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# STRESSORS OF VARYING PROPERTIES INDUCE DIFFERENCES IN THE MODULATION OF BEHAVIOR AND BIOLOGY

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ABSTRACT

Stress induces enduring behavioral and biological consequences. Despite its pervasiveness, the biological and neural processes underlying its effects remain largely unknown. The existing literature generally examines the repercussions of stress from the aspect of a single stressor; however, stressors are not uniform. Rather, stressors may intrinsically differ in their properties such as intensity, duration, and quality. Since animals regularly encounter various types and levels of stress throughout their lifetime, this study compares the differentiation of behavior and biology across inherently different stressors, specifically social isolation (SI) and foot shock (FS). FS dispenses a series of intense acute stressors, whereas SI presents an incessant stressor lacking reprieve. While the current literature recognizes SI and FS for producing variations in behavior such as anxiety, sensitivity, and aggression, research comparing these overlapping behavioral consequences is lacking; particularly from the aspect of the dorsal bed nucleus of the stria terminalis (dBNST), a brain region essential for stress, anxiety, and fear response. As such, this research examines the role of the dBNST in mediating behavioral and biological effects across the stressors SI and FS. Varying behaviors were produced when male C57BL/6N mice were treated with SI or FS, suggesting that repercussions from these stressors are nuanced and not interchangeable. SI mice significantly practiced more avoidant behaviors and dissociable attack behaviors such as approaching male BALB/c intruders from the front and directing bites toward the head area during resident intruder (RI) assays. Although not statistically significant, FS mice exhibited elevated avoidance and aggression. Moreover, behaviors instigated from SI and FS appear to derive from differing brain regions. Chemogenetic inactivation of the dBNST influenced subsequent aggressive and avoidant behavior in FS mice, but not SI mice.

## INTRODUCTION

Stress instigates enduring behavioral and biological repercussions. Despite its pervasiveness, the biological and neural processes underlying its consequences remain largely unknown. The existing literature generally examines the behavioral and biological effects of stress from the aspect of a single stressor, lacking crosstalk across stress induced consequences. Since animals encounter various types and levels of stressors throughout their lifetime, this research compares the differentiation of behavior and biology across intrinsically different stressors, specifically social isolation (SI) and foot shock (FS).

### Stressors of Varying Properties

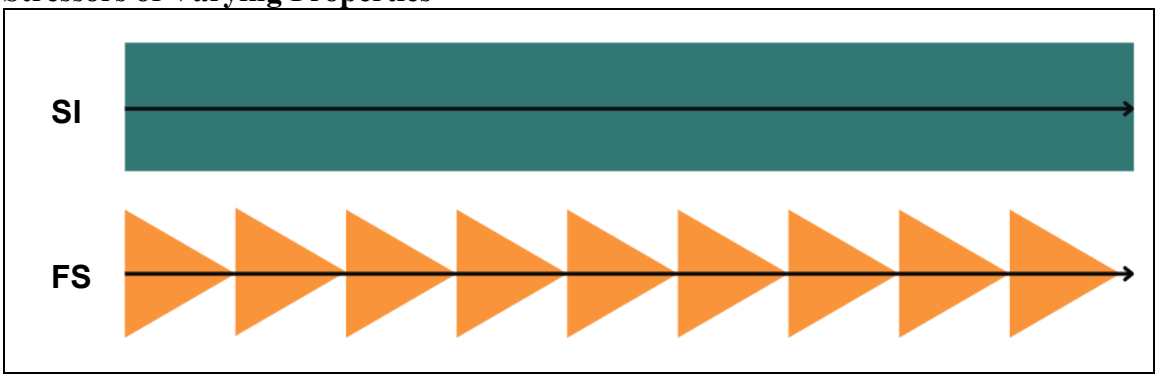


**Figure 1.** Paradigm of stressors varying in properties.

The stressors SI and FS present stress paradigms that vary in properties such as duration, intensity, and quality. Chronic SI characterizes an uncommon and distinctive paradigm where

stress is dispensed persistently without cessation (Zelikowsky et al., 2018). While levels of SI induced stress may marginally vary at times, stress from chronic SI is generally sustained (Figure 1). Extended periods of SI have been especially relevant recently, ascribable to the COVID-19 pandemic.

FS portrays a paradigm where stress is experienced physically and emotionally (Bali et al., 2015). Stress from FS is endured in intense bursts (Figure 1) and habituation of FS is uncommon (Bali et al., 2015). Even though the effects of FS persist long after the stressor is no longer presented, there is a period of reprieve unlike SI.

*Social Isolation*

Mice are social animals, forming interpersonal relationships and structures beyond the individual level. As such, mice generally prefer group housed (GH) conditions (Kappel et al., 2017). When animals are chronically isolated, they exhibit distinctive behavioral changes namely, elevated aggression and anxiety (Wiberg et al., 1963; Matsumoto et al., 2005; Ieraci et al., 2016; Zelikowsky et al., 2018). Moreover, chronic SI induces biological modifications such as an altered metabolism (Sun et al., 2014), reduction in the reactivity of 5-HT neurons for mood regulation (Sargin et al., 2016), and upregulation of the neuropeptide tachykinin 2 (Zelikowsky et al., 2018).

*Foot Shock*

Lasting behavioral and biological consequences arise from exposure to FS (Cao et al., 2007; Bali et al., 2015; Wu et al., 2020). Mice treated with FS generally exhibit depressive, anxious, and post-traumatic stress disorder (PTSD) behaviors (Bali et al., 2015). Biological modifications such as bladder (Wu et al., 2020) and thermal (Wu et al., 2021) sensitivity are produced after FS exposure.

**Stress Mediating Brain Regions**

The dorsal bed nucleus of the stria terminalis (dBNST) is fundamental for integrating and monitoring limbic information (Lebow et al., 2016). At times regarded as an extension of the amygdala (Lebow et al., 2016), the dBNST is integral for mediating behavioral and biological stress responses (Kash et al., 2015). The dBNST plays a role in anxiety (Walker et al., 2009; Jennings et al., 2013; Kim et al., 2013) and fear learning (Sullivan et al., 2004; Walker et al., 2009).

**Research Objectives**

Stressors are not identical and inherently harbor varying properties, such as intensity, duration, and quality. FS dispenses a series of unhabitual acute stressors (Bali et al., 2015) whereas SI presents an unceasing stressor lacking reprieve (Zelikowsky et al., 2018). While the current literature recognizes SI and FS for producing variations in behavior such as anxiety, sensitivity, and aggression (Bali et al., 2015; Ieraci et al., 2016; Zelikowsky et al., 2018), research comparing these overlapping behavioral repercussions is lacking; particularly from the aspect of the dBNST, a brain region essential for stress, anxiety, and fear response (Sullivan et al., 2004; Walker et al., 2009; Jennings et al., 2013; Kim et al., 2013; Kash et al., 2015; Lebow et al., 2016). This study aimed to address the following objectives:

i. Evaluate if behavioral differences exist across stressors varying in properties, specifically SI and FS. Compare the presence or absence of behavioral variability across SI and FS.

ii. Examine if behavioral repercussions from SI and FS are modulated through the dBNST. Determine which behaviors are mediated through the dBNST.

## METHODS

### General Conditions

While mice are social animals and generally favor GH conditions, some male mice strains are overly aggressive, form rigid social hierarchies within their cages, and foster social defeat for other male occupants (Kappel et al., 2017). Male C57BL/6 mice normally refrain from engaging in aggressive behaviors when GH with other males (Kappel et al., 2017); thus, male C57BL/6N mice were used for this study.

Wildtype male C57BL/6N mice aged 8-12 weeks from Charles River were GH for 7 days for their acclimation period before the study commencement. C57BL/6N mice were maintained on a reverse 12-hour light and 12-hour dark cycle. Resident intruder (RI) behavioral assays were conducted during their dark cycle. Food and water were accessible *ad libitum* throughout the entire study across treatment groups. The temperature was sustained at 22 °C to 23 °C. The mice were regularly assessed for their physical and mental health. Cages were cleaned weekly and environmental conditions remained the same unless specified otherwise.

### Treatment Groups

*General Behavior Differentiation*

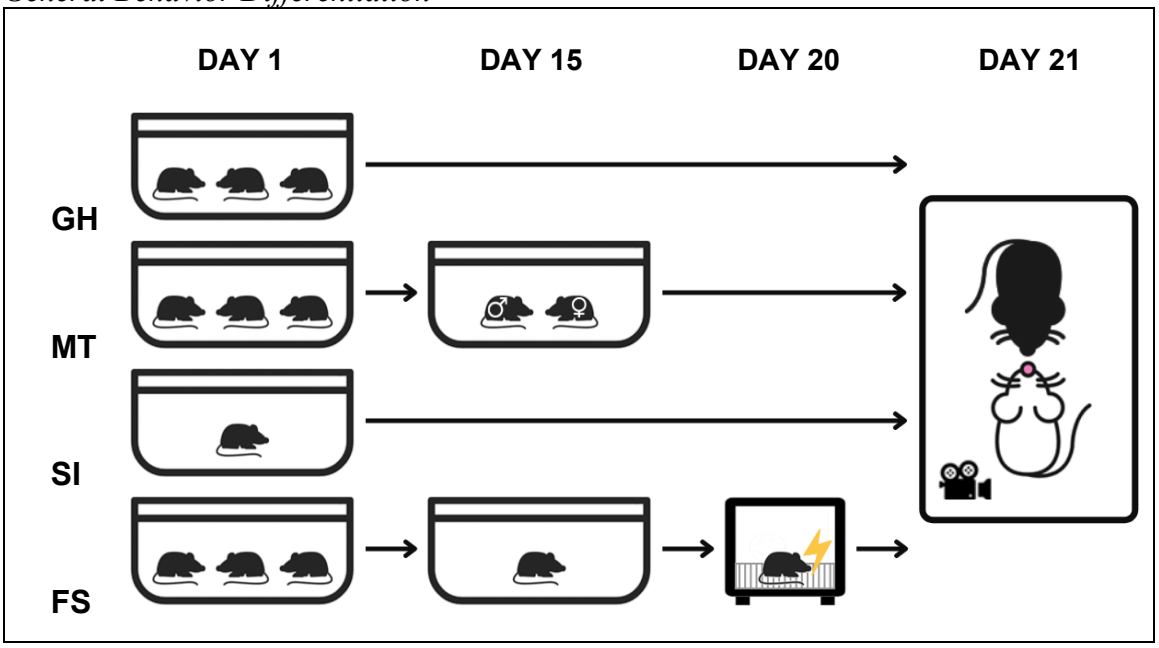


**Figure 2.** Delineation of treatment groups for general behavior differentiation.

For GH cohorts, 3 male C57BL/6N mice were placed in each cage and GH for the entire duration of the study (Figure 2). GH cohorts were not treated with stressors, acting as the context control (Figure 2). On Day 21, RI assays were conducted on GH mice (Figure 2).

In mated (MT) cohorts, 3 male C57BL/6N mice were GH for 15 days then relocated to separate cages containing a female C57BL/6N mouse (Figure 2). The female C57BL/6N mice were GH in sets of 3 for 15 days as well. MT male mice generally become more aggressive and territorial of their home cage when mated with female mice (Albert et al., 1988); as such, MT male mice served as the positive control for aggression. RI assays were performed on MT mice on Day 21 (Figure 2).

For the SI condition, male C57BL/6N mice endured SI for 20 days (Figure 2) since SI longer than 14 days induces the characteristics associated with chronic SI stress (Zelikowsky et al., 2018). On Day 21, RI assays were conducted for SI mice (Figure 2).

In FS cohorts, 3 male C57BL/6N mice were group housed then separated into different cages on Day 15 (Figure 2). FS mice were isolated before the FS context because some practice excessive and in some cases, fatal attacks following the FS context. Since acute SI fosters social cravings, encourages prosocial behaviors (Tomova et al., 2020), and behavioral repercussions from chronic SI arise only after 14 days (Zelikowsky et al., 2018), mice were SI on Day 15 to reduce confounding influences from SI and FS stress (Figure 2). On Day 20, FS mice were placed into boxes with metal electric bars. The FS mice were issued 15 pseudo-randomly spaced 1.0 mA shocks over the span of 1.5 hours (Figure 2). The same series of randomly spaced shocks were administered for each FS mouse (Figure 2). RI assays were performed for FS mice on Day 21 (Figure 2).

*dBNST Mediating Behavior Differentiation*

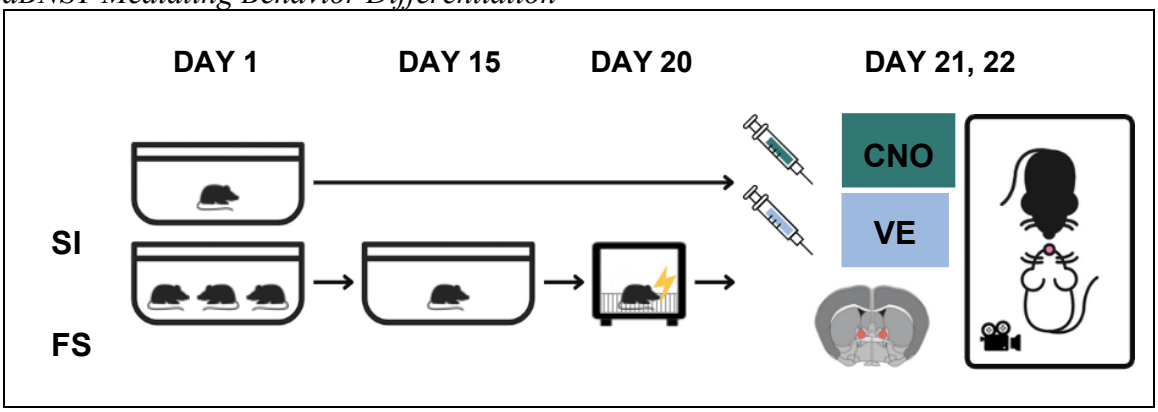


**Figure 3.** Delineation of treatment groups for dBNST mediated behavior differentiation.

3 weeks prior to the study, mice received surgery for viral expression of the inhibitory hM4D DREADD (Designer Receptors Exclusively Activated by Designer Drugs) into the dBNST. Mice were administered isoflurane anesthesia until unconscious. Mice were then placed onto a stereotaxic instrument and supplied with 1.5-2% isoflurane throughout the surgery. 200 nL of virus was backfilled into glass capillaries with a 50 µm diameter tip and bilaterally injected into the dBNST at a rate of 50 nL per minute. Capillaries remained at the site of injection for 2 minutes to prevent backflow and encourage diffusion of the virus.

For SI cohorts, male C57BL/6N mice were isolated in their cages for 20 days (Figure 3). On Day 21, SI mice were intraperitoneally injected with either vehicle (VEH) or clozapine-N-oxide (CNO) 20 minutes before conducting RI assays (Figure 3). VEH consisted of a saline solution. CNO is an activating agent for DREADDs receptors and elicits chemogenetic inactivation when delivered to animals expressing the hM4Di receptor. On Day 22, SI mice previously administered VEH were dispensed CNO and vice versa (Figure 3).

In FS cohorts, 3 male C57BL/6N mice were GH then isolated on Day 15 (Figure 3). On Day 20, FS mice were placed into boxes with metal electric bars. FS mice were administered a series of 15 randomly spaced 1.0 mA shocks over the course of 1.5 hours (Figure 3). On Day 21, FS mice were intraperitoneally injected with VEH or CNO 20 minutes prior to the RI assays (Figure 3). On Day 22, FS mice were either dispensed VEH or CNO depending on their injection the day before (Figure 3).

**Behavior Evaluation**

*Resident Intruder Assays*

Mice behavior is commonly examined through RI assays, a standardized experimental protocol where the behavior of an offensive resident mouse is evaluated when a defensive intruder mouse enters their home cage territory (Koolhaas et al., 2013). Male resident mice become offensive against male intruders because residents consider and establish their home cage as personal territory after a couple of days (Koolhaas et al., 2013). As such, when male intruders invade their home cage territory, male residents generally present aggressive behaviors toward male intruders (Koolhaas et al., 2013).

Male BALB/c intruders were deployed for the RI assays (Figure 2, 3). BALB/c mice are considered less aggressive than C57BL/6N mice strains (Kappel et al., 2017); as such, male BALB/c intruders were used because aggression instigated from only C57BL/6N residents were analyzed. BALB/c intruders were counterbalanced among the treatment groups.

Treatment groups were counterbalanced during the RI assays. Male C57BL/6N mice from each treatment group were placed alone in their home cage for 2 minutes. Resident mice from treatment groups with more than 1 mouse in their home cage were briefly separated into clean cages and placed into their original home cage during the RI assays. Ensuing the 2-minute acclimation baseline, male BALB/c intruders were introduced into the home cages and subsequent behaviors from male C57BL/6N residents were observed for 10 minutes. Several cohorts of each treatment group were conducted and evaluated.

Behaviors from male C57BL/6N residents during the RI assays were recorded and scored on the program Noldus Observer XT. Behaviors were classified as attacking, mounting, chasing, freezing, escaping, digging, investigating, anogenital sniffing, and grooming. These behaviors were broadly categorized into aggressive, avoidant, and prosocial behaviors. Attacking, mounting, and chasing were considered as aggression. Freezing, escaping, and digging were regarded as avoidance. Investigating, anogenital sniffing, and grooming were grouped as prosocial. Male C57BL/6N mice engaged in cage exploration outside these behaviors. The duration of individual and grouped behaviors in seconds from the recorded RI assays were totaled.

*Attack Behavior Differentiation*

Attacks conducted by different treatment groups from the general behavior differentiation cohorts (Figure 2) during the RI assays were further deconstructed into attack approach and bite location.

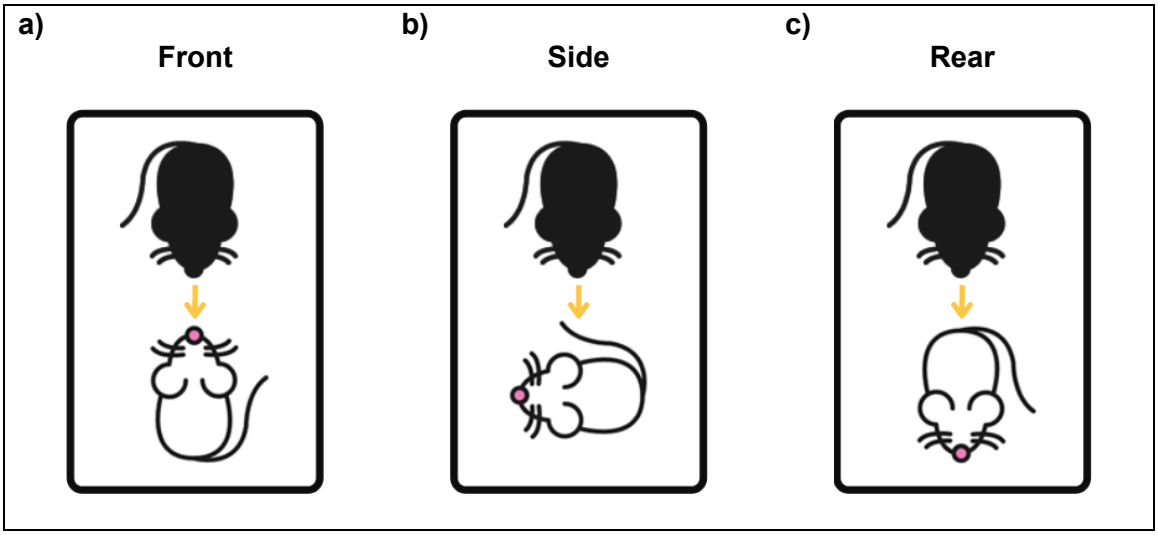


**Figure 4.** Different approaches residents practiced when attacking intruders.

Male C57BL/6N residents approached male BALC/c intruders from the front (Figure 4a), side (Figure 4b), and rear (Figure 4c) when attacking. The proportion of attack approaches across treatment groups was analyzed.

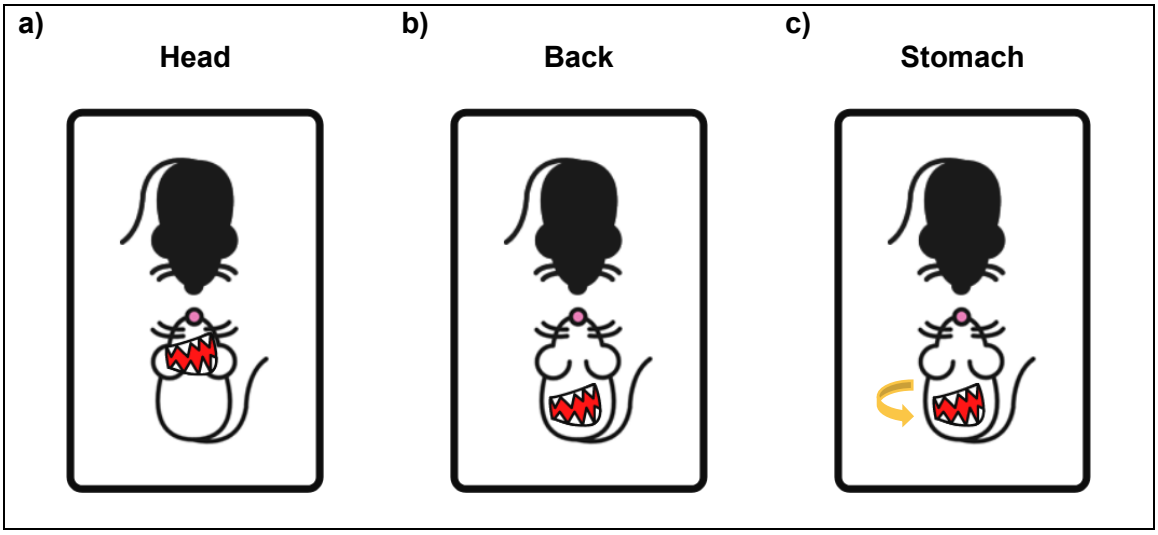


**Figure 5.** Different bite locations residents targeted when attacking intruders.

Male C57BL/6N residents directed their bites toward the head (Figure 5a), back (Figure 5b), and stomach (Figure 5c) area when attacking male BALB/c intruders. The proportion of bite locations across treatment groups was evaluated.

**Statistical Analysis**

The duration of individual and grouped behaviors in seconds as well as the proportion of attack approaches and bite locations from the recorded RI assays were analyzed through one-way ANOVA tests. A value of $p < 0.05$ was regarded as significantly different and are depicted through asterisks. The data and graphs are portrayed as mean ± standard error.

RESULTS

**General Behavior Differentiation**

Individual and grouped behaviors in male C57BL/6N residents were examined across treatment groups when presented with male BALB/c intruders during RI assays (Figure 2).

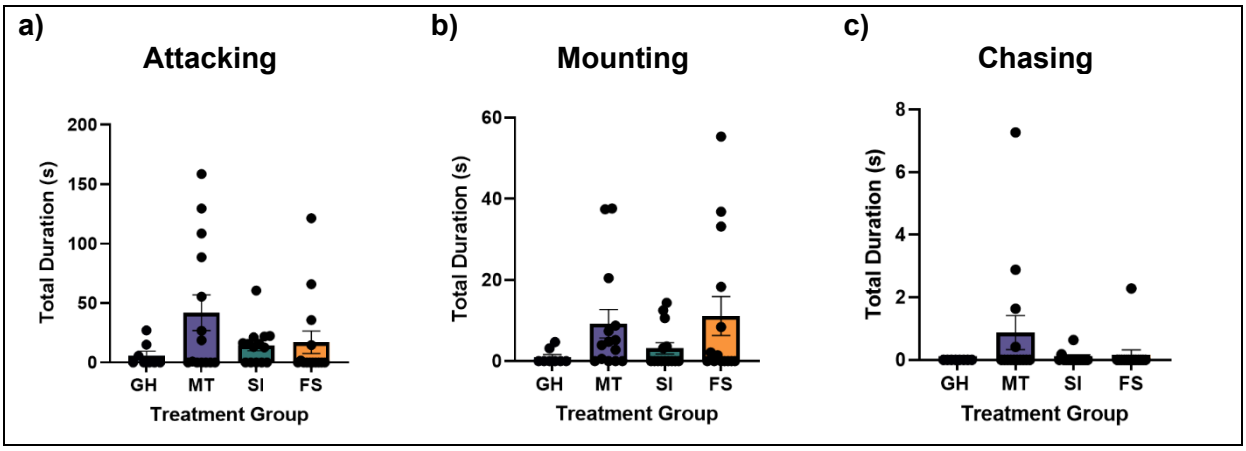


**Figure 6.** Total duration in seconds of aggressive behaviors across treatment groups.

Although not statistically significant, there was a trend of MT, SI, and FS mice attacking male BALB/c intruders more than GH mice (Figure 6a) who generally demonstrated an absence of aggressive behaviors (Figure 6a, 6b, 6c). Moreover, there was a trend of MT cohorts attacking BALB/c intruders more than SI and FS mice (Figure 6a). Although not statistically significant, MT, FS, and SI mice engaged in mounting more than GH mice, with MT and FS cohorts mounting more than SI mice (Figure 6b). There was a trend of MT cohorts chasing more than GH, SI, and FS mice, who practiced minimal chasing (Figure 6c).

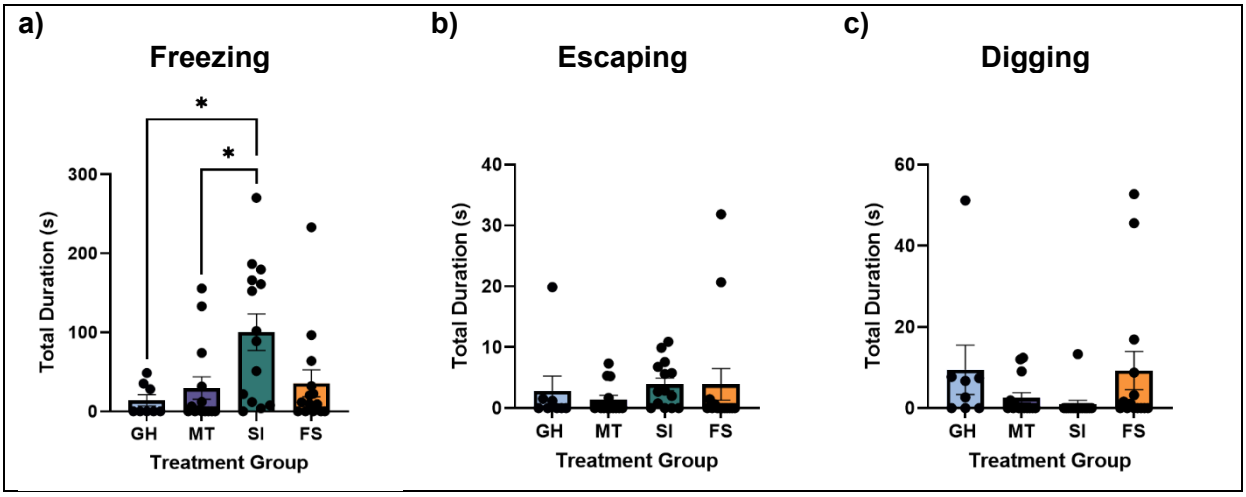


**Figure 7.** Total duration in seconds of avoidant behaviors across treatment groups.

SI cohorts exhibited freezing significantly more than GH and MT mice, but not significantly more than FS mice (Figure 7a). While MT cohorts demonstrated escaping less than GH, SI, and FS mice, the differences are minimal and not statistically significant (Figure 7b). There was a trend of GH and FS mice engaging in digging behaviors more than MT and SI treatment groups (Figure 7c).

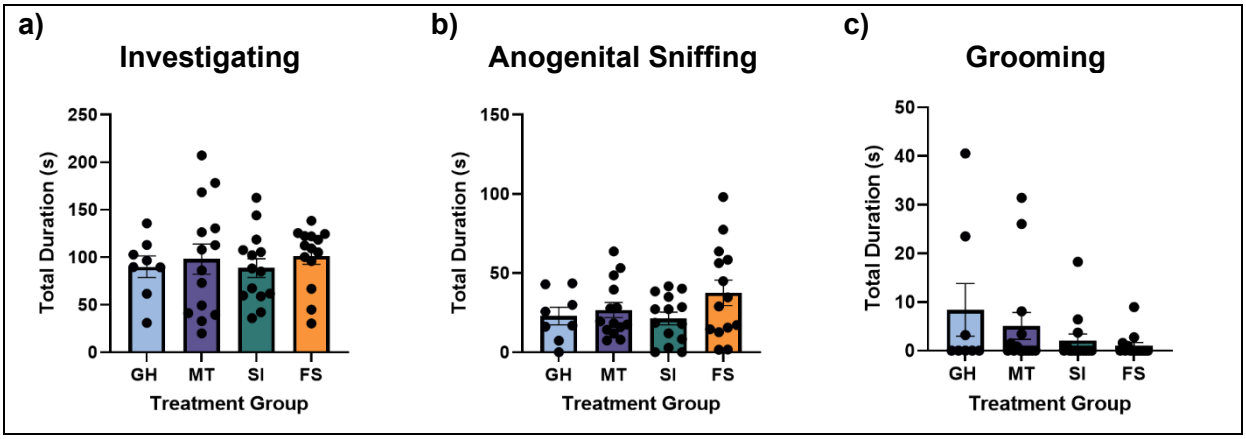


**Figure 8.** Total duration in seconds of prosocial behaviors across treatment groups.

Distinct differences were not observed for investigation across treatment groups (Figure 8a). There was a trend of FS cohorts practicing anogenital sniffing more than other treatment groups (Figure 8b). Although not statistically significant, GH and MT mice demonstrated more grooming than SI and FS mice, who rarely engaged in grooming (Figure 8c).

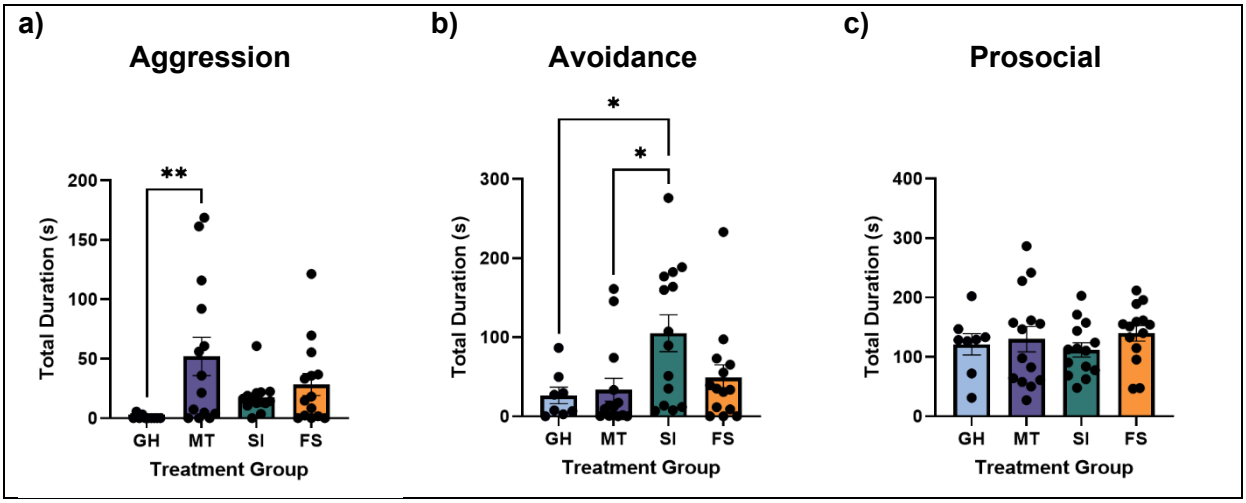


**Figure 9.** Total duration in seconds of grouped behaviors across treatment groups.

As anticipated, MT cohorts engaged in aggressive behaviors significantly more than GH mice (Figure 9a). Although not statistically significant, FS and SI mice demonstrated more aggression than GH mice as well (Figure 9a). SI cohorts practiced significantly more avoidant behaviors than GH and MT mice, but not significantly more than FS cohorts (Figure 9b). While not statistically significant, FS cohorts exhibited more avoidance than GH and MT treatment groups (Figure 9b). Minimal differences for prosocial behaviors were observed across treatment groups (Figure 9c).

**Attack Behavior Differentiation**

Attacks conducted by male C57BL/6N mice against male BALB/c intruders from the RI assays evaluating general behaviors (Figure 2) were further assessed based on attack approach (Figure 3) and bite location (Figure 4). Only attacks accompanied with bites were evaluated. As such, attacks from GH were not examined since none of their attacks involved biting.

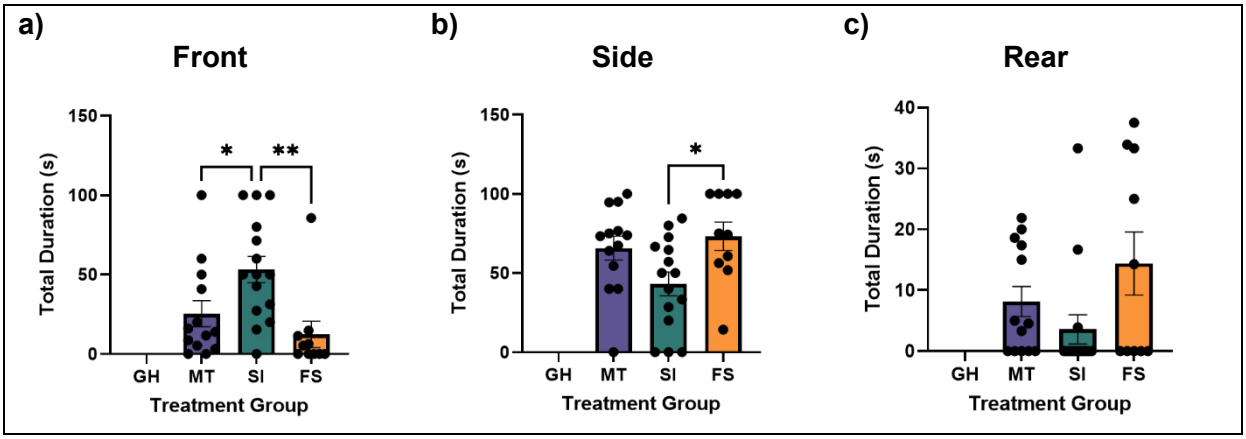


**Figure 10.** Proportion of attack approaches across treatment groups.

SI mice approached BALB/c intruders from the front significantly more than MT and FS cohorts (Figure 10a). Although not statistically significant, MT mice attacked BALB/c intruders from the front more than FS mice (Figure 10a). FS cohorts attacked from the side significantly more than SI, but not significantly more than MT mice (Figure 10b). There was a trend of MT mice approaching BALB/c intruders from the side more than SI mice (Figure 10b). While not statistically significant, FS cohorts advanced from the rear more than MT and SI treatment groups, with MT cohorts engaging in rear attacks more than SI mice (Figure 10c).

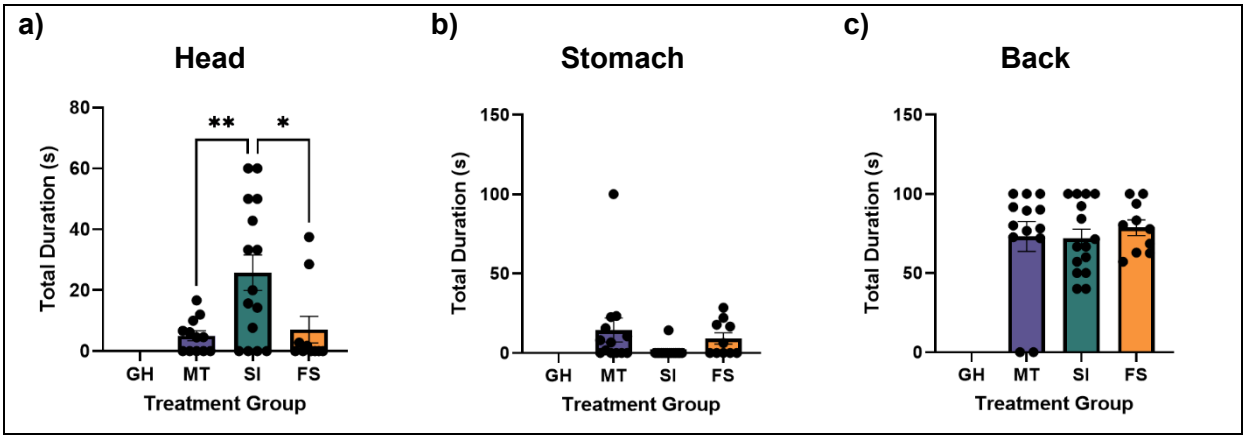


**Figure 11.** Proportion of bite location across treatment groups.

SI cohorts targeted the head area significantly more than MT and FS treatment groups (Figure 11a). Although not statistically significant, MT mice directed their bites toward the stomach more than FS and SI mice (Figure 11b). While FS mice practiced biting the stomach at times, SI cohorts rarely engaged in stomach attacks (Figure 11b). Minimal differences were observed across treatment groups for bites conducted on the back area (Figure 11c).

**dBNST Mediated Behavior Differentiation**

Grouped behaviors in male C57BL/6N residents were examined during RI assays when injected with VEH and CNO on different days (Figure 3). VEH acted as the context control while CNO inactivated the dBNST in SI and FS treatment groups (Figure 3).

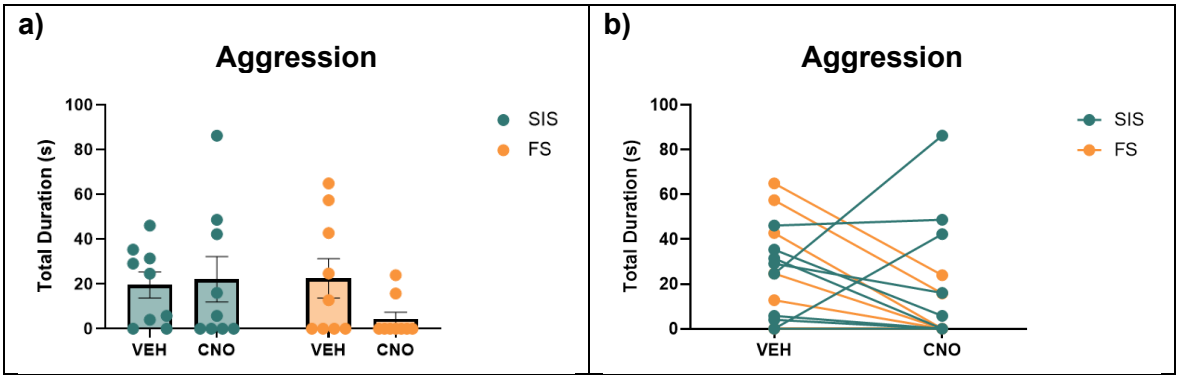


**Figure 12.** Total duration in seconds of aggressive behaviors after chemogenetic inactivation of the dBNST.

As expected, FS mice persistently demonstrated aggressive behaviors when administered VEH (Figure 12a); however, when FS cohorts were injected with CNO, aggression notably decreased (Figure 12b). In contrast, no remarkable differences in aggression were distinguished from SI cohorts dispensed VEH or CNO (Figure 12a). When FS mice were injected with CNO, a general downward trend in aggression was exhibited whereas SI mice presented a more random pattern (Figure 12b).

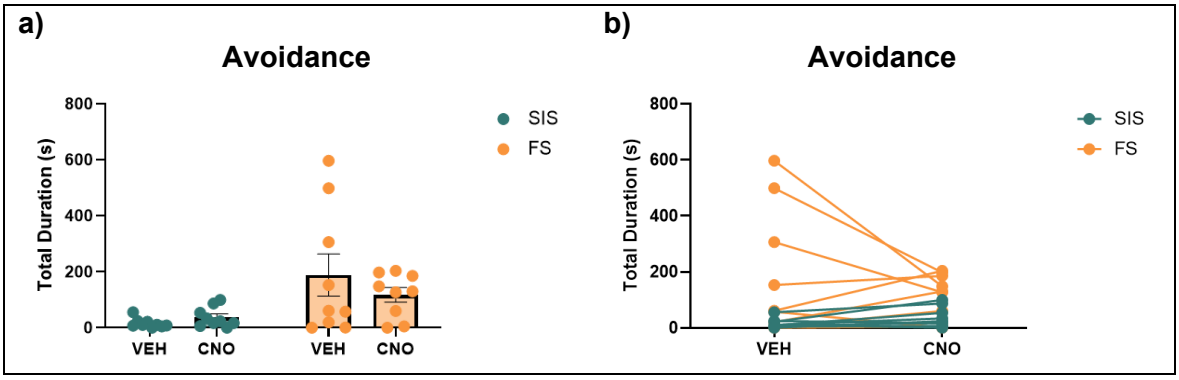


**Figure 13.** Total duration in seconds of avoidant behaviors after chemogenetic inactivation of the dBNST.

Baseline avoidant behaviors were minimal in SI mice regardless of VEH or CNO administration (Figure 13a, 13b). FS mice practiced less avoidant behaviors when injected with CNO rather than VEH (Figure 13a, 13b) and a general downward trend in avoidance was presented (Figure 13b).

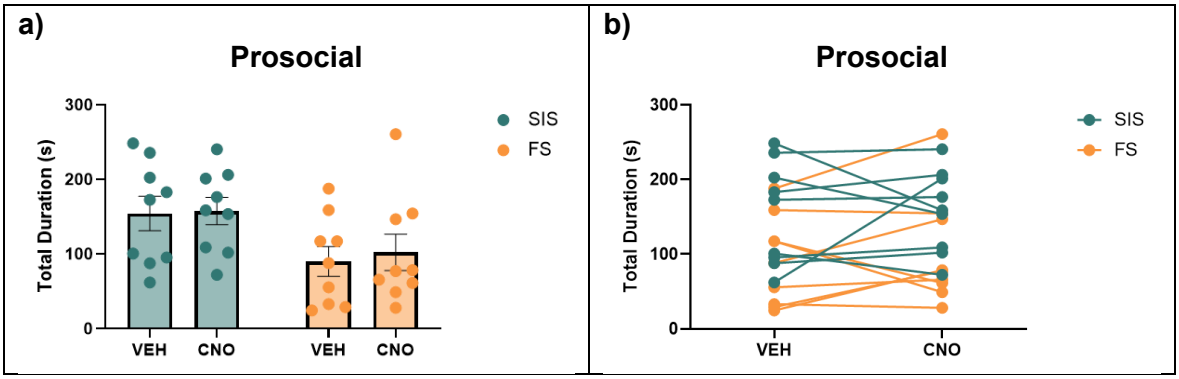


**Figure 14.** Total duration in seconds of prosocial behaviors after chemogenetic inactivation of the dBNST.

The total duration of prosocial behaviors remained relatively similar within SI and FS cohorts when administered VEH or CNO (Figure 14a, 14b). As such, no discernible trends were observed for SI and FS treatment groups in terms of prosocial behaviors after chemogenetic inactivation of the dBNST (Figure 14b).

## DISCUSSION

### General Behavior Differentiation

Differentiation of behavior was observed in male C57BL/6N mice across treatment groups during RI assays. Different treatment groups exhibited varying levels of aggressive and avoidant behaviors (Figure 9a, 9b). GH mice demonstrated practically no aggression, whereas aggressive behaviors were elevated in MT, SI, and FS treatment groups; however, only increased aggression in MT mice were considered statistically significant (Figure 9a). While SI and FS mice practiced more avoidant behaviors than other treatment groups, only SI cohorts were statistically significant (Figure 9b). No distinctive differences were observed for prosocial behaviors across treatment groups (Figure 9c).

These results are suggestive of a relationship between different stressors and subsequent behavior. The stressors SI and FS seem to influence aggressive and avoidant behaviors to varying degrees. SI and FS appear to lack an influence on prosocial behaviors evaluated in this study.

### Attack Behavior Differentiation

Aggression based on attack approach and bite location differed across treatment groups (Figure 10, 11). SI cohorts approached male BALB/c intruders from the front and attacked the head area significantly more than MT and FS treatment groups (Figure 10a, 11a). In turn, SI mice attacked from the side and rear less than MT and FS cohorts, although not statistically significant (Figure 10b, 10c). While there was a trend of MT and FS treatment groups attacking the stomach more than SI mice, the differences were minimal (Figure 10b). No distinctive differences were observed for bites directed toward the back area across treatment groups (Figure 11c).

These results indicate that SI and FS induces dissociable attack behaviors, specifically attack approach and bite location; however, the stressors SI and FS may lack an influence in mediating back targeted bites.

**dBNST Mediated Behavior Differentiation**

The role of the dBNST in modulating behavior differentiation for SI and FS was evaluated through the administration of CNO, a dBNST antagonist. Chemogenetic inactivation of the dBNST through CNO influenced the expression of aggression in FS cohorts, but not SI mice (Figure 12a, 12b). Moreover, chemogenetic inactivation of the dBNST reduced avoidant behaviors in FS mice, but not SI mice (Figure 13a, 13b); however, this may partially arise from SI lacking baseline avoidant behaviors in the dBNST cohorts. As such, drawing concrete conclusions about the role of the dBNST in avoidant behaviors for SI mice were refrained. No discernible differences were exhibited in SI and FS mice when injected with either VEH or CNO (Figure 14a, 14b).

These results are suggestive of a relationship between the dBNST and behavior differentiation in FS mice, but not SI mice. The dBNST appears to mediate aggressive and avoidant behaviors in FS mice, demonstrated through changes in aggression and avoidance after administration of VEH and CNO. Generally, the dBNST seems to lack influence over prosocial behaviors in SI and FS mice.

**Conclusive Thoughts**

The variations in behavior observed across different stressors suggest that SI and FS stress are not identical, nor interchangeable. SI and FS stress are intrinsically different in their properties and induce distinct behavioral consequences. These results indicate that behavioral differences from SI and FS are mediated through different brain regions; specifically, the dBNST appears to play a role in behavior differentiation in FS stress, but not SI. Since animals are subjected to various types and levels of stress throughout their lifetime, research evaluating the behavioral and biological consequences from different stressors is paramount.

**Future Direction**

When examining the role of the dBNST in behavior differentiation, the SI cohorts hardly demonstrated any avoidant behaviors during the RI assays (Figure 12a), contrary to the results presented in earlier cohorts when observing general behavior differentiation (Figure 8b). Discrepancies in avoidant behavior for SI cohorts may arise from variability among cohorts or perhaps avoidant behaviors in SI mice are not robust. In either case, more SI cohorts should be conducted to address these disparities. Supplemental FS cohorts should be performed as well to affirm these results.

Future studies should examine the role brain regions other than the dBNST in mediating stress since the dBNST appeared to lack influence over chronic SI. Subsequent research should also consider stressors other than FS and SI.

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