of chronic restraint. Here, we replicated the findings of increased norepinephrine and histidine in the hippocampus (CA3) following stress. It should be noted that only single-housed stress subjects exhibited an increase in histidine and only double-housed stress subjects had increased levels of norepinephrine. At this time, the nature of the stress-induced histidine and norepinephrine effects remains somewhat unclear, insofar as to whether these levels represent increases or decreases in histamine and norepinephrine synthesis and metabolism. It appears, though, that the changes are influenced by both the stress histories of the subjects and the housing condition. Moreover, others (Stolk et al., 1974; Thoa et al., 1977; Weinstock et al., 1978) have

shown that noradrenergic activity can be affected by housing status when reactivity is assessed using pharmacological challenges. For norepinephrine, our chronic stress paradigm seems to also serve as a challenge, thus producing similar effects. Prefrontal dopamine was lower in stress subjects, but dopamine metabolism (HVA) was only lower in single-housed stress subjects. The double-housed stress group exhibited higher HVA levels than the single-housed stress group. Past research into the interaction between stress and housing (Holson, Ali, & Scallet, 1988) found that frontal dopamine metabolism (HVA) immediately after a forced swim is lower in rats that are single-housed compared to those pair-housed. Our results suggest a continuation of this pattern of less stress-induced dopamine activity when subjects experience a chronic regimen of stress.

The neurochemical profiles of these subjects could underlie the behavioral differences between the stress groups. Higher CA3 norepinephrine levels were previously found in chronically restrained subjects that exhibited poor performance in object recognition (Beck & Luine, in press). Interestingly, in that study, food-deprived stress subjects, who did not exhibit as poor a performance (as stress only), did not show higher levels. Those results appear to parallel the differential performance here between our two housing conditions with double-housed restrained males having higher norepinephrine in CA3 and object recognition impairments. However, we need to also address possible role of the prefrontal dopamine changes to these behaviors. The double-housed stress group also showed a greater amount of prefrontal HVA than the single-housed stress, although the HVA/DA ratio in this study was not statistically significant. But the HVA/DA ratio for the double-housed stress group was 1.39 (single-housed stress was 1.22 and both naïve groups were 1.10) which is approximately the same as we found in our previous study (1.31) for the impaired stress group (Beck & Luine, in press). The question, then, is whether changes in norepinephrine or dopamine could explain the behavioral differences in the tests for object recognition and object placement.

Both object recognition and object placement recognition (or location recognition) tasks have been studied in prefrontal and fornix-lesioned rats (Ennaceur et al., 1997; Ennaceur & Aggleton,

1994; Rothblat & Kromer, 1991). Neither prefrontal nor fornix-lesioned rats showed behavioral impairments that differed from controls in tests requiring memory for the object (Ennaceur et al., 1997; Ennaceur & Aggleton, 1994; Rothblat & Kromer, 1991). Similar results are even found if a new object is compared to the sample object in a rotated configuaration (Ennaceur & Aggleton, 1994). Subjects with adrenalectomy-induced tissue damage in the dentate gyrus of the, hippocampus also perform as well as controls, if they are given CORT supplements (McCormick, McNamara, Mukhodphyay, & Kelsey, 1997). Yet, fornix-lesioned rats fail to show a preference to explore an object in a novel location (Ennaceur et al., 1997). Ennaceur et al. (1997) purpose that these results illustrate the importance of the hippocampus to the mnemonic processes of object spatial location, whereas, neither the prefrontal cortex or hippocampus appears necessary for recognition of object features (provided they are in the same location). Lesion studies, though, do not completely explain the possible ramifications of over or under-activity in intact neural connections that can lead to changes in affect, attention, or memory that may bias this form of behavioral testing. For instance, the pharmacological studies using these procedures (Willig, Van de Velde, Laurent, M'Harzi, & Delacour, 1992; Ennaceur & Meliani, 1992) suggest dopaminergic and cholinergic manipulations affect object recognition behavior but only when drugs are given in high doses (scopolamine) or to impaired populations (D2 agonist RU 41656 to Roman high-avoidance rats). Still, Cavoy and Delacour (1993) failed to find any significant affect of aging on object recognition in intact rats, while showing spatial deficits across age using a t-maze. However, an important point of distinction between the past object recognition / object placement studies (cited above) and the current work needs to be addressed. The delay intervals here ranged from 2.5 to 4 hours, in contrast to those typically used in the past that rarely extended beyond one hour. In fact, the longest delay was 15 minutes in the prefrontal and fornix lesion comparison study (Ennaceur, Neave, & Aggleton, 1997) and 5 minutes in the aging study (Cavoy & Delacour, 1993). Ennaceur and Aggleton (1994) attempted a 2-hour delay, but their controls failed to exhibit significant preferences for the novel object. Although not stated in their report

(single versus group housing), we raise the question of whether housing conditions (as shown here) could explain the lack of naïve subject sample recognition after a 2-hour delay. McCormick et al. (1997) did show a adrenalectomy (ADX) effect at a 1hour delay, with control and ADX+CORT groups showing clear discrimination, but their next delay length was 24-hours where rats do not show any preference (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992; Richards, Beck, & Luine, 1997). Thus, our previous report (Beck & Luine, in press) is the only other study that has performance of naïve rats on object recognition at delays beyond 1 hour. In addition, our neurochemical profile of impaired stress groups here closely approximates the impairments in that study.

The nature of the behaviors exhibited in these tasks need to be viewed with reference to the unquestionable effect the home-cage environment had upon the object oriented tests. Our previous studies in males and females showed that non-stressed Sprague-Dawley rats show a preference for a novel object following a 4-hour delay. In the current study, both double-housed naïve and stress rats failed to show reliable object preference (although the stress group did approach significance in the shorter delay). At the same time, the single-housed stress group (which performed equally as well as single-housed controls in object recognition) did not show a preference for the sample in the novel location following a 2.5 or 4-hour delay while the double-housed stress group did (up through a 4-hour delay). Consequently, we are left with two alternatives that most readily explain this dissociation in object memory tasks. Stress under different housing conditions lead to different memory dysfunctions; alternatively, the housing conditions could affect exploratory drive thus moderating behavioral patterns in these "'pure' working memory" tasks (Ennaceur & Delacour, 1988, p. 57), leading to behaviors that do not necessarily reflect memory functioning.

The hypothesis that chronic stress impairs different forms of memory (i.e. spatial versus nonspatial) depending on the context of the subjects housing environment has not been investigated. However, since we have already found differences in performance across sex,

males showing impairments in object recognition while females do not, it is possible that differences could exist within sex. Restraint in our previous studies was under double-housed conditions and individual housing was used during testing. In addition, the first behavioral decrements reported using the 21-day restraint paradigm (Luine et al., 1994) were spatial in nature (using the radial-arm maze) and occurred under individual housing conditions. Conrad et al. (1996) used pair and triplicate housing and found Y-maze decrements after a 4-hour delay in stress subjects, however, two important procedural differences must be noted between that study and the current work. First, Conrad et al. had their subjects inspect the maze for 15 minutes during the sample trial (in contrast to our 3 minutes). They reported that performance in controls was stable at 2 hours but began to wane at the 4-hour mark, as did our controls. Our subjects though could only experience the sample objects for one-fifth the time (assuming maximum exploration). Second, restraining and testing is conducted during the rats' active period (dark phase) in our lab; Conrad et al. restrained and tested during the light phase. Although these variables have not been thoroughly scrutinized under this paradigm, others (Holson & Walker, 1986) have considered this in similar multi-behavior assessment studies. Depending on the timing of testing (for ours it is 2-3 hours after lights out), basal CORT levels could explain differences observed in both naïve and previously stressed subjects. Thus, our results, suggesting both spatial and nonspatial memory impairments, are feasible under the previous conditions used in our laboratory. Spatial memory deficits are present in males single-housed during the stress period and non-spatial memory deficits are present in males double-housed during stress. The failure of naïve double-housed males to show similar behavior as single-housed controls will be addressed in context to the second alternative.

The housing situation of rodent subjects can influence general open-field activity, exploratory activity, and feeding (Holson, Scallet, Ali, Sullivan, & Gough, 1988); thus subject reactivity to the testing situation, as influenced by housing condition, needs to also be considered as a possible explanation these results. Our behavioral measures showed groups could be dissociated by

housing status. Previous research has shown that individual housing will tend to increase subject activity in an open field (Holson, Scallet, Ali, & Turner, 1991) and decrease the latency to enter a novel environment if the subjects have also been stressed (Holson et al., 1988). We did not see any differences in "forced" open-field activity (i.e. sector visits), but single-housed stress subjects did spend more time in the "free" open-field when given the option to explore the field versus remaining in the shaving-filled tub. The single-housed stress group was also the quickest to enter the field and also approach the novel objects. This essentially replicates the findings of Holson et al. (1988) while also showing that the interaction between isolation and stress reactivity is generalizable beyond an acute shock paradigm. Moreover, if we digress back to the "forced" open field results, we saw that chronic stress generally decreased the amount of rearing during the trial (regardless of housing status). This may reflect a decrease in exploratory drive or initiative to orient in the environment. Now, if we consider the "free" open field results showing the single-housed stress group spending more time in the field, it appears that any exploratory impediment created by chronic stress is not global and is, in fact, interrelated to the housing status of the subjects. Taking this argument further, double housing the subjects clearly influenced their performance in the object recognition paradigm (an exploration dependent task). If we look at the total trial exploration times, double-housed subjects generally explored objects less than their single-housed counterparts. This was the case in both object recognition and object placement tasks. It appears that a threshold of object exploration is necessary for these subjects (male Sprague-Dawley rats) to show the prototypical pattern of novel-object preference, and double-housing them decreases their drive to explore the objects to the needed degree. It appears that single-housing subjects counteracts any exploration decrements caused by chronic restraint, leading them to show behavior comparable to single-housed controls.

It is also possible that both of these scenarios are occurring with housing affecting drive or attention, which decreases the quality of the exploration of the sample object, thus leading to an apparent memory deficit for that object. In the single-housed situation, the rat experiences

arousal from being taken out of the cage and placed on the field. We know this because they even show a different neurochemical profile, compared to the home-cage, when they are sacrificed immediately after a trial. The important point here is that there is no immediate arousing stimulus in the home-cage. This contrasts the double-housed rat's situation. It is constantly being aroused because of the presence of another male. In fact, the only time the double-housed subject is away from its counterpart, is when its being placed in the novel testing environment. Thus, after a sample trial, the double-housed subject is placed into an arousing environment (with another male) while the single-housed subject is not. The presence of arousing stimuli, during the delay, in the local environment could be causing interference in double-housed males. Moreover, since these memory tests are not based upon drive reduction (i.e. reinforcement), then the quality or importance of the information (the objects) may not be given much "priority" in the encoding process. Therefore, it is more readily forgotten when other stimuli are given priority (during the delay). Although not tested directly, by such means as placing cage-mates in individual tubs during the delay, the general exploration of the objects, both during the sample and test trials, was lower in double-housed groups. Thus, if it is a memory-related process that is involved, it is most likely occurring in conjunction with decreased sample exploration. Either a failure to properly encode the information regarding the sample or proper maintenance of that information could be viewed as valid hypotheses. In both cases, though, general exploration time during both sample and test trials cannot be ruled out as a major factor influencing the object recognition results. Conversely, sampling time appears to be critical to object recognition but may not be as critical in object placement. The difference in housing / stress effects on performance between these two tasks needs to be addressed in light of this discussion. First, housing-status also played a role in this task. After the 4-hour delay, the double-housed stress group showed a significant preference and the double-housed naïve group showed a preference that was marginally significant. Clearly, the influence of housing is not as evident as in the object recognition task, both naïve groups show a significant degree of

preference after the 2.5-hour delay, while stress only affected the single-housed stress subjects. This stress effect also does not appear to be dependent on exploration time. Again, single-housed stress subjects are exhibiting different behavior than their naïve counterparts and the double-housed groups, but we cannot completely rule out the possibility that they are simply reacting to the testing situation differently than the other groups. For instance, it is possible that the single-housed stress group does recognize the object as being in a new location, but because they are more active in exploring the field, they fail to exhibit a clear-cut preference for one over the other. This notion supports the argument of inefficient exploration as the underlying behavior for these effects, but unfortunately, this interpretation does not explain the differences in the object recognition trials unless this purposed reactivity is focused by novel stimuli (not by relocated sample stimuli). Thus, in a task involving new objects, single-housed stress subjects focus upon the novel object, but in a task involving simple object relocation they do not. In any case, it is clear that housing status greatly influences exploratory activity and consequently, must be regarded as an important variable able to moderate these field-based memory tasks.

At his time, we should reiterate that the previously observed neurochemical changes also occurred in the double-housed stress group, thus supporting the fact that 21 days of restraint under double-housed conditions does lead to impairment in object recognition, while single-housing does not. The importance of this fact cannot be emphasized enough for it suggests that two concurrent environmental conditions, namely restraint and social-arousal, may be required to cause these deficits in object recognition. Others (Blanchard, Sakai, McEwen, Weiss, & Blanchard, 1993; Brown & Grunberg, 1995) have described the behavioral and endocrine profiles of multi-housed male rats, such as the increased levels of CORT in both dominant and subordinate rats (compared to individual controls). This suggests, in group-housed chronic stress experiments, that the stressful nature of the environment, as assessed by CORT levels or sympathetic activity, could generally be higher than if the same procedure is conducted in isolated rats. Giralt and Armario (1989) tested this hypothesis using a 14-day restraint paradigm

but found no differences in CORT levels, but Armario, Luna, and Balasch (1983) showed individual and group-housed rats do not show differential increases in CORT levels when placed in a testing situation. It should be noted that, although we did not measure CORT levels, our double-housed stress males were the only group to exhibit significantly lower weights throughout the study (from stress through sacrifice). Other studies have shown that group-housed males are more likely to exhibit activity-induced ulcers (Pare, Vincent, & Natelson, 1985; Pare & Valdsaar, 1985) and have a greater sensitivity to pain (Schwandt, 1993). Nevertheless, Mormede, Lamaire, Castanon, Dulluc, Laval, and Le Moal (1990) have suggested that the CORT response does not fully characterize the stress response unless the sympathetic nervous system response is also considered, especially when testing over different social-dominance systems. This may explain why we observed select catecholamine changes predominantly in the double-housed stress group over the single-housed stress group.

In conclusion, the current work further characterizes the nature of the 21-day restraint paradigm under several activity, exploration, and memory tests. Chronic stress appears to decrease reactivity in the "free" open-field while also decreasing field scanning (rearing) in the "forced" open-field. Housing and stress interacted in producing differential patterns of effect in the spatial (object placement) and non-spatial (object recognition) tests for object memory. Currently, it appears that housing influenced the level of reactivity or exploration of objects, thus, biasing the test results. Single-housed stress subjects showed impairments in the spatial version of the test, and double-housed stress subjects showed impairment in the non-spatial version of the test. Moreover, increases in hippocampal CA3 norepinephrine and prefrontal dopamine turnover, in the double-housed stress group, replicate previous findings (Beck & Luine, in press) that conducted restraint under double housing conditions. Therefore, we conclude that the deficits observed in object recognition are dependent on stressing the animals under double-housed conditions. Further investigation into the general housing effects on object placement and

recognition are warranted since each task appears to be influenced by the housing conditions of the subjects.

Chronic Stress and Housing Condition Differentially Affect Limbic Neurochemistry, Object Memory and Open-Field Behaviors in Females

Chronic restraint-stress, as defined by 21-days of 6-hour restraint, has been extensively studied for its behavioral and physiological effects by our lab (as review by Luine, 1997; Beck & Luine, in press) and by others (as reviewed by McEwen, 1998; Galea, McEwen, Tanapat, Deak, Spencer, & Dhabhar, 1997; Conrad, Galea, Kuroda, & McEwen, 1996). The results of this and other studies show that males and females react differently to this stress paradigm. Both Luine (Luine et al., 1994a; Luine, Villegas, Martinez, & McEwen, 1994b; Luine, Martinez, Villegas, Magarinos, & McEwen, 1995) and Conrad (Conrad et al., 1996) have shown stress-induced spatial memory impairments in male rats. Beck and Luine (in press) also found that object recognition is impaired by the same stress regimen, thus suggesting a general memory deficit (not solely spatial) in male rats. Females however did not show such impairment (previous chapter). In addition, we did not find the same neurochemical changes in females, as we did in males. However, we only assessed basal (homecage) levels in the previous female study, not arousal levels (immediately after testing).

In this study, we also assessed the role of housing condition (single versus double) in addition to stress in females. Other research (Viveros, Hernandez, & Gallego, 1990; Dalrymple-Alford & Benton, 1981) has shown that novelty and aversive stress behavior tests dissociate males and females, but these effects are also influenced by the housing status of the subjects. Such an interaction might be expected since males and females show different basal corticosterone (CORT) levels depending on the number of cagemates (Brown &Grunberg, 1995; 1996). Males increase their CORT levels as more males are added to the homecage, while females (who have higher levels under single housing) decrease their levels when more females are added. In our previous study (last chapter), males were greatly influenced in their response to stress by their housing status, both behaviorally and neurochemically. Based on previous

research, we expected to find a different behavioral and neurochemical pattern of effect in females.

The current study also comprehensively characterizes female field behavior using several field-based tests. A forced open-field is used to assess basic novelty-induced behaviors (peripheral versus inner sector visits, rears, and wall climbs) that have previously been shown to dissociate behavior among male stress subjects. A free open-field is used to assess a subject's approach/avoidance to the field and novel objects in that field. After the first two days of open-field testing, the remainder of the post-stress interval involves object-related memory assessment. Object recognition (Ennaceur & Aggleton, 1994) assesses non-spatial memory by measuring subject exploration preference for a novel object placed in a position of a previous sample object. Object placement recognition (Ennaceur, Neave, & Aggleton, 1997) is used to assess the preference of a subject for a previously explored object that has been moved to a new location in the field (spatial memory). By using these non-reward dependent tasks, we can assess several behavioral parameters within a single week, following the termination of stress. Monoamine and amino acid tissue levels in select limbic system structures (prefrontal cortex, hippocampus, and amygdala) following a field trial were assessed to provide a neurological profile that may underlie the observed behavioral differences.

Methods

Subjects

Thirty-four female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), 50-60 days old upon arrival, were used. The housing status of each subject was determined upon arrival (single or double) and remained constant throughout the experiment. Room lighting followed a 12/12 reversed light/dark cycle (lights on at 8:00 p.m.). Baseline weight measures from the initial acclimation period (6 days) were used to match tubs to either naïve or stress conditions. Double-housed subject pairs were placed in the same condition. For 21 consecutive

days (following acclimation and initial baseline weight measures), 8 single-housed and 8 double housed (4 pairs) were taken daily to an adjacent room where they were placed into Plexiglas restrainers and housed in a sound-attenuating chamber (white noise was supplied by the inflow and outflow fans). Subjects remained restrained in the chamber for 6 hours (during the dark phase, approximately (9:30am – 3:30pm). All subjects were weight (in the a.m., prior to stress) on stress days 1, 7-8, 14-15, and 20-21.

Behavioral Apparatus

For all open-field trials (forced open-field, free open-field, object recognition, and object placement), we used an 88 X 88cm square open-field (with 60cm high walls). The experimenter was seated approximately 1 m from the field, and observed subjects using a monitor connected to a Panasonic video camera that faced the field from above the north wall. All trials were coded at a later date from the recorded tape. An additional tub and cardboard tunnel were used in the free open-field trial.

Behavioral Measures

In the first two days post-stress, two open field trials were conducted. For the forced open-field trial (post-stress day 1), subjects' activities (sector visits, rears, wall climbs) were recorded both in reference to time (occurring in either the first or second 3 minutes of the 6 minute trial) and location (occurring in either inside or perimeter sectors). In the free open-field (day 2), instead of being placed at the midpoint of the south-end wall (as in the forced), each subject was placed in the plastic transport tub, and the tub itself was connected to a cardboard tunnel that opened to the field (from the midpoint of the south wall). Thus, the location of entry is the same as the starting point of the previous days forced open-field trial. However, two objects were added to the far (north) end of the field (equidistant from the corners). This task served three purposes: 1) as an assessment of the degree of approach/avoidance shown toward the field (total field time and latency to enter the field); 2) as a measure of the degree of

approach/avoidance shown toward the objects (object exploration latency); and 3) an initial object-exposure habituation trial (before object recognition / object placement trials).

Over the next 5 days, 4 object-exploration memory trials were conducted. Object recognition sessions consisted of two 3-minute trials (in the forced open-field): a sample trial (T1) and a recognition trial (T2), each separated by an inter-trial interval of either 2.5 hours or 4 hours. The total time each subject interacted with each of the objects was recorded. In these trials, two identical objects were placed equidistant from the north corners during T1, but for T2, one of them is switched with a novel object. The left/right locations of the novel object (and which object was the sample or the novel) were fully counterbalanced within each separate delay session across groups. For object placement trials, the procedures in T1 are identical, but in T2 one of the objects was moved to the SW corner of the field. Because subjects typically begin trials along the south wall, we placed the subjects (for these trials in particular) closer to the SE wall (facing the east wall) so that they would not be biased to the closer proximity of the newly located, sample object. Again, total time exploring each object was recorded. In both tasks, exploration was defined as facing the object (within 2cm of the object), handling the object (while facing it), sniffing the object, or whisking the object.

Neurochemical Analyses

On post-stress day 9, subjects were each placed on the field for a single T1 (sample) trial. Immediately after the 3-minute trial, subjects were taken to a separate room and sacrificed by decapitation (without anesthesia). Their brains were quickly removed and placed immediately in dry ice. The brains were subsequently stored at -70 °C until HPLC analysis.

Thirty-two of the forty-nine subjects used in behavioral analyses were used in the neurochemical analysis for monoamines and amino acids. All procedures were identical to those described previously (Beck & Luine, in press).

Data Analysis

For forced open-field measures, a mixed 2 X 2 X 2 (stress X housing X time) ANOVA was used to determine differences in sector visits and rears. The same procedures were used for free open-field measures of total field time and object exploration time. Differences in the latency to enter the field were assessed by a 2 X 2 (stress X housing) ANOVA. Correlated t-tests were used to determine significant object exploration differences (novel vs. sample) during the T2 recognition trial for both object recognition and object placement. Inter-group comparisons (in both tests) for object preference (during T2) were conducted using a between subjects 2 X 2 (stress X housing) ANOVA. These comparisons included indexes of habituation (T1/2 - T2 novel), novel preference (T2 novel – T2 sample), and discrimination ratio [(T2 novel-T2 sample)/ T2]. General exploration differences due to condition and trial were determined by a mixed 2 X 2 X 2 (stress X housing X trial) ANOVA.

Neurochemical tissue levels for monoamines are expressed as pg / ug protein and amino acids as ng / ug protein. A between subjects 2 X 2 ANOVA tested group differences for all neurochemicals. Fisher's LSD was used for all post-hoc analyses. All statistical analyses were conducted using GB-STAT (Dynamic Microsystems Inc).

Results

Weight Gain Analysis

As shown in figure 25, all subjects began with equal baseline weights and then gained weight between stress day 1 and post-stress day 9 [time $F(4, 128) = 489.91$, $p < .0001$]. Stress groups exhibited lower weights than controls following the first week of restraint through the end of the study [stress X time $F(4, 128) = 2.63$, $p < .04$]. After the baseline period, double-housed naive subjects were consistently heavier than both groups of stress subjects. The single-housed naïve group was also significantly heavier than the stress groups over the second two weeks of

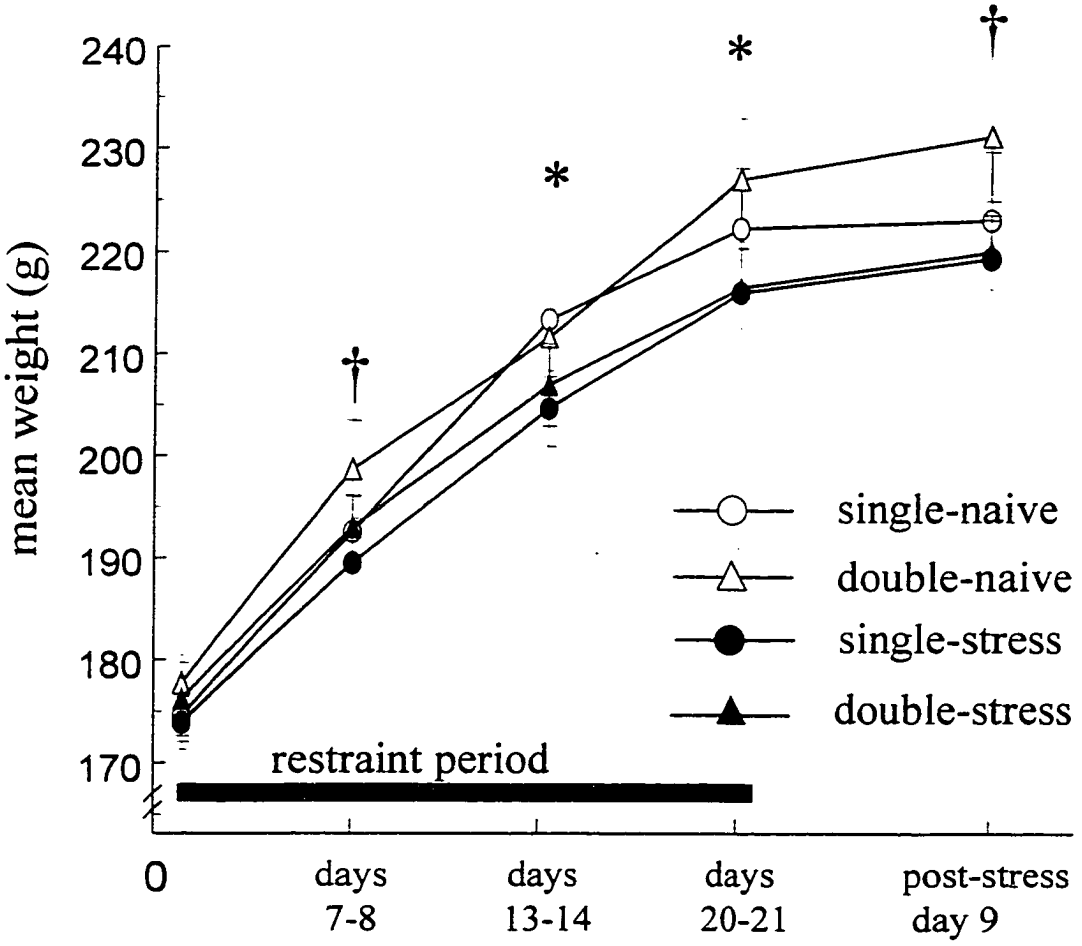


fig. 26

stress. At termination, however, the single-housed naïve subjects had similar weights to the stress groups, whereas the double-housed naïve subjects were still heavier.

Behavioral Measures

Generally subjects showed no groups differences in the forced open-field. As illustrated in figure 26, the same number of outer-sector visits were made across groups, with a general decrease in visits over time [$F(1, 32) = 39.53$, $p < .0001$], but inner-sector visits did vary between groups and across time [stress X housing X time $F(1, 32) = 5.50$, $p < .03$]. Both stress groups (single and double-housed) showed significantly less activity than single-naïve subjects (double-housed stress subjects were also less than double-naïve subjects). Yet, in the second half of the trial, the single-housed stress group showed similar activity in the middle of the field as the two naïve groups, while the double-housed stress group did not show such an increase; they remained at the same level. As shown in figure 27, there was a general increase in rearing across the two halves of the trial for all groups [time $F(1, 32) = 12.86$, $p < .001$], but wall climbing did not change across time or between conditions.

The "free" version of the open field also differentiated the double-housed stress group from the other groups. As shown in figure 28, the double-housed stress group showed the longest mean latencies both to enter the field and approach the objects for the first time. However, because of the large degree of variability within the groups (especially the double-housed stress group), an extended median test was performed on both data sets, which suggested marginal difference between groups [$\chi^2 (N = 34, 3) = 6.60$, $p = .07$] for each measure. Only one of the subjects in the double-housed stress group had field entrance and object exploration latencies below the overall median value for the groups (15.44 sec. and 44.15 sec., respectively). All other groups showed a more normal distribution around the median. Thus, it appears that the double-housed stress group did represent a different population on this measure. The total time spent exploring the field was also influenced by the stress status of the group. The stress groups spent

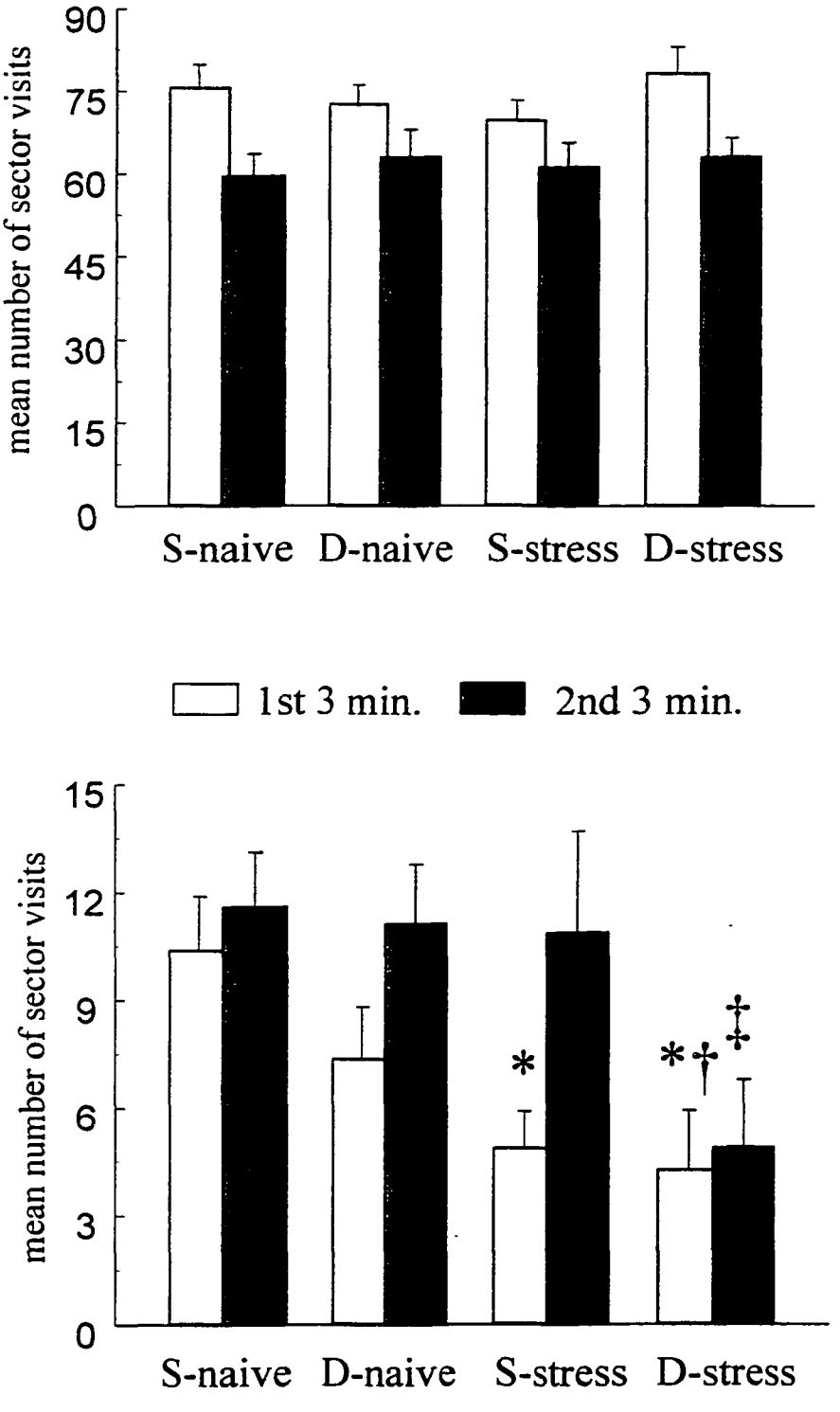
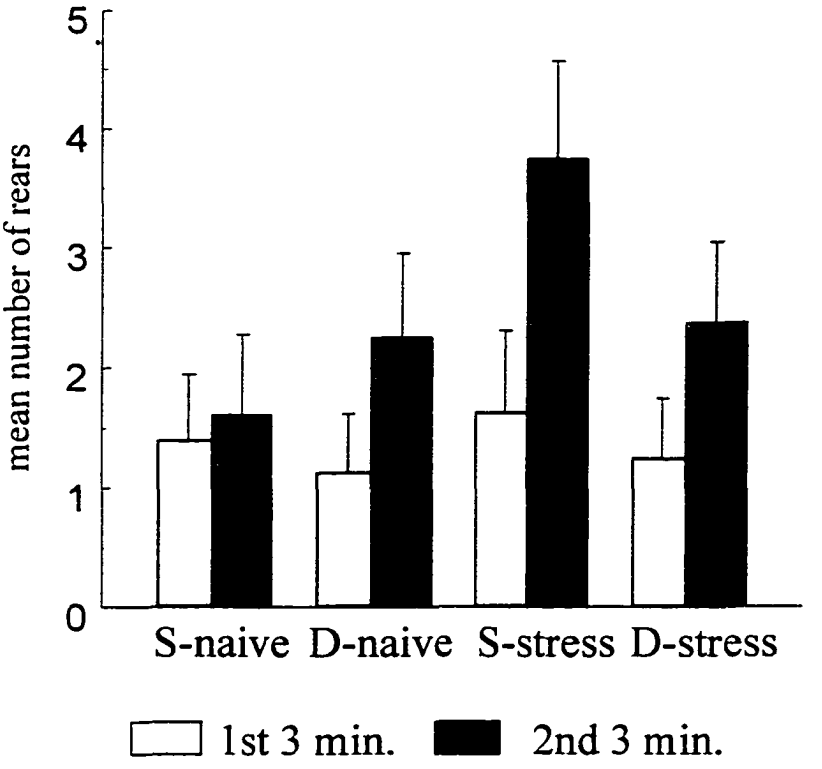


Fig. 27



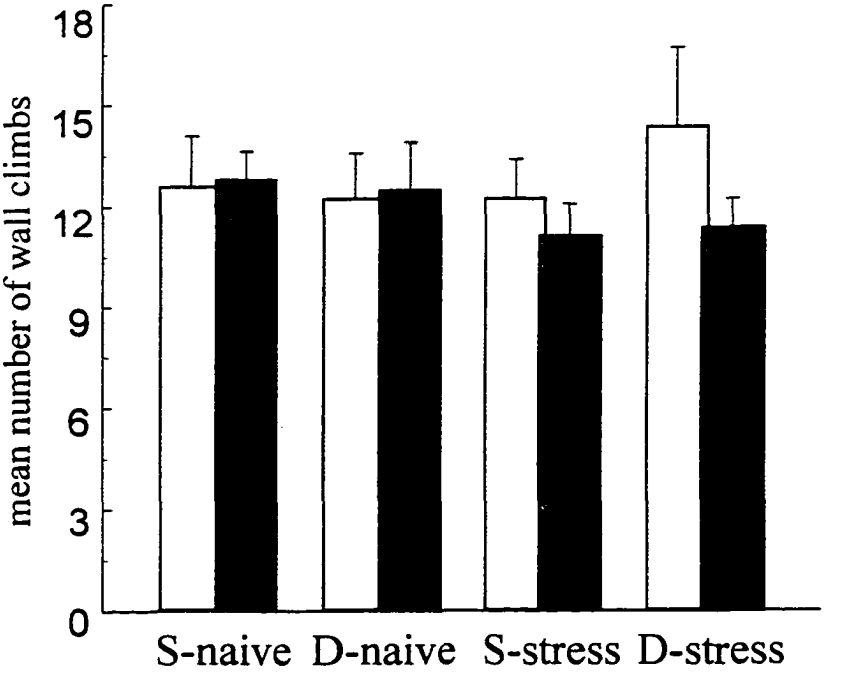
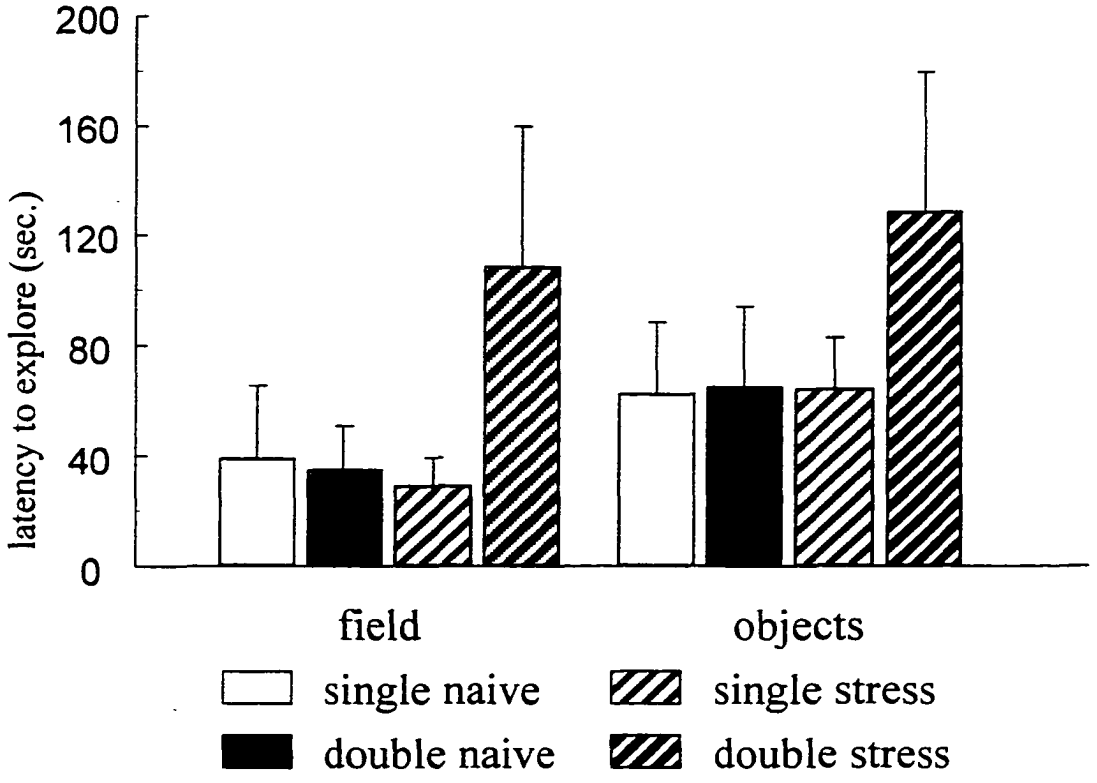


fig. 28



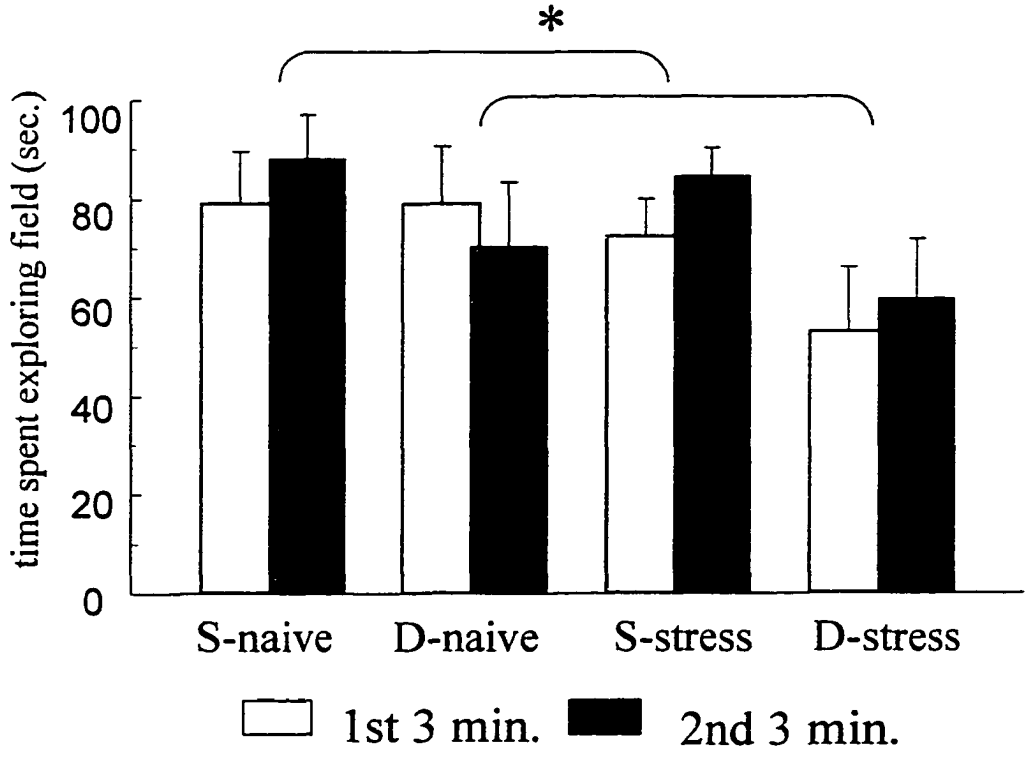
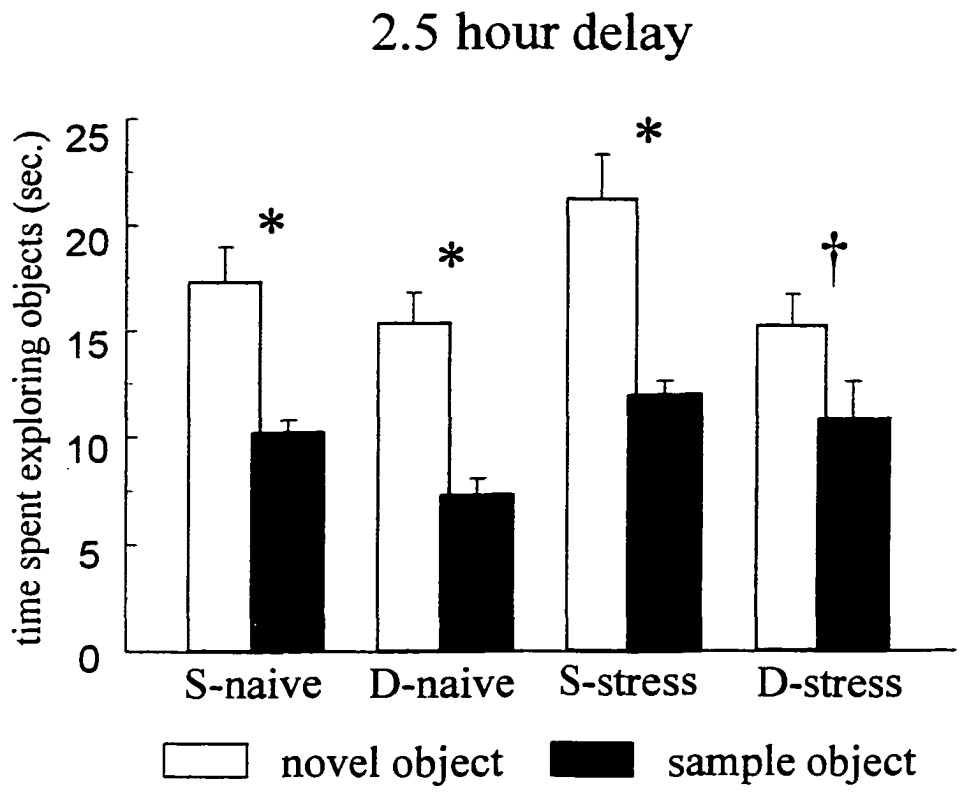


fig. 29

less time in the field as compared to naïve groups [stress $F(1, 32) = 4.28$, $p < .05$]. The stress groups did not differ from each other.

All groups performed similarly in the object recognition trials. As shown in figure 29, all groups showed a preference for the novel object after either a 2.5 or 4-hour delay, with one exception. In the 2.5-hour delay, the double-housed stress group showed a difference that was only marginally significant ($p = .08$), but they did show a significant discrimination after a 4-hour delay, leading us to believe there is not an impairment. The discrimination ratio also reflected this effect in the 2.5-hour delay [stress X housing $F(1, 29) = 5.43$, $p < .03$]. It also showed that the double housed naïve and stress groups significantly differed from each other ($.36 \pm .04$ versus $.13 \pm .08$), with the others showing intermediate ratio values ($.24 \pm .05$ and $.27 \pm .05$). The habituation index for both the 2.5-hour [stress $F(1, 29) = 6.34$, $p < .02$] and 4-hour [interaction $F(1, 30) = 7.81$, $p < .009$] delay trials further show that the double-housed controls showed the greatest amount of habituation. As illustrated in figure 30, the total exploration times, for the 2.5-hour delay trial, also increased from T1 to T2 except in the double-housed controls [stress X time $F(1, 32) = 5.64$, $p < .02$]. In the 4-hour trial, the single-naïve group showed consistent exploration while the other 3 groups decreased their exploration from T1 to T2 [stress X housing X time $F(1, 32) = 4.77$, $p < .04$]. There were no significant differences in the novel preference index. Thus, neither stress nor housing status caused impairment in subjects' discrimination of novel objects from sample objects, furthermore it also appears that the double-housed naïve subjects showed the most habituation to the sample objects.

The object placement test produced a pattern of results that differed from the object recognition test. As shown in figure 31, naïve groups did not show any preference toward the newly located object following a 2.5-hour delay. The pattern continued in the 4-hour delay trial, although only the single-housed stress group continued to show a preference. This difference is also reflected in the 4-hour trial novel preference index [housing $F(1, 29) = 5.38$, $p < .03$] and



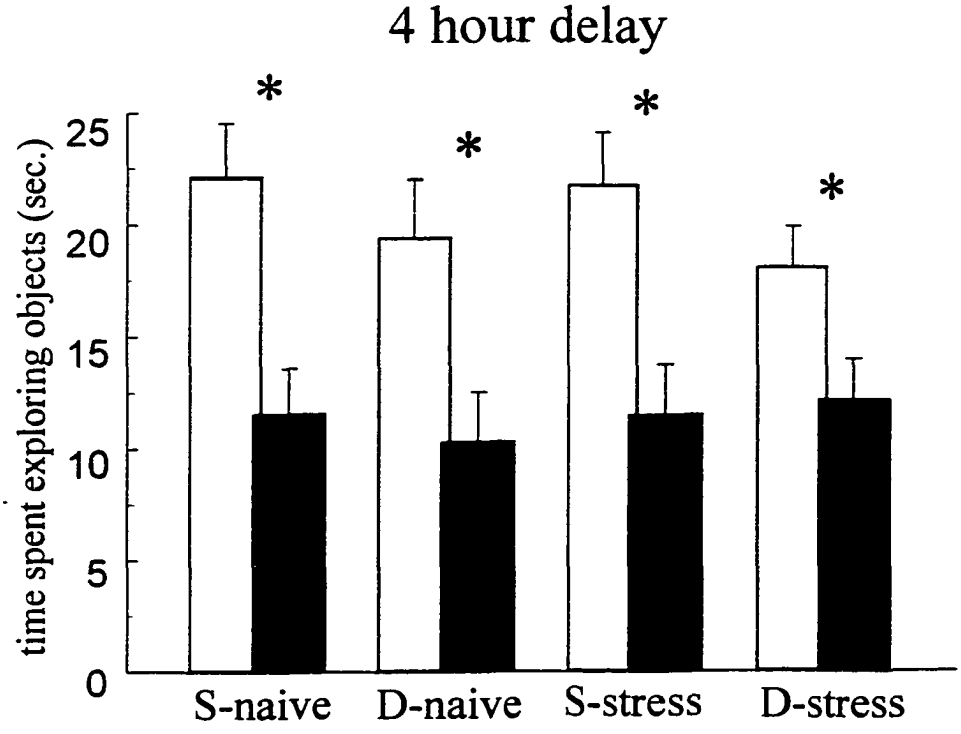


fig. 30

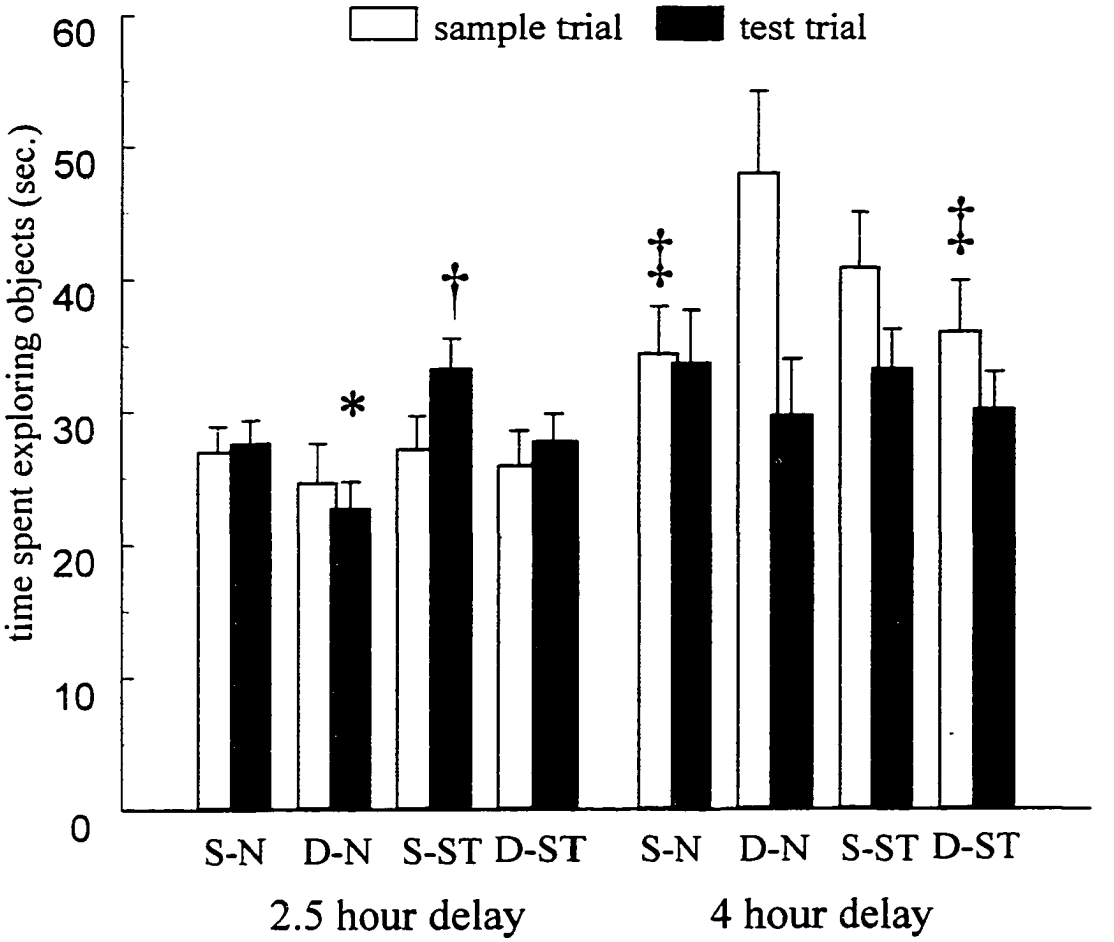


fig. 31

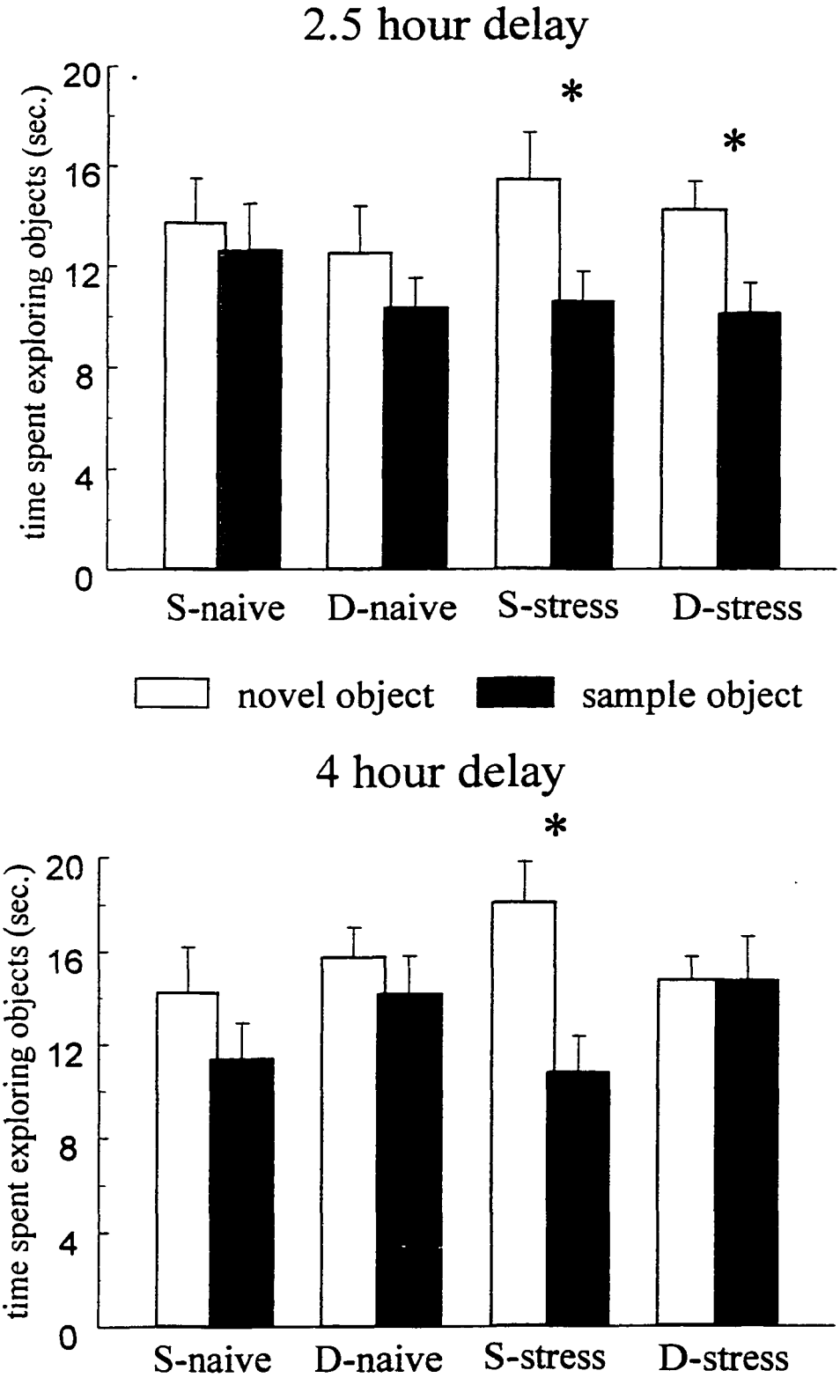


fig. 32

discrimination ratio [housing $F(1, 29) = 4.76$, $p < .04$]. Essentially, the single-housed subjects showed the greatest difference in exploring the two objects, although only the stressed group showed a significant preference when comparing times within each group. Habituation indexes did not differ between groups at either delay. Total exploration times did not differ between groups (see figure 32). All groups decreased exploration time from T1 to T2 in both the 2.5-hour trial [time $F(1, 31) = 84.59$, $p < .001$] and the 4-hour trial [time $F(1, 31) = 137.03$, $p < .0001$].

The behavioral data illustrate the complex interaction between housing and chronic stress on female performance. The forced open-field appeared to dissociate stress from naïve subjects. Double-housed stress subjects had the least number of inner sector visits. Single-housed stress subjects only showed a transitory decrease in inner sector visits (in the first 3 minutes of the trial). The double-housed stress group was last to enter the free open-field and approach the novel objects. They also spent the least amount of time in the free open-field. In the object placement test, the stress groups showed a preference for the newly located object after 2.5 hours, although, only the single-house stress group continued to show the preference after a 4-hour delay. The naïve groups did not show any preference after either delay. No differences were seen in general object exploration time between groups. Interestingly, no group differences were evident in object recognition, despite some group differences in trial-specific exploration of the objects.

Neurochemical Measures

Monoamine levels were affected by both experimental manipulations in the prefrontal cortex and the hippocampus (figure 33). Generally, the serotonergic system was most affected between groups. Serotonin levels [housing $F(1, 30) = 7.25$, $p < .01$] were higher in single-housed groups as was serotonin metabolism (5HIAA levels) [housing $F(1, 30) = 3.52$, $p < .07$] in hippocampus CA1. Housing and stress both influenced serotonin metabolism in prefrontal cortex [stress X housing $F(1, 30) = 4.14$, $p = .05$], with stress in double-housed subjects decreasing levels to

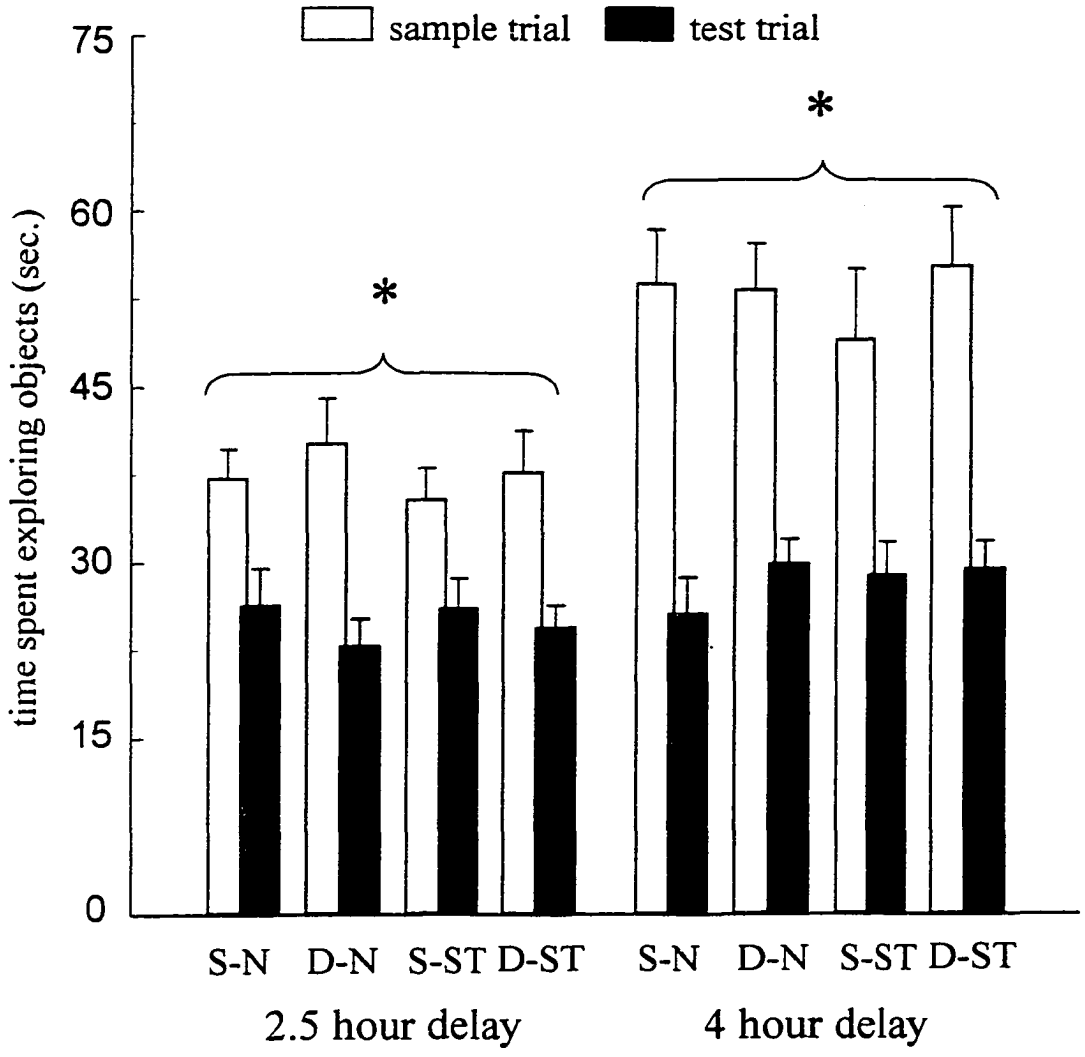


fig. 33

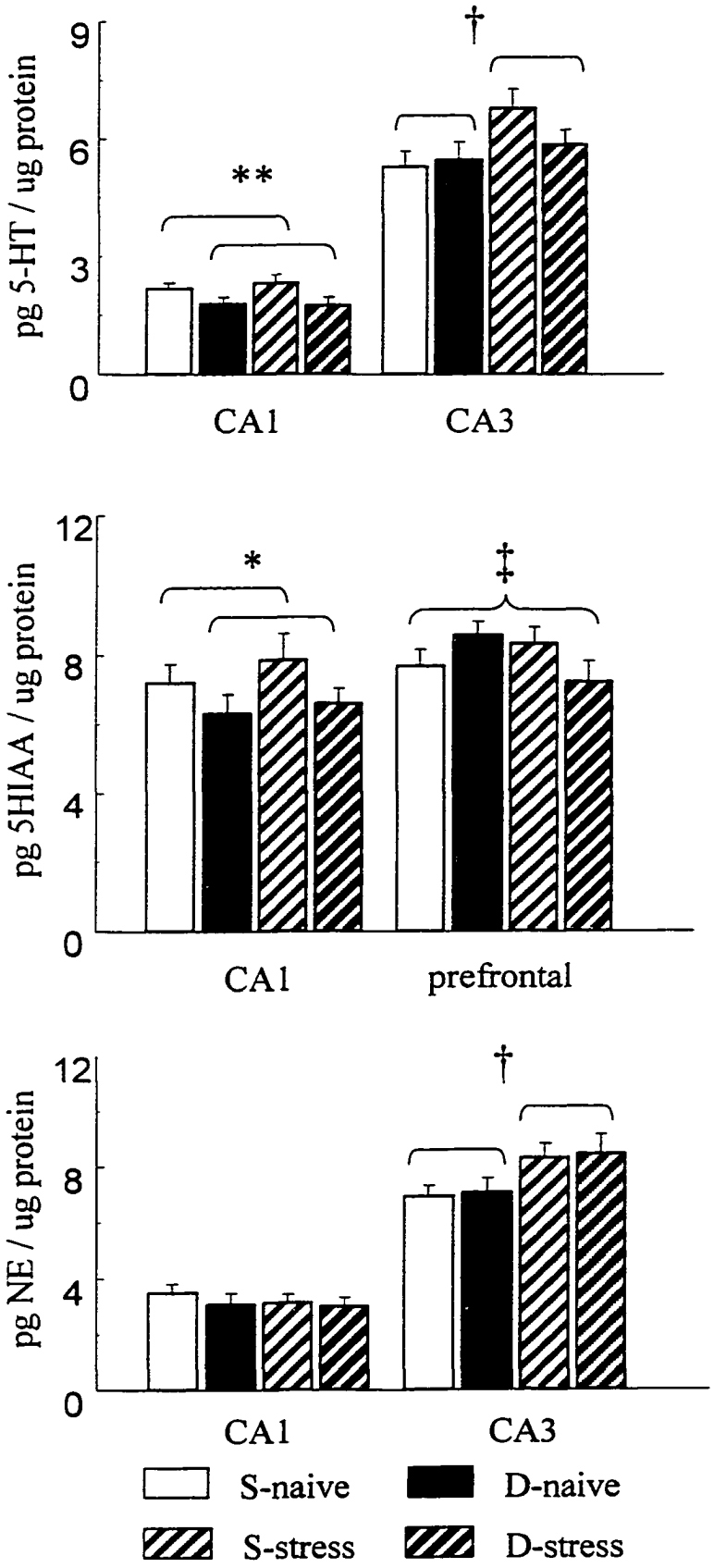


fig. 34

single-control levels and stress in single-housed subjects increasing metabolism to double-control levels. Serotonin levels were also changed in hippocampus CA3 due to stress condition. Chronic stress was associated with higher serotonin levels [$F(1, 30) = 4.98$, $p < .03$]. A similar effect was found in norepinephrine levels. Stress groups had higher levels [stress $F(1, 30) = 7.11$, $p < .01$].

Amino acid levels were significantly altered in prefrontal cortex and hippocampus CA1 (see figure 34). Histidine was lower in the prefrontal cortex in both stress groups [stress $F(1, 30) = 5.27$, $p < .03$]. In CA1, glycine and GABA were affected by housing status with glycine [housing $F(1, 29) = 4.81$, $p < .04$] and GABA [housing $F(1, 29) = 5.12$, $p < .03$] lower in double-housed groups, regardless of stress condition.

Stress and housing changed neurochemistry rather selectively in either the prefrontal cortex or hippocampus. Housing influenced the changes cited above in CA1regarding serotonin, glycine, and GABA. Chronic stress increased serotonin and norepinephrine in CA3. The prefrontal cortex showed stress-induced histidine changes, and a complex interaction between stress and housing in serotonin metabolism.

Discussion

As we found in our previous female-stress experiment, chronic restraint did not cause object recognition impairment, but unlike the previous study, it did affect open-field behaviors (inside visits). The pattern of increased object preference to the novel location, in the object placement test, by stress groups was unexpected, but still, the task did differentiate the stress groups from the naïve in the 2.5-hour delay. The fact that single and double-housed stress subjects showed different preference after a 4-hour delay also suggests that chronic stress did affect the two stress groups differently. The free open-field results also support this notion; double-housed stress subjects generally had longer latencies to enter the field and explore the novel objects. At the same time we found stress affecting prefrontal and CA3 neurochemistry

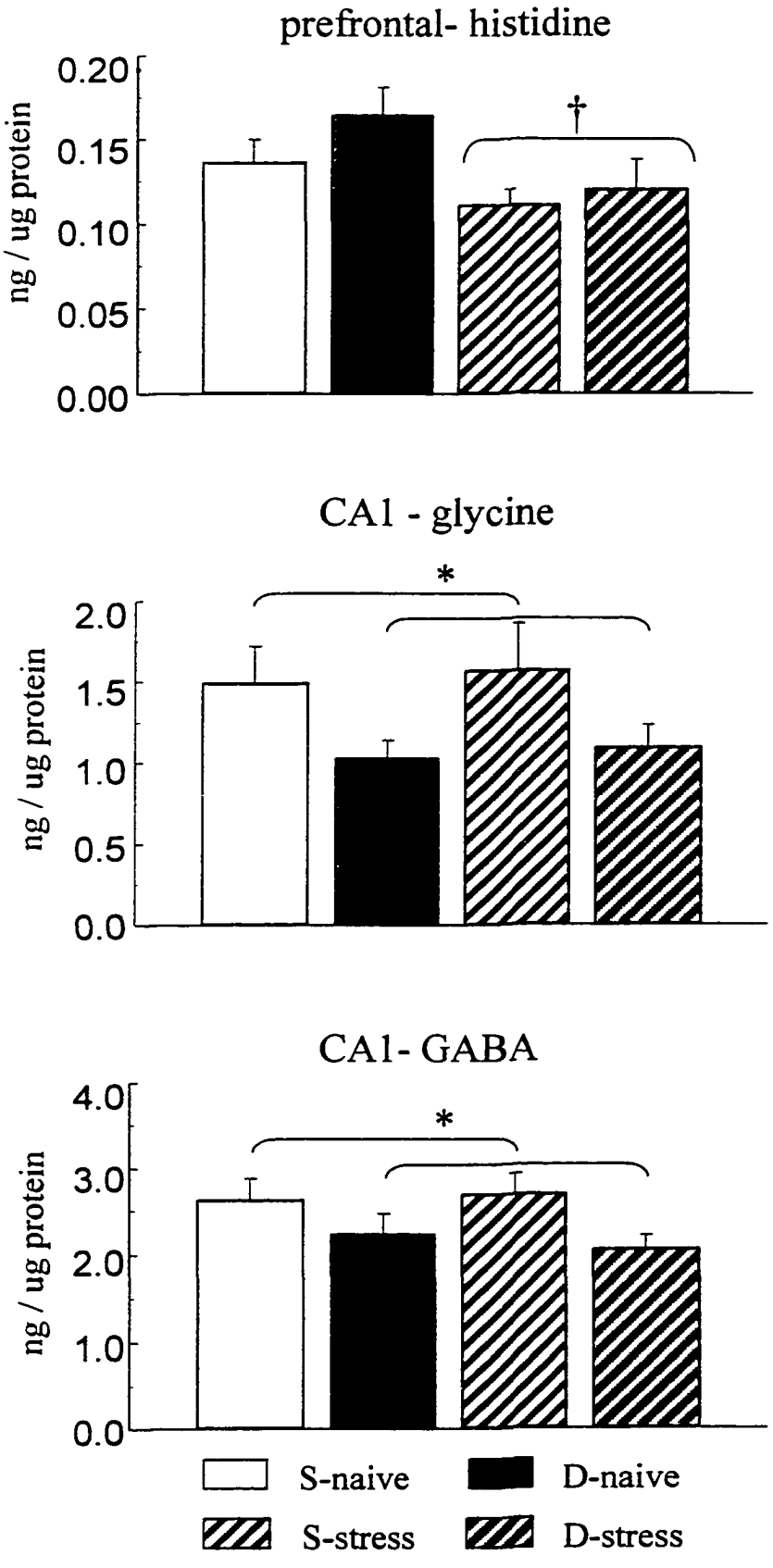


fig. 35

and housing affecting CA1 neurochemistry in these subjects. Thus, we show here that housing condition (single-versus double) influences both chronic restraint-stress induced behavioral changes, most notably in the free open-field and object placement, and limbic neurochemistry.

Comparing the neurochemical results here to the previous female-stress chapter, some parallels in amino acid changes are evident. In the previous study, prefrontal histidine was lower in subjects that had experienced one of two possible stressors (restraint or food deprivation). Here, we found that stress groups had less prefrontal histidine. In the current study, we found higher glycine levels in single-housed subjects, while our previous study found food-deprivation or chronic restraint increased glycine levels (the combination did not). Based on the current results, the groups in the past study may have had elevated glycine levels, at the time of sacrifice, because they had been single-housed for 8 days. Since the time-lines of housing are not the same in the two studies, it is a factor that needs further investigation. We also found GABA in CA1 to follow the same pattern (higher levels in single-housed groups). In the previous study, we did see, an unreported, trend ($p = .10$) of food deprived groups having higher CA1 GABA. It appears GABA is higher in subjects that are currently in a different environmental situation than what is typically considered "normal" housing conditions – double housing. Interestingly, we did not see a decrease in dopamine activity (HVA / DA) due to stress or housing, as reported in the previous chapter. This could be because the timing of sacrifice. Subjects, in the previous study, were not sacrificed immediately after a sample trial as done here. Together, these results would mean that "basal" (homecage) levels of dopamine activity are decreased in stressed females, but "arousal" (field-activity) levels are not different. This pattern is different than what we observed in males (higher activity in stress groups) and could explain why chronic stress appears to more readily affect certain male rat behaviors and not the same female rat behaviors (i.e. object recognition).

Still, there are some common neurochemical alterations evident between the males and females in these housing-stress studies. First, in both studies prefrontal histidine and CA3 norepinephrine levels were changed due to stress. The most notable effect is with hippocampal

CA3 norepinephrine. Both double-housed stress males and all female stress subjects showed an increase in CA3 norepinephrine levels. We already established, in the previous male study, that double housing appeared important in the chronic restraint-induced object recognition memory deficits in males and suggested that the imbalance in CA3 norepinephrine could be a factor in this effect. The female results suggest similar CA3 change in norepinephrine, due to stress, do not lead to object recognition impairments, at least in females. Moreover, these changes in norepinephrine levels are not affected by housing condition in females. Yet, other regions, CA1 in particular, show a clear housing affect on neurochemistry, which may explain why we do not have consistency in behavioral performance in both female stress groups. As observed in the food deprivation – stress experiments, the effects of a secondary variable (in his case housing), upon those caused by stress, can lead to different neurochemical changes in males versus females. The single-housed females showed changed levels of serotonin, 5HIAA, glycine and GABA in CA1 while single-housed males showed no such changes in CA1. Double-housed males had higher dopamine activity in amygdala while females did not. Histidine changes were evident in both males and females but the distribution and the influence of housing were different. The combination of stress with either housing manipulation or food deprivation leads to prefrontal or hippocampal histidine level changes. The extent to which these changes could underlie the behavioral differences following stress need further study, but finding these different chemical changes, across sex, support the notion that the housing environment is a powerful variable upon the neurochemical milieu within the limbic system, and its influence is clearly different in males versus females.

Previous studies, illustrating the housing – sex interaction on physiology, support the notion that housing is a critical factor and must be taken more into account when trying to model physiology and / or behavior across sex. Holson (Holson et al., 1988) reported a select prefrontal dopaminergic interaction between acute stress and the housing condition of male rats. Isolate males do not show the same amount of increase in HVA following stress. Our previous study

saw a similar pattern in males. No such effect occurred in the females here. We also did not see a housing effect on amygdala dopamine activity, as we did in males. Holson, Scallet, Ali, Sullivan, and Gough (1988) also found a similar discrepant pattern in pituitary beta-endorphin. Social males have higher levels while females show no increase (if anything their levels appear to decrease slightly). The females in this study exhibited the greatest changes in regards to serotonin, glycine, and GABA. Interestingly, increases in serotonin metabolism are commonly found in colony-housed subordinate males (Blanchard, Cholvanich, Balnchard, Clow, Hammer, Rowlett, & Bardo, 1991; Blanchard, Sakai, McEwen, Weiss, & Blanchard, 1993). Male basal CORT levels also increase with the addition of other males, but in females, CORT levels decrease (toward male levels) as females are introduced to the environment (Brown & Grunberg, 1996; 1995). The fact that our single-housed females had higher serotonin levels with increased metabolism suggests that this effect is CORT mediated. Although we did not assess CORT levels here, we did find a similar pattern in our food-deprived males (Beck & Luine, in press). Food-deprived males also had higher serotonin activity in CA1, and they also had higher circulating CORT levels. Thus, we are then left with the scenario that, at times, housing conditions should be considered an added stressor since it appears to elicit changes in neurochemistry that are associated with CORT activity. This becomes a methodological problem since it does not affect males and females in the same manner.

Behavioral researchers, characterizing performance differences between males and females, have also had to address this issue. Some have found consistent patterns of housing status across the behavioral sex-difference. For instance, Seymoure, Dou and Juraska (1996) showed that, although males perform better than females (less errors) on the radial-arm maze, rearing in a social environment enhanced performance in both sexes. However, others have found less consistency. Robinson (1976) found increasing the numbers of females from 1 to 4 per cage generally led to a progressive decrease in the number of trials to reach criterion in a Y-alternation paradigm. Males did not show any significant performance enhancement, and, if they came from

a larger litter, their performance actually was worse if they were also group housed. Viveros, Hernandex, and Gallego (1990) found male active-avoidance performance was sensitive to social housing. Isolate males did not learn or retain the conditioned response as well as social or crowded males, yet females, who generally performed better than males, did not show a housing effect. This result suggests that isolate males either have some form of performance deficit or that they are reacting to the shock (a stress-evoking stimulus) differently, and females are not affected by their housing environment in the same manner. The most used measure of environment-reactivity, the forced open-field, also yields sex differences (i.e. Dalrymple-Alford & Benton, 1981). Although, in this task, it appears that the sex-difference tends to override the sex X housing interaction if only general activity counts are recorded (Dalrymple-Alford & Benton, 1981). Here, since we segregated peripheral field visits from inner-sector field visits, we were able to show a stress X housing X time interaction. In our previous male version of this study, we did not see such an effect. Latency to enter the free open-field was influenced by both housing and stress in females, but only by stress in males. Conversely, time spent in the free open-field was influenced by both housing and stress in males, while differences were primarily influenced by stress in females. Still, regardless of stress, we now have also shown that the housing effect on object recognition is specific to males. Clearly, housing is an issue of utmost importance in the study of behavioral sex-differences, and these results should lead us to question whether differential housing conditions should be used across sex to control for this effect.

Thus, females and males appear to show different behavioral effects following chronic stress. Female performance in the object recognition task is quite stable and apparently not effected by chronic restraint stress. Since we have found this before (in the food deprivation experiment), it appears that female object recognition behavior is not easily manipulated by environmental conditions (as males behavior is). The results from the object placement task suggest that naïve females do not discriminate previously explored spatially rearranged objects. Furthermore, stress females show the male pattern of preferentially exploring the newly located

object (at a 2.5-hour delay). Whether this represents an enhancement in spatial memory, it is interesting that single-housed stress females and double-housed stress males show this behavioral preference through a 4-hour delay. It is difficult to fully explain these results since the only other study that utilized this task (Ennaceur et al., 1997) only used male subjects. Object placement, however, is sensitive to fornix lesions (Ennaceur et al., 1997), thus reflecting changes in hippocampal processing in stressed subjects; possibly an over-sensitivity to the spatial relations of stimuli, which naïve females have little reactivity. In both the previous and the current housing study, the groups that showed a new placement preference had higher NE levels in CA3. The additional up-regulation of CA1 inhibitory neurotransmitters, in single-housed stress subjects, may limit this reactivity as compared to double-housed stress subjects who only show the CA3 NE change. These differential neurochemical effects may also underlie the apparent open-field behavioral reactivity in double-housed stress subjects. Obviously, the stress and housing induced neurochemical changes selectively influence certain aspects of female field-tested behavior that does not include non-spatial memory for object recognition.

In conclusion, this study has replicated our previous work showing that chronic restraint does not impair female object recognition performance, but appears to enhance object placement performance over controls (especially in single-housed stress subjects). These effects are accompanied by results in the open-field tests that appear to dissociate double-housed stress females from single-housed stress females (longer exploration latencies and less inner-sector activity). The neurochemical analyses further support that housing and chronic stress both affect monoamine and amino acid levels in the hippocampus and prefrontal cortex. Yet, similar to the differential task-specific behavioral effects, the neurochemical changes were region-specific involving different transmitter systems. Consequently, this study illustrates the complexity of housing-stress interactions and that the resultant effects can be delineated differently on both a behavioral and neurochemical level depending on the paradigm and brain region of study.

General Discussion

Chronic Restraint Stress

The studies presented here, together with past studies (Luine et al., 1994a, Luine et al., 1994b; Luine et al., 1995; Conrad et al., 1996; Galea, et al., 1997; Magarinos & McEwen, 1995; Magarinos, et al., 1997), show that chronic restraint stress leads to behavioral and physiological changes in rats. In addition, the effects of 21-day restraint on rat physiology and behavior appear sexually dimorphic. Both Galea's study (Galea et al., 1997) and the current work show that males and females respond to chronic restraint differently; they show different behavioral changes, monoamine and amino acid level changes, corticosterone level changes, and CA3 dendrite reorganization. With a particular focus on behavior, forced open-field behaviors, free open-field exploration, and object placement recognition show changes following stress in both males and females. Interestingly, in some cases, the changes are similar (i.e. less inner sector visits in the forced open-field after stress), but usually the changes differ across sex (i.e. free open-field latencies are increased in stress females but are decreased in stress males). Yet, the current work also implicates the homecage environment as an important moderating variable of these effects across sex. Both food depriving post-stress males and single housing males during the stress period buffer object recognition impairments elicited by chronic restraint. Single-housed males, however, show the least object placement recognition while double-housed males show the most (as long as a 4-hour delay). Females exhibit a different pattern of stress effects. Only female stress groups show object placement recognition at 2.5-hour delays, and only single-housed stress females continue to show placement recognition following a 4-hour delay. Moreover, in the free open-field, only double-housed stress females selectively show longer field and object exploration latencies. Hence, it appears that both the behavioral change being modeled and the sex of the subjects (serving as the models) are indelibly interrelated when attempting to use 21-day restraint as a chronic stress paradigm.

A major finding regarding the housing-stress interaction in males is the impact of double housing during stress. First, using object recognition as a male behavioral model for non-spatial memory, we observed stress dependent impairments in males in both sets of studies. Males double-housed during the 3-weeks of restraint period were impaired; males single-housed during that time were not. Furthermore, the impaired double-housed males experienced somewhat different stress environments. Double-housed stress subjects in the food deprivation experiments were restrained in their homecages. Double-housed stress subjects (from the housing study) were restrained in a separate chamber, in a separate room, ironically with the single-housed stress subjects (who later exhibited no object recognition impairment). Therefore, at least for males, double housing appears to be a necessary condition for subsequent object recognition deficits to be triggered by chronic restraint. In contrast, it is interesting that housing did not affect free open-field latencies. This suggests the aforementioned impairment is not due to an increased fear of novelty. However, exploration differences were noted, single-housed groups explored objects (in both object recognition and object placement) more than double-housed groups. Thus, we could view single housing during restraint as having similar effects as post- stress food deprivation – an exploration-inducing condition.

It appears that the restraint-induced neurochemical changes in CA3 norepinephrine and prefrontal dopamine were also influenced by the housing status and food availability. Males double-housed during the behavior week (naïve and stress) had higher dopaminergic activity in the amygdala and also had object recognition impairments. Yet, the naïve controls did not show stress-induced changes in CA3 norepinephrine and prefrontal dopamine. These results suggest that amygdala-dopamine activity is affected by housing status (in the male rat) and may also underlie either the proposed mnemonic "interference" or decrease in exploratory drive. The double-housed males may have had higher basal CORT levels, compared to single-housed males (i.e. Brown & Grunberg, 1995; 1996), because of the inherent male-to-male dominance struggle (i.e. Blanchard, Cholvanich, Balnchard, Clow, Hammer, Rowlett, & Bardo, 1991; Blanchard,

Sakai, McEwen, Weiss, & Blanchard, 1993) which could then lead to the common effect in the amygdala. Those that were also restrained had additional CA3 and prefrontal cortex changes. On the other hand, single-housing males during chronic restraint does not "protect" them from any effects. They spend more time in a free open-field and show impairments in object placement recognition. Without a discernable neurochemical effect, present solely in that group, the possible neurochemical basis for these behavioral effects is still unknown. Others have suggested that behaviors dependent upon 3-dimensional object relationships in space are not primarily hippocampal dependent (Save, Poucet, Forman, & Buhot, 1992); thus, there may be other cortical areas, such as posterior-parietal cortex, that are affected by chronic restraint but have not been investigated by anyone at this time. Basically, chronic restraint affects double-housed males in a different manner than single-housed males both behaviorally and neurochemically; thus, whatever psychological process (spatial memory, non-spatial memory, anxiety etc.) one wants to model using chronic restraint stress in males, the housing status must also be seriously considered.

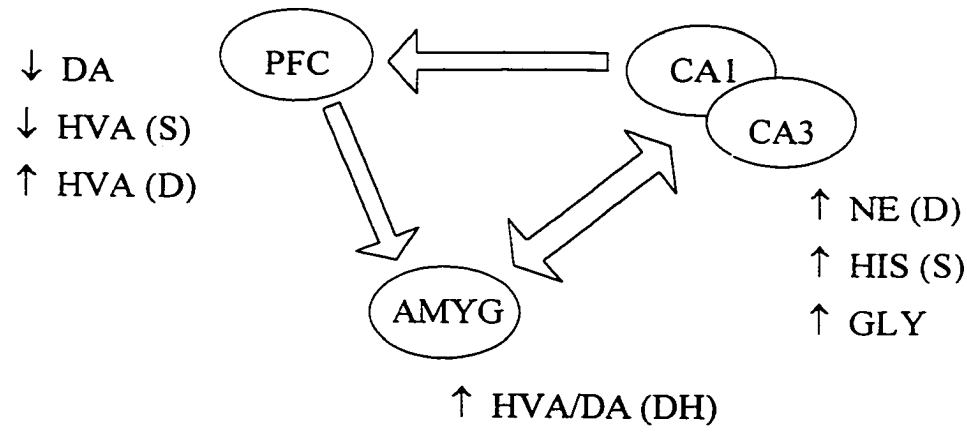
Determining whether the 21-day restraint paradigm appropriately models chronic stress effects in female rats will have to involve further study. These studies are the first to assess behavioral and neurochemical effects in females following 21 days of restraint and show females are effected differently than males. Females do not show any object recognition impairment following chronic restraint, nor do they show object placement recognition impairment beyond that of controls. In fact, stress females performed better than controls in the object placement task. However, we did find a similar housing interaction, as we did in males, with only single-housed stress females showing novel-location preference after a 4-hour delay. Still, unlike males, the effect appears to reflect an enhancement, not impairment. The female data thus far do not suggest that either double or single housing, during stress, is necessary for restraint stress-induced behavioral changes, outside of increasing the fear of entering and exploring objects in a free open-field (only seen in the double-housed stress females).

Monoamine and amino acid levels further show females respond differently to housing and chronic stress (see figure 36). It appears that housing does not influence stress-induced changes in CA3 norepinephrine in females (both stress groups had higher norepinephrine), but, it does influence chemical activity in other regions, such as hippocampus CA1. How the increased serotonin, GABA, or glycine levels in CA1 are influencing single-housed females cannot be determined at this time from the present data. However, the combination of CA1 and CA3 changes in single-housed stress subject could reflect the performance differences between the two stress groups in object placement recognition, but others (Cressant, Muller, & Poucet, 1997; Save, Poucet, Forman, & Buhot, 1992) have downplayed the hippocampus as a critical region for processing 3-dimensional objects. The presence of fewer behavior changes in females is somewhat puzzling since they tended to show more reactivity to being restrained. Females resist being nudged into the restrainers and, on occasion, attempt to eat through the Plexiglas to get out (personal observation). Males rarely resist crawling into the restrains (personal observation). Moreover, females show less habituation over time, as assessed by CORT levels (Galea et al., 1997). Consequently, female rats do not appear to be as reactive to the lasting (albeit transitory) effects of chronic restraint stress, as male rats, despite showing less habituation to being placed daily in restraints.

Sex Differences in Object-Related Memory Tasks

The second major finding of these studies is sexual dimorphisms in baseline behaviors or environment-induced behavioral change. For object recognition tasks naïve males and females exhibited different performance on each version of the object-memory tasks. Females perform object recognition consistently and reliably, regardless of housing situation, food deprivation, or chronic restraint. Single-housed males perform object recognition, but double-housed do not (at least at delays 2.5 hours and longer). Stress also impaired male object recognition, but food restriction increased exploration (in both stress and naïve males), thereby buffering some of the stress-induced deficits. The opposite was observed in object placement. Males show object

Male Stress Effects



Female Stress Effects

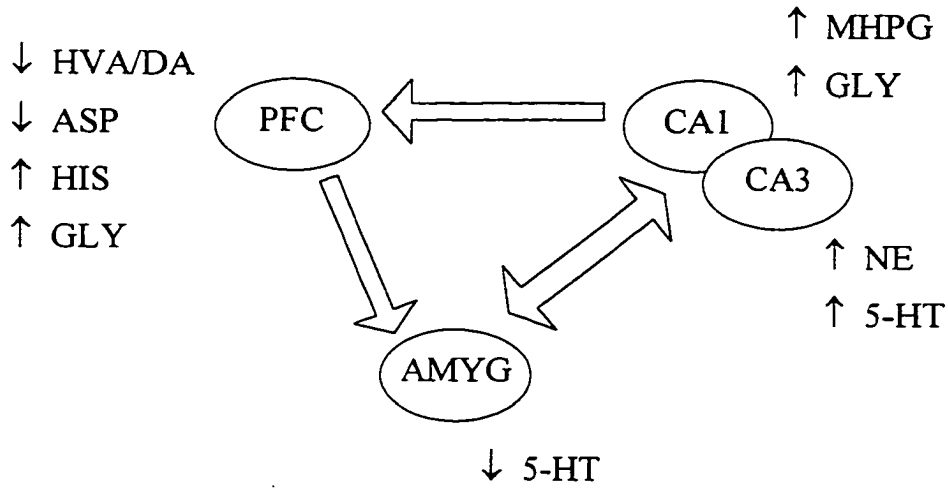


fig. 36

placement recognition after a 2.5-hour delay, but their naïve female counterparts do not. In both sexes, we saw stress affect object placement recognition, but this was housing dependent (single-housed stress females and double-housed stress males showed preferences even after a 4-hour delay). Together, these results suggest that females show more stability in object recognition performance whereas males show more stability in object placement recognition performance.

The sex differences found in other stress-learning/memory paradigms might aid us in understanding how stress exacerbates the behavioral differences between males and females in these object memory tests. In addition to object recognition, males show greater stress-induced detriments in avoidance and escape learning compared to females (Kirk & Blampied, 1985; Davis, Porter, Burton, & Levine, 1976; Steenbergen, Heinsbrock, Van Hest, & Van de Poll, 1990; Steenbergen, Heinsbrock, Van Hest, & Van de Poll, 1989). Yet, females show more impairment in eye-blink conditioning following stress (Shors, Lewczyk, Pacynski, Mathew, & Pickett, 1998), which is dependent upon the presence of gonadal hormones (Wood & Shors, 1998). Stressed females appear to avoid unknown or novel environmental stimuli. Thus, they readily avoid or escape shock, explore open fields predominately by ambulating in the periphery, and enter and explore free open fields later than controls. Stress males show more difficulty in tasks that involve a very specific or directed, ambulatory response to a stimulus cue (whether a tone or an object). Therefore, in contrast to females, stress males could be actually showing disinhibition (essentially a failure to focus). Our free open-field would also support this idea. Stress males tended to explore the field and novel objects quicker than naïve males. In tasks that involve directed behaviors (in specified locations), a male that is disinhibited may appear to have impairments because the activity is not focused. Such a relationship would also explain why addition of food deprivation or single housing could apparently "reverse" the impairment. Both of these conditions tend to increase male activity, but they may also increase exploratory drive toward novelty (perhaps forcing them to focus). This could explain why Luine et al. (1994) did not see the same type of spatial memory deficits as Conrad et al. (1996). The prior study used

food deprived single-housed males, while the latter used double-housed, ad lib fed males. On the other hand, hypothesizing that stress female behavior is biased by novelty avoidance, stress females should be less diverse in their exploratory behavior toward the objects, thus leading to impairment. This was not the case. Stress did not impede and, at times, it aided (placement recognition) test performance, suggesting that whatever avoidance or inhibition toward fearful or novel stimuli is elicited, it is not detrimental enough to effect these tasks. Whether the multiple-task format of these studies affects the habituation of males and females to the testing arena differently, thus leading to diverse results, also needs further study. Increasing or decreasing the subjects' reactivity to the stimuli in our testing environment may exaggerate some of the subtle sex differences already present in these behavior tests.

If the resultant effects of stress are different in across sex and are further differentially influenced by environmental conditions, then modeling the behavioral effects of chronic stress should be done separately in males and females. This was the approach of the current work. As two populations that have different patterns of reactivity to the environment, males and females continue to show different behaviors after experiencing a chronic regimen of restraint stress. Since early hormone organizational effects lead to different neural networks involved in general limbic system activities (such as memory and the stress response) as well as the HPA-axis (such as secretion patterns and sex behaviors), we might expect different effects when using the same behavioral and stress paradigms in males versus females. Recent studies have also illustrated these differences in humans (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). Regulation of the HPA-axis (including cortisol, adrenocorticotropic hormone, and corticosteroid-binding globulin levels), in reaction to a situation deemed stressful by the subject, is not the same in men and women. Like the results from the current work, the differences that have been documented in humans (Kirschbaum et al., 1999) suggest that the physiological and the psychological response to stress results from a complex interplay of factors that are gender and estrous cycle dependent. Hence, the same issues of organizational and activational actions of

adrenal and gonadal hormones in the stress response are evident in humans as they are in rodents, suggesting that these two populations should be modeled and studied as two distinct populations.

Conclusion

The experiments discussed here exemplify the complex problems associated with modeling how environmental stimuli or conditions can influence behavior. Whether chosen conditions appear stressful (restraint) or not (double housing) to the researcher, does not necessarily guard us from imposing an unknown bias in behavioral reactivity to our manipulations or to our assessment techniques. Consequently, when we decide to test the generalizability of our models to other populations, we may, on the surface, observe an apparent enhancement, buffering, or even total reversal of the behavioral and / or physiological pattern seen in the original model. Yet, when the other environmental conditions are changed, the results may better approximate the original model. The question then is, which condition is the appropriate baseline? The answer depends on what is being modeled. The 21-day restraint stress model clearly allows us to study how chronic stress can lead to (at least transitory) behavior changes in male Sprague-Dawley rats. These effects on behavior may stem from general disinhibition in exploring the testing environment. The generalization of the effects on males to female Sprague-Dawleys is still questionable. The basic differences in the free open-field suggest that stress could decrease general exploration, while object memory tests further implicate an increased focus upon object stimuli. Whether increasing the length of restraint per day (7-8 hours) or increasing the number of restraint days (to 28 or 30 days) in females would lead a pattern of effect more like that of males, is an issue that needs to be addressed. As previously reviewed by Luine (1997), the duration of the restraint period influences the stress effect seen in radial arm-maze performance in males. Moreover, we still need to determine if double housing is a necessary condition for female stress effects, as it appears to be in males (using object memory tests).

From a neurochemical perspective, we still have to determine whether the CA3 norepinephrine increases, prefrontal dopamine activity increases, or amygdala dopamine increases are critical in object recognition impairments. Recent work (Bussey, Muir, & Aggleton, 1999; Wan Aggleton, Brown, 1999) would suggest neither the prefrontal cortex nor the hippocampus are as critical as perirhinal regions of the temporal lobe, but as shown by Myhrer (1988), the tracts from perirhinal cortex to the hippocampus (both dentate gyrus and CA3) are involved in processing information regarding exploration of novel stimuli. This was also the notion of McCormick et al. (1997) when they set out to study if dentate degeneration leads to object recognition impairments. In a paradigm like the current one, which uses long delays to assess memory (on the range of hours), a disruption in the proper level of norepinephrine in CA3 could have an affect on encoding the novelty of presented stimuli, thus, leading to abnormal explorations of previously viewed items. A similar finding was shown with delayed alternation impairment and dopamine over-utilization in prefrontal cortex (Zahrt, Talyor, Mathew, & Arnsten, 1997). Moreover, Wood, Mumby, Pinel, and Phillips (1993) found local damage to CA1 could affect non-spatial delayed-non-match-to-sample. Studies using specific pharmacological manipulations of local neural regions are needed to determine if either CA3 norepinephrine or prefrontal dopamine is a possible neurochemical underpinning of the behavioral changes caused by chronic restraint.

Further investigations into the nature of the female rat response (behaviorally and hormonally) to chronic restraint should help us determine if chronic restraint is the best paradigm to use in attempting to model the neurological and behavioral changes occurring with chronic stress in females. Shors (Shors et al., 1998; Wood & Shors, 1998) has already shown that female classical conditioning is quite sensitive to prior exposure to multiple shocks, while males are not, but, again, this effect is estrous cycle-dependent (it is not always apparent). Hormone manipulations during or following the 21 days of stress may provide more information that further implicates the pituitary-hypothalamic-gonadal axis in these differences (i.e. Diano,

Naftolin, Horvath, 1997; Burgess & Handa, 1992; Handa, Burgess, Kerr, & O'Keefe, 1994; Viau & Meaney, 1991; Toufexis, Rochford, Walker, 1999; Patchev, & Almeida, 1996; Patchev, Hayashi, Orikasa, & Almeida, 1995). Still, these studies provide a basic behavioral and neurochemical profile of the post-chronic stress intact male and female rat. Stress-induced sex differences occur in object-memory tests following 21-day restraint. In spite of exhibiting several changes in monoamines and amino acids, stress females were the least impaired in object-memory tests. Sex differences were also evident in open field activities, thus, possibly reflecting a stress effect on fear or anxiety. We also found housing and food deprivation were critical variables, especially in males, in moderating the stress effects on field-based object-memory tests. On the whole, these tests have dissociated the type of lasting-responses, both behavioral and neurochemical, in males and females following chronic restraint using non-reward mediated (non training-dependent) tasks of object memory that can be used in the hypothesized "window" (Luine, 1997; Conrad et al., 1996) of impairment.

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