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ULTRASONIC VOCALIZATIONS AND STRESS RESILIENCE: BEHAVIORAL AND NEUROBIOLOGICAL CORRELATES

Nathaniel P. Stafford
University of New Hampshire, Durham

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BY

NATHANIEL P. STAFFORD

BS, Psychology, Wright State University 2012

MA, Psychology University of New Hampshire, 2015

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NATHANIEL P. STAFFORD

This dissertation has been examined and approved in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in Psychology by:

Dissertation Director, Robert C. Drugan, Ph.D., Professor of Psychology

Sergios Charntikov, Ph.D., Assistant Professor of Psychology

Thomas Foxall, Ph.D., Professor of Animal Science

Wm. Wren Stine, Ph.D., Associate Professor of Psychology

Barbara Prudhomme-White, Ph.D., OTR/L, Associate Professor of Occupational Therapy

On April 6, 2018

Original approval signatures are on file with the University of New Hampshire Graduate School.

DEDICATION

This dissertation and the work it represents is dedicated to my family and friends that supported me throughout my time in university. Without the love and support of my Nana, Rosemary Aber, and to my parents, Jerry and Cindy Stafford, I would not have even attempted university. Without the love and support of my wonderful partner, Lindsey Cole, I would not have made it through graduate school.

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ABSTRACT

ULTRASONIC VOCALIZATIONS AND STRESS RESILIENCE: BEHAVIORAL AND NEUROBIOLOGICAL CORRELATES

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Nathaniel P. Stafford

University of New Hampshire, May, 2018

When we are exposed to a traumatic or stressful life event, some individuals may develop symptoms of anxiety or depression while others may appear unaffected. In humans and nonhuman organisms, the ability to cope plays a large role in how an organism responds to a stressor, and this coping may be influenced by innate mechanisms. We have identified the use of ultrasonic vocalizations during intermittent swim stress (ISS) to forecast innate behavioral differences in stress reactivity. Vocalizing rats are resilient as they exhibit less cognitive impairment, motivational changes, and fewer anxiety-like behaviors typically observed post-ISS. Resilience should be associated with an active, stress buffering coping strategy during ISS, whereas non-vocalizing should exhibit more passive behaviors. These active or passive behaviors are driven by a corticolimbic serotonergic circuit originating in the dorsal raphe nucleus. Active coping is associated with reduced dorsal raphe serotonin activity, which leads to reduced post-stress impairment. We hypothesized vocalizing rats would engage in active coping responses, display fewer anxiety-like behaviors, and exhibit decreased serotonergic activation in the dorsal raphe nucleus compared with non-vocalizing rats. We found vocalizing rats exhibited reduced post-stress social anxiety, but engaged in passive coping during stress. Vocalizing rats

further exhibited increased serotonergic activity in stress-responsive subregions of the dorsal raphe nucleus compared to non-vocalizing and unstressed controls. These data are the first to verify the coping strategy and associated serotonergic activity of vocalizing rats as a novel model of stress resilience.

I. INTRODUCTION

Exposure to traumatic psychological stress is a significant risk factor in the etiology of depression, anxiety, and trauma-related disorders, such as posttraumatic stress disorder (PTSD) (Kessler et al., 2009). No one individual is "stress-free," and most will experience a significantly stressful situation at some point in their lifetime (Bonanno, Westphal, & Mancini, 2011). Furthermore, nearly 40 million in the U.S. will suffer from a major depressive episode or symptoms of anxiety as a result of acute distress (Kessler et al., 2009). Unfortunately, nearly 50% of these stress vulnerable individuals will also fail to respond to many of the currently available pharmacological therapeutic agents (Barlow, Allen, & Choate, 2016; Berton & Nestler, 2006). It is therefore critical that novel treatment options are considered. One of these options is a shift toward promoting proactive coping strategies to build resilience and buffer against the negative consequences of stress (Waugh & Koster, 2015). Some persons espouse a clear *innate* resilience or resistance to stress (Bonanno, 2008) and understanding the innate neurobiological mechanisms that afford stress resilience may direct toward novel treatment paradigms for those non-responsive to current therapeutic strategies.

Animal models of stress provide invaluable insight into the neurobiology of stress vulnerability and resilience, and the recent increase of the study of resilience is considered by some as a needed paradigm shift (Drugan, Christianson, Warner, & Kent, 2013; Ebner & Singewald, 2017; Koolhaas, de Boer, Buwalda, & Meerlo, 2017; Pfau & Russo, 2015). Understanding resilience requires a behavioral reference, or performance metric, such as a change from pre-stress to post-stress functioning. However, behavioral reactivity *during* stress, such as adopting or learning a behavioral strategy to alleviate the stressor, is critical to interpreting post-stress functioning. These behavioral changes during stress are considered to

model coping strategies (Commons, Cholanians, Babb, & Ehlinger, 2017; Puglisi-Allegra & Andolina, 2015). The inability/ability to cope with a stressful experience is critical to the development of, or resistance to developing, depressive-like, anxiety-like or PTSD-like symptoms (Baker & Berenbaum, 2007; Waugh & Koster, 2015).

Coping

Clinical research has identified two forms of coping that are critical with respect to adaptation and reaction to challenge; problem-focused or emotion-focused coping (Lazarus, 2000; Taylor & Stanton, 2007). Resilient individuals tend to adopt problem focused strategies to attenuate and control the stressor, while vulnerable individuals tend to employ emotion focused strategies to suppress negative emotions or distract from the situation when they perceive the situation is uncontrollable (Bonanno et al., 2011). Preclinical stress paradigms model coping by assessing the ability of an organism to learn a stress control response (i.e., terminate the stressor) or by assessing struggling/escape attempts and dominant behaviors. The inability to learn or escape results in a passive behavioral strategy that is considered analogous to "giving in" to the stressor (Baker & Berenbaum, 2007; Staiger, Melville, Hides, Kambouropoulos, & Lubman, 2009).

Behavioral Models

The way in which an individual or organism responds to a stressor is influenced both by prior experience and innate traits. Learning from prior experience is adaptive to adopt behavioral strategies and mitigate negative consequences when faced with subsequent stressful situations (Maier, 2015). However, in order to survive that initial stressor exposure, an organism must engage in an innate adaptive strategy (Koolhaas et al., 2017). Adaptive behavioral strategies in nonhuman animals, particularly in rodents, can be revealed during an initial stressful

experience (Drugan, Basile, Ha, Healy, & Ferland, 1997; Minor, Dess, Ben-David, & Chang, 1994; Paul et al., 2011; Warden et al., 2012), which is stable across non-stress periods (Castro et al., 2012; Drugan, Skolnick, Paul, & Crawley, 1989), but may also shift in response to repeated challenges (Nishimura, Tsuda, Oguchi, Ida, & Tanaka, 1988; Paul et al., 2011; Roche, Commons, Peoples, & Valentino, 2003).

Learned Resilience

Stressor controllability is one rodent model of active coping that provides an ability to learn an instrumental response to terminate a shock or swim stressor (see Drugan et al., 2013 or Maier and Seligman, 2016 for review). If an escape response is provided, learning the escape responses protects against development of behavioral depression (cognitive/motivational deficits; (Christianson, Paul, et al., 2008; Drugan et al., 2005, 1997; Maier, Albin, & Testa, 1973; Weiss, 1968)), inhibits fear responses (Maier, 2001), and reduces post-stress anxiety-like behavior such as neophobia (Jackson, Alexander, & Maier, 1980; Paré, 1994) or social avoidance (Christianson, Paul, et al., 2008; Christianson, Drugan, Flyer, Watkins, & Maier, 2013; Christianson, Thompson, Watkins, & Maier, 2009; Short & Maier, 1993) and prevents the activation of endogenous opioid-mediated stress-induced analgesia (Drugan, Ader, & Maier, 1985). The ability to control the initial stressor is considered analogous to resilience as controllability "immunizes" against developing depressive-like or anxiety-like behaviors when the organism is faced with subsequent inescapable stress (Amat, Aleksejev, Paul, Watkins, & Maier, 2010; Christianson et al., 2013; Williams & Maier, 1977) and may last for several weeks (Lucas et al., 2014; Maier, 2001; Moye, Hyson, Grau, & Maier, 1983).

Innate Resilience

Although controllability clearly promotes stress resilience, the opportunity to actively control a stressful situation is not always present. Exposure to an inescapable stressor can result in a bimodal distribution of approximately 50/50 resilient/vulnerable to developing post-stress depressive-like or anxiety-like behaviors post-stress (Drugan, 2000; Drugan et al., 1989; Levay et al., 2006). Rats develop innate preferential active or passive behavioral strategies during stress exposure. During an acute (single, short duration) forced swim, resilient rats engage in active escape attempts (struggling against cylinder wall, swimming and diving) (De Kloet & Molendijk, 2016). During social stress (intraspecific agonistic encounters), resilient rats exhibit dominant or aggressive behaviors. Vulnerable rats are considered to adopt more passive behaviors indicated by floating during swim stress or exhibit subordinate behaviors during social stress (Commons et al., 2017; De Kloet & Molendijk, 2016; Koolhaas et al., 2017; Wood & Bhatnagar, 2015). Defensive burying is considered an active coping strategy to defend against the painful stimulus, while immobility and avoidance are passive strategies (Cohen et al., 2017). An initial active behavioral strategy is associated with resilience, because these organisms continue to "resist" developing depressive-like behaviors in subsequent posttests (Drugan et al., 1989; Wood, Walker, Valentino, & Bhatnagar, 2010).

Neurobiology

Coping Circuits

One of the primary neurobiological circuits associated with coping involves a reciprocal cortico-limbic serotonergic pathway originating in the pontine dorsal raphe nucleus. Active behaviors are generally considered a product of reduced serotonergic activity while passive behaviors are produced by enhanced serotonin activity (Puglisi-Allegra & Andolina, 2015).

Active behavioral output is a product of initial corticotropin releasing factor (CRF) binding to CRF type-I receptors. CRF-I receptors are highly selective to CRF during periods of acute release as part of an initial fight or flight response, and have inhibitory properties on dorsal raphe serotonin neurons (Donner et al., 2016) via binding to and causing release of GABA from local GABAergic interneurons colocalized with serotonin neurons (Homberg & Contet, 2009). The experience of learning an adaptive escape response that terminates the stressor inhibits further serotonergic output. Under these learning conditions, or as arousal decreases, prelimbic glutamatergic projections to dorsal raphe reduce activity of serotonin neurons through tonic inhibition via inhibitory γ-amino-butyric acid (GABA) interneurons (Weissbourd et al., 2014; Zhou et al., 2017). This prelimbic glutamatergic input on dorsal raphe GABAergic interneurons inhibits serotonin activity (Amat, Paul, Watkins, & Maier, 2008; Baratta et al., 2009), thereby inhibiting release in forebrain projection areas, such as basolateral amygdala (Cabib & Puglisi-Allegra, 2012; Puglisi-Allegra & Andolina, 2015).

The inability to adapt to the stressor by learning or resisting results in continued CRF release, and a shift of CRF binding to type-II receptors. CRF-II receptors are excitatory on serotonin neurons, and as such continued CRF-II binding results in increased extracellular serotonin within dorsal raphe (Amat, Matus-Amat, Watkins, & Maier, 1998; Hammack et al., 2003; Waselus, Nazzaro, Valentino, & Van Bockstaele, 2009). Stress-induced serotonin release binds to serotonin type -1A receptors on prelimbic glutamatergic cell bodies inhibiting them, which removes prefrontal inhibition of dorsal raphe serotonin cell bodies. Continued serotonin release ultimately leads to desensitization of inhibitory serotonin type -1A and 1B autoreceptors on serotonin cell bodies and excitatory serotonin type-1A and type-2C on GABA neurons, which

disrupts the local negative feedback and leads to over activation of serotonergic output from dorsal raphe (Hassell et al., 2017; Liu, Jolas, & Aghajanian, 2000; Rozeske et al., 2011).

Dorsal Raphe Neurobiology

The circuitry of active or passive behaviors is generally considered in a holistic context of the dorsal raphe nucleus. Reduced serotonin activity is associated with active behaviors, while passive behaviors are associated with increased serotonin activity. These effects are noted in specific areas of dorsal raphe, and it is well established that dorsal raphe is uniquely functionally topographically organized into distinct subregions (Hale & Lowry, 2011). These regions are functionally organized based upon afferent input and efferent projections via tracing and stimulation studies (McDevitt et al., 2014; Warden et al., 2012), as well as via functional activation measured using cFos (an immediate early gene widely regarded as an index of neuronal activity) protein product expression in the serotonin cell nucleus (Kelly, Donner, Hale, & Lowry, 2011; Kovács, 2008), and mRNA expression of the serotonin synthesis rate limiting enzyme, tryptophan hydroxylase (Donner et al., 2018).

Dorsal raphe serotonergic populations can be subdivided into 5 subregions (dorsal, ventral, ventrolateral, caudal, and interfasicular nucleus) extending to different points along its rostral-caudal axis (Abrams, Johnson, Hollis, & Lowry, 2004; Paxinos & Watson, 1998). Local inhibitory GABA interneurons are distributed throughout all of these subregions, with few to none in the interfasicular nucleus (Roche et al., 2003; Soiza-Reilly & Commons, 2014; Stamp & Semba, 1995). Furthermore, dorsal raphe serotonin neurons may be sensitive to stimulus intensity, as traumatic stressors, such as inescapable tail shock or shock prod induce more robust widespread activation than swim stress or a mild stressor, such as open field exposure (Bouwknecht et al., 2007; Cohen et al., 2017; Grahn et al., 1999; Roche et al., 2003).

Particular dorsal raphe subregions are responsive to different stressor types, implicated in active/passive coping, as well as post-stress anxiety. Inescapable tail shock produces increased serotonergic activity of the caudal subregion, while active coping inhibits serotonin release in the rostral extent of the dorsal subregion and in the caudal subregion (via local GABAergic mechanisms (Amat, Paul, Zarza, Watkins, & Maier, 2006; Rozeske et al., 2011)). Rats exposed to a shock prod that engage in active coping strategies (defensive burying) have higher activation in the caudal extent of dorsal subregion, while activity is reduced in the midrostral extent (Cohen et al., 2017). Acute continuous swim stress with cold (19ºC), ambient (25ºC), or warm (35ºC) water activates mid-rostral levels of dorsal, ventral, and ventrolateral subregions as well as the caudal subregion (Drugan, Hibl, et al., 2013; Kelly et al., 2011), and ventrolateral subregions appear to be involved in passive behaviors induced by swim and other inescapable stressors. A single 5 min forced swim in ambient water (25ºC) results in greater activation of serotonin neurons in dorsal and ventrolateral subregions (Kelly et al., 2011). Acute social defeat results in widespread activation of dorsal, ventral, ventrolateral, and caudal subregions with increased serotonergic activity in ventrolateral wings associated with passive behaviors during defeat (Paul et al., 2011).

The response of dorsal raphe serotonergic neurons to stress is partially dependent upon the stressor type, but dorsal, caudal, and ventrolateral subregions are consistently implicated in coping. Furthermore, desensitization of serotonin type-1A and 1B inhibitory autoreceptors to produce passive coping, as well as prelimbic glutamatergic modulation of GABAergic interneurons, occurs primarily in dorsal and caudal subregions (Donner et al., 2018; Rozeske et al., 2011). Learning an active coping strategy “quiets” serotonin neurons in those regions.

Therefore, the serotonergic origins of the "coping circuit" described above likely reside within one of those regions.

Novel Model of Resilience

Recently, our laboratory has identified a novel model of innate stress resilience to intermittent swim stress (ISS). ISS was developed as a hybrid of the inescapable tail shock (learned helplessness) and forced swim (behavioral despair) models of stress (Brown, Hurley, Repucci, & Drugan, 2001). As a model of inescapable stress, ISS exposes rats to a series of forced swims in cold (15ºC) water. ISS produces a bimodal distribution of post-stress behavioral differences with some rats exhibiting vulnerability while others exhibit resilience (Christianson & Drugan, 2005; Levay et al., 2006). Vulnerable rats demonstrate cognitive deficits post-ISS in the form of impaired instrumental escape learning (Drugan, Christianson, Stine, & Soucy, 2009; Levay et al., 2006; Stiller, Drugan, Hazi, & Kent, 2011), compromised spatial learning (Drugan, Warner, Papallo, Castracane, & Stafford, 2014), reduced active coping behavior in a subsequent forced swim (Drugan et al., 2014), and enhanced social avoidance (Stafford, Jones, & Drugan, 2015). Resilient rats do not display these deficits and exhibit superior learning and pro-social behaviors.

In several early ISS studies, our laboratory found some rats emitted ultrasonic vocalizations (USVs) (Christianson & Drugan, 2005), and formal analysis of these effects demonstrated rats that emitted 22-kHz vocalizations did not display expected instrumental learning deficits. Rather, these vocalizing rats exhibited superior learning (Drugan et al., 2009). The generality of this putative "resilience" was tested in three subsequent experiments that found superior spatial learning (Drugan et al., 2014), protection from ISS-induced behavioral despair (Drugan et al., 2014) and social anxiety (Stafford et al., 2015). In each of these studies,

approximately 25-50% of ISS subjects vocalized, which is consistent with the behavioral expression of innate differences in resilience and vulnerability in shock (Drugan, 2000) and swim (Drugan et al., 1989) paradigms.

The emission of 22-kHz USVs as a behavioral marker of resilience is a novel interpretation of these vocalizations. Aversive stimuli such as mild handling (Brudzynski & Ociepa, 1992), air puff (Brudzynski & Holland, 2005), fever inducing prostaglandin E_2 administration (Blumberg & Moltz, 1987) or lipopolysaccharide administration (Bassi et al., 2012), benzodiazepine receptor inverse agonists (Beckett, Aspley, Graham, & Marsden, 1996; Miczek, Weerts, Vivian, & Barros, 1995), foot shock (Jelen, Soltysik, & Zagrodzka, 2003), and intraspecific agonistic encounters (Portavella, Depaulis, & Vergnes, 1993) all result in 22-kHz USVs and are considered to convey an anxiety-like state. Emission of 22-kHz USVs serve an as ethological long-range tool to communicate the presence of immediate threat or danger to conspecifics (Endres, Widmann, & Fendt, 2007; Litvin, Blanchard, & Blanchard, 2007). Rats do not emit these vocalizations in the direct presence of a threat (Litvin et al., 2007). Therefore, if the rat is vocalized it likely successfully fled or defended itself against the threat. In the context of resilience to ISS, an alternative interpretation is that the emission of 22-kHz USVs may occur concomitantly with activation of neurobiological pathways associated with a particular coping strategy that is beneficial to the swim stress model.

Several lines of research have also demonstrated that 22-kHz vocalizations serve a thermoregulatory function and are involved with hypothalamic cooling after central pro-inflammatory (e.g., prostaglandin E_2) administration (Blumberg & Moltz, 1987) or fever due to peripheral lipopolysaccharide injection (Bassi et al., 2012). Furthermore, one study found rats that recovered quickest from fever uniquely emitted 22-kHz USVs, and these rats engaged in

dominant social behaviors during a subsequent social interaction (Bassi et al., 2012). These studies suggest rats that emit these vocalizations during ISS may be physiologically capable of recovering from threat exposure as a form of stress resilience.

Current Study

Several studies from our laboratory demonstrated that ISS produced groups of rats that are behaviorally vulnerable or resilient when challenged in a post-stress test. Resistance to developing ISS-induced depressive-like or anxiety-like behavior is consistent in rats that emitted 22-kHz USVs during ISS. Given that 22-kHz vocalizations are indicative of an anxiety-like state (Brudzynski & Chiu, 1995; De Vry, Benz, Schreiber, & Traber, 1993; Jelen et al., 2003; Naito, Nakamura, Inoue, & Suzuki, 2003), it is not immediately clear what mechanism affords resilience to ISS in vocalizing rats. One hypothesis for USV-associated resilience is that vocalizing rats respond to ISS by adopting a more active behavioral strategy compared with non-vocalizing rats. Others have found in response to an interoceptive stressor (fever), 22-kHz USVs were emitted by dominant and less socially anxious rats that exhibited quicker recovery period from fever (Bassi et al., 2012). USV-emitting rats engaged in greater social exploration post-ISS, therefore, it is likely these rats exhibited an active phenotype.

In order to test the active coping hypothesis, two experiments were conducted to test the central hypothesis that active coping is employed during ISS by rats that emit 22-kHz vocalizations, and this active behavioral strategy is associated with reduced activation of serotonin neurons within dorsal raphe. Experiment 1 tested the hypothesis (hypothesis 1) that USV-emitting rats engage in active behaviors during ISS, which serve to protect against the anxiety-inducing effects of the stressor. Experiment 2 tested two hypotheses that active coping during ISS is associated with specific function of dorsal raphe. Specifically, experiment 2 tested

the hypotheses that resilient rats engaging in active coping would exhibit reduced serotonergic activation (hypothesis 2) and greater GABAergic activation (hypothesis 3) of dorsal and caudal subregions of dorsal raphe.

II. GENERAL METHODS

Materials

Subjects

A total of 143 adult male Sprague Dawley rats (SAS Derived, Charles River Labs, Kingston, NY, USA) were used as experimental subjects in the study. Rats weighed 275-400 g and were approximately postnatal day 75 (±5 days) at the time of testing. Juvenile Sprague Dawley males (PD 28-32) served as social exploration stimuli in the social exploration tests. The vivarium was maintained on a 12-hour light/dark cycle (lights on 07:00) with cool fluorescent bulbs that produced ambient light of 366-400 lx. For all rats, food and water were available *ad libitum* for the duration of the experiment. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011) and were reviewed and approved by the University of New Hampshire Institutional Animal Care and Use Committee (appendix A).

Surgical procedures

Subjects were anesthetized with ketamine/xylazine (70/7 mg/kg) and a 2 cm lateral incision was made along the midline at the border between the abdomen and lower abdomen and a core body temperature datalogger was inserted free-floating. The peritoneal muscle wall was sutured closed using dissolvable suture followed by nonabsorbable suture to close the skin. Rats received a 1ml/kg injection of 5mg/ml atipamezole to reverse xylazine sedation. Burtorphanol tartrate at 0.5mg/kg was administrated i.p. immediately following surgery to manage post-operative pain and lidocaine was applied around the surgical site to reduce manipulation of sutures by the animals.

Apparatus

Social exploration pretest and posttest were conducted in identical test chambers, which consisted of a plastic tub cage 40.6×20.3×20.3 cm (*l*×*w*×*h*), wire lid, and 3 cm of wood shaving bedding free of food and water. The room was lit by cool fluorescent bulbs and light penetration into the test chamber was 200-300 lx. A camera that was mounted above the apparatus recorded behavior during each test session.

Intermittent swim stress was conducted in two acrylic cylinders 21×42 cm (*d*×*h*) with a ¼-inch galvanized wire mesh at the bottom of each cylinder suspended over a tank 80.6×45.7×28.6 cm (*l*×*w*×*h*) filled with water maintained at 15±1 °C (Figure 1). The apparatus was controlled by a custom Arduino software. The room was lit by cool fluorescent bulbs positioned directly above the swim cylinders (subjects were shadowed by the ceiling of the apparatus) resulting in ambient light of 200-300 lx. A camera that was mounted above the apparatus recorded behavior during each test session.

Figure 1. Intermittent swim stress

Vocalizations were recorded using an Ultramic 200K high frequency ultrasound microphone with integrated digital to analog converter (Dodotronic, Pavia, Italy) into the Sound Emission Analyzer program (Centro Interdisciplinare di Bioacustica e Ricerche Ambientali, Pavia, Italy). Recordings were later visualized and analyzed using Raven (Cornell Lab of Ornithology, Cornell University, Ithaca, NY, USA).

Methods

General Procedures

All rats arrived at the vivarium at postnatal day 21 and were allowed to acclimate to the vivarium for 5 weeks prior to experimentation. Rats were group housed 4 per cage, weighed weekly, and handled twice weekly (to mark tails for identification) randomly by all experimenters. Rats were individually housed post-op for the remainder of the experiment and not manipulated except for transport to the experiment room. Cage bedding type/brand remained consistent and was changed twice weekly by the same husbandry staff individual throughout the duration of the experiment. All surgical procedures occurred between 09:00-14:00. In order to limit potential cohort effects on vocalizations and behavioral endpoints, both experiment 1 and experiment 2 were conducted in tandem (rats arrived in cohorts approximately n=20 and randomly assigned to experiment 1 or experiment 2 procedures prior to manipulation). Social exploration tests were conducted between 09:00-12:00 and ISS was conducted between 08:00-14:00. Adults were transported to the test chamber and acclimated for 1 h after which a juvenile was placed into the chamber and behavior scored for 3 min. Juvenile stimuli were utilized for a maximum of 4 tests and adults were never exposed to the same juvenile more than once.

Rats were weight-matched and assigned to ISS, confined conspecific, sham-ISS, or home cage control. In order to emit USVs, ISS rats require a confined conspecific, which were paired (weight matched) with the ISS rat and were exposed to the apparatus in tandem in the absence of water. Sham-ISS were paired (weight matched) and experienced identical treatment as ISS in the absence of water. Home cage control subjects were left undisturbed in the vivarium. ISS rats were exposed to 80 swim trials. Each swim trial consisted of a 5 s forced swim in which the

cylinder was submerged to a depth of 25 cm. Trials were presented at a variable 60 sec (10-110 s) inter-trial-interval. USVs were recorded from ISS, confined conspecific, and sham-ISS rats during the entirety of apparatus exposure.

Behavioral Analyses

Social exploration tests were scored in real time by a single trained experimenter blind to group membership that has previously met interrater reliability of $r = 0.95$. Social exploration was scored via custom written open-source reaction time counter in Python v2.2.7 on OS X. Exploratory adult behaviors directed at the juvenile were quantified. Total times the adult engaged in the following behaviors were summed and each subject was assigned a total social exploration score: sniffing (direct snout contact against any portion of the juvenile and primarily observed directed at anogenital region, pinning (minimum of two fore-paws against juvenile), allogrooming (adult grooming the juvenile), and chasing.

Intermittent swim stress recordings were manually analyzed for instances of immobility, swimming, or climbing during each of the 5 s swim trials by a single trained experimenter blind to vocalization condition that has previously met interrater reliability of $r = 0.95$. Immobility was defined as remaining stationary in the cylinder with minimal movements to keep the head above water and the body adopting a horizontal posture. Swimming was defined as 1) lateral movements across the radius or perimeter of the cylinder with the body adopting a horizontal posture, or 2) diving in which the subject submerged part of all of the body, or 3) any movement while submerged. Climbing was defined as vigorous forelimb movement against the side of the cylinder in which the fore paws must break the surface of the water and the body adopted a vertical posture. Behaviors were scored using a binary scheme (1=behavior occurred, 0=behavior did not occur).

Per our previous experiments, ISS rats that emitted any 22-kHz USV were designated into the "ISS/USVs" group, while remaining rats were included the "ISS/No-USVs" group (Drugan et al., 2014; Stafford et al., 2015). Vocalizing rats emitted between 10-150 separate USVs throughout the ISS session. USVs were manually marked as separate, discrete calls based upon a downward frequency sweep indicating the onset of a vocalization with an interval vocalization interval of 10 ms. A typical vocalization consisted of 19-kHz – 26-kHz bandwidth emitted in long-pulse trains of 300 ms – 1000 ms in duration.

Core body temperature was recorded via SubCue dataloggers (Canadian Analytical Technologies Inc., Calgary, Alberta, CA) at 10 min intervals. Recorded temperatures were calibrated using the formula (*measured temp - intercept*)*(*slope*) using specific intercept and slope values each datalogger provided by the manufacturer. Data were then manually exported and matched to a 1 h baseline (24 h before testing), and social exploration pretests and posttests (acclimation period to 1 h after testing). For both experiments body temperature was assessed 1 h prior to ISS. In experiment 1 body temperature was assessed for 8 h post-ISS, while in experiment 2 temperature was assessed up to perfusions (90 min) post-ISS.

Data Analyses

All behavioral and temperature data were analyzed using Prism version 7.0 (GraphPad Software, La Jolla, CA, USA), while cell count data were analyzed using SPSS version 25.0. Statistical significance was accepted at $p < 0.05$ for all tests. Family-wise error for multiple comparisons were adjusted using the Holm's-Sidak method (Abdi, 2010).

III. EXPERIMENT ONE

Experiment 1 was designed to replicate our previous finding that 22-kHz USVs emitting during ISS predicted increased post-stress social exploration and extend our previous report by investigating coping strategies during ISS that may afford stress resilience. Similar to others (Christianson, Paul, et al., 2008; Christianson et al., 2013, 2009), we interpreted in our previous report and the current study a reduction in social exploration from baseline as a social anxiety response and as vulnerability to the negative consequences of ISS, while a positive change or no change from baseline was interpreted as a lack of social anxiety and considered stress resilience (Stafford et al., 2015). We hypothesized following these previous studies that vocalizing rats engaged in an active coping strategy during ISS that protected against post-ISS anxiety. Exposure to inescapable stress, such as tail shock results in social anxiety, while active behavioral control to terminate the stressor protects against it (Christianson, Paul, et al., 2008; Christianson et al., 2013, 2009). During a forced swim, rats typically engage in three behavioral subsets: immobility, swimming, and climbing. The latter two behaviors are considered active behaviors associated with resilience, while immobility is considered an analogue of behavioral depression (Drugan, Christianson, et al., 2013; Drugan et al., 2014). Therefore, we hypothesized that vocalizing rats are protected against ISS-induced anxiety and related deficits by engaging in active coping behaviors during stress exposure.

Methods

Procedure

The timeline for experiment 1 is depicted in Figure 2. A total of 70 adult rats were used in this study consisting of ISS (ISS/USVs, n=7; ISS/No-USVs, n=16), confined conspecific (CC, n=23), sham-ISS (S-ISS, n=12), or home cage control (HCC, n=12). Two CC rats were removed

after the ISS session due to computer malfunction. A subset of rats underwent datalogger implantation (ISS/USVs, n=4; ISS/No-USVs, n=8; CC, n=12; S-ISS, n=8; HCC, n=8) and were allowed to recover for 1 week before experimentation. After recovery, rats were tested in the social exploration, followed 24 h later by ISS or control conditions. Following ISS, rats were towel-dried and returned to the vivarium (controls were briefly handled in a towel). Twenty-four hours after ISS or control, rats were again tested in the social exploration.

Figure 2. Timeline for experiment 1

Statistical Analyses

Social exploration pretests were analyzed via one-way analysis of variance (ANOVA). To capture the degree of change in social exploration from pretest to posttest, the percent change from baseline [((posttest-pretest)/pretest)*100] was calculated and analyzed via ANOVA. Behaviors during ISS trials were analyzed by first calculating 10 trial blocks for active (sum of swimming and climbing) or passive (immobility) behaviors. Active and passive behaviors between ISS/No-USVs and ISS/USVs were tested using two-way repeated measures ANOVA with treatment (USV emission) as the between-subjects variable and trial block as the within-subjects variable. All temperature data were analyzed via repeated measure ANOVA with treatment as the between-subjects variable and time as the within-subjects variable. Tables presenting pairwise comparisons of means for behavioral data are available in appendix B.

Results

Social Exploration

Pretest values were equivalent between groups [F(4,65) = 0.079, $p = 0.988$], but there was a significant effect of treatment on social exploration percent change from baseline after ISS [F (4,65) = 4.167, $p = 0.005$]. Group differences were further tested with all possible pairwise comparisons. Rats that did not vocalize exhibited a significant change in social exploration time (Figure 3) compared with ISS/USVs ($p < 0.001$), confined conspecific ($p < 0.001$), sham-ISS ($p = 0.015$), and home cage controls ($p = 0.02$). Non-vocalizing rats exhibited an 11.48% decrease from baseline, while vocalizing rats increased 11.02% from baseline.

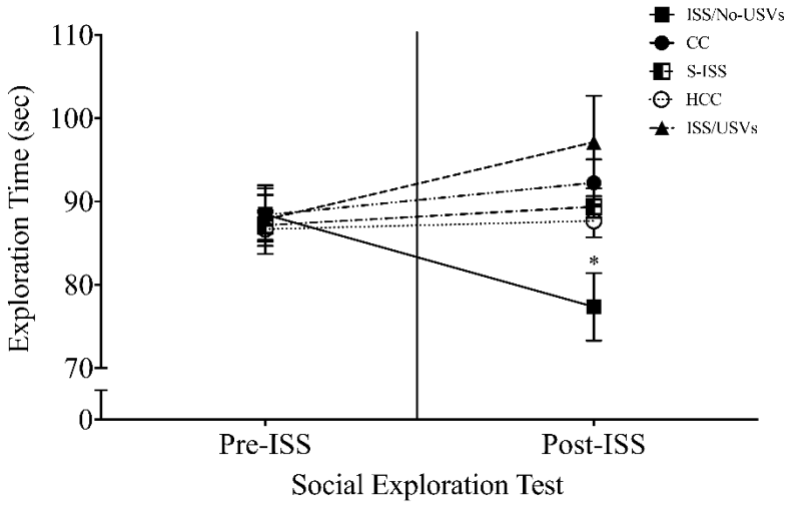

Figure 3. Mean (± SEM) social exploration (in s) during the 3 min test. * indicates significantly different from all other groups ($p < 0.05$).

Intermittent Swim Stress

There was a significant effect of treatment [F(1,18) = 14.79, $p < 0.001$] and trial [F(7,126) = 2.339, $p = 0.028$] and nonsignificant treatment x trial interaction on active behaviors during ISS [F(7,127) = 1.604, $p = 0.140$]. Planned comparisons were conducted to assess differences in active or passive behaviors at each trial block. Vocalizing rats specifically engaged in significantly less active behaviors (Figure 4A) than non-vocalizing rats between trial blocks 2-6 (trials 11-60; all *p's* < 0.05). For passive behaviors (Figure 4B), there was also a

significant effect of treatment [F(1,18) = 13.69, $p = 0.002$] and time [F(7,126) = 2.319, $p = 0.029$], but the interaction was nonsignificant [F(7,126) = 1.206, $p = 0.305$]. Vocalizing rats engaged in greater passive behaviors between trials 31-40 compared with non-vocalizing rats ($p = 0.022$).

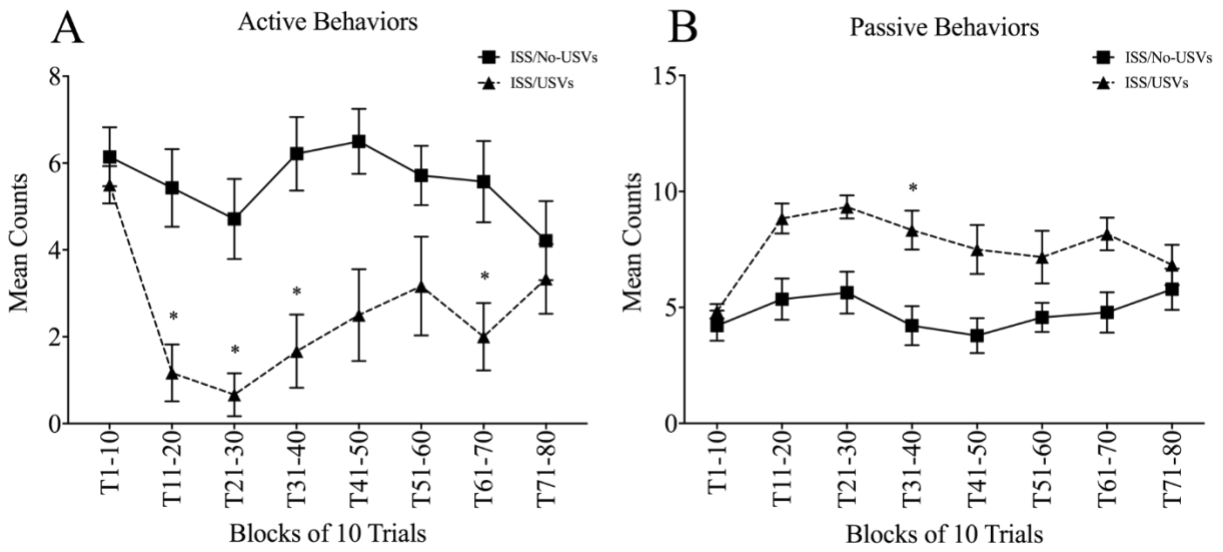

Figure 4. Mean (± SEM) active behaviors (A) and passive behaviors (B) during ISS aggregated into 8 blocks of 10 trials. * indicates significantly different from ISS/No-USVs group ($p < 0.05$).

Core Body Temperature

Baseline measurements indicated all rats had equivalent core body temperature prior to manipulation as there was no effect of treatment [F(4,36) = 0.601, $p = 0.664$], time [F(5,180) = 1.586, $p = 0.166$], however the treatment x time interaction was significant [F(20,180) = 2.11, $p = 0.005$], which reflected a change due to the end of the dark cycle.

Temperature fluctuated during the social exploration pretest (Figure 5A) as a significant effect to time was observed [F(12,432) = 43.42, $p < 0.001$]. Importantly, there was no effect of treatment [F(4,36) = 0.600, $p = 0.665$], indicating rats in each experimental group were equivalent on pre-stress social exploration. In addition, the interaction was significant [F(48,432) = 1.52, $p = 0.017$]. Within subjects contrasts revealed a mild core body temperature

increase within the first 20 min of the acclimation and again 20 min after the social exploration test (all *p's* < 0.05). Body temperature during the social exploration posttest also fluctuated, indicated by a significant effect of time [F(15,525) = 33.17, $p < 0.001$]. Within subjects contrasts revealed mild core body temperature increase in the first 40 min of the acclimation and again 30 min after the social exploration test (all *p's* < 0.05). There was no effect of treatment on core body temperature during the posttest [F(4,35) = 0.372, $p = 0.826$], and the treatment x time interaction was nonsignificant [F(60,525) = 0.735, $p = 0.931$] (Figure 5B).

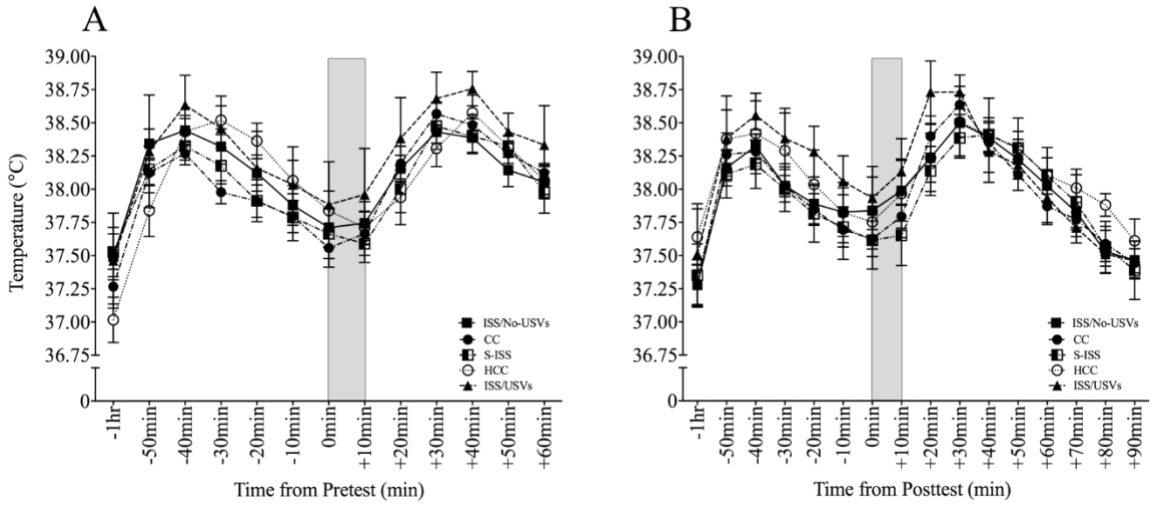

Figure 5. Mean (± SEM) core body temperature during the social exploration test. Measurements were collected during the 1 h acclimation period (-1hr to 0min) and 1 h post-testing (+10min to +60min). Gray highlights indicate the testing period.

During ISS, a significant effect of treatment [F(4,36) = 114, $p < 0.001$], time [F(30,1080) = 138.9, $p < 0.001$] and treatment x time interaction [F(120,1080) = 88.96, $p < 0.001$] was observed. There was no significant difference in body temperature until the onset of ISS, confined conspecific, or sham-ISS exposure. Planned comparisons were conducted to compare the following: change in temperature between the two ISS conditions and all control conditions, temperature differences between the ISS/USV and ISS/No-USV group, and temperature changes in confined conspecific and sham-ISS relative to each other and the home cage control.

Compared with the home cage control condition, the confined conspecific condition resulted in significant decrease of core body temperature for the first 40 trials, (0-40min) and again at the end of the 80 min session (all *p*'s < 0.05). Sham-ISS was not significantly different from confined conspecific or home cage control. ISS resulted in significant decrease of core body temperature for both vocalizing and non-vocalizing rats compared with all control groups from 10 min to 180 min after the onset of swim stress (Figure 6). Non-vocalizing ISS rat exhibited a further decrease in core body temperature compared to vocalizing rats at 60 min and sustained until 120 min after the onset of ISS (all *p's* < 0.05).

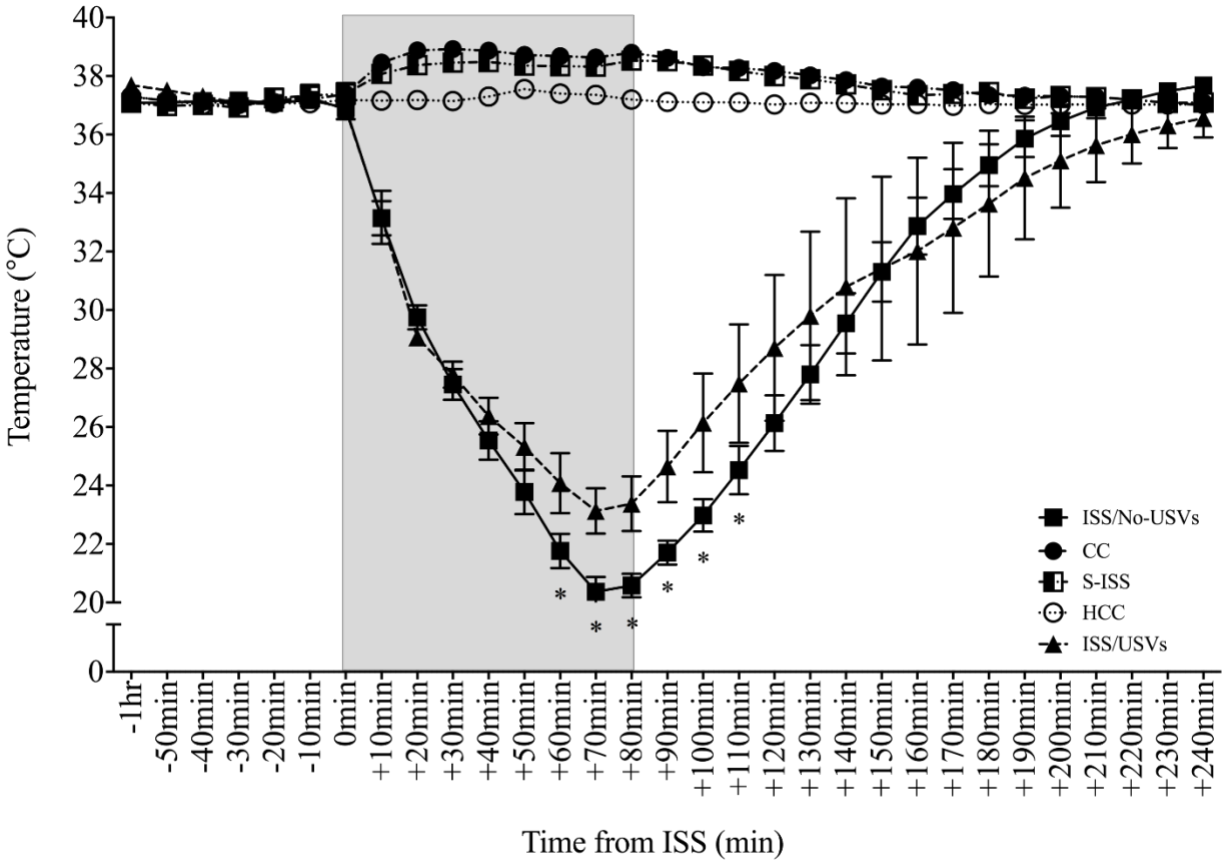

Figure 6. Mean (± SEM) core body temperature 1 hr prior to ISS, CC, S-ISS, or equivalent HCC time point until 240min post-ISS. Gray highlights indicate the period of ISS exposure. *indicates significantly different from ISS/USVs ($p < 0.05$).

Furthermore, the non-vocalizing group exhibited a significant core body temperature rebound and increase compared with all other groups at 250 min that sustained until 290 min after the onset of ISS (Figure 7) (all *p's* < 0.05). There were no differences in weight on the day of ISS between vocalizing and non-vocalizing rats [$t(18) = 0.428$, $p = 0.673$, Mean Diff = -5.071 ± 11.84].

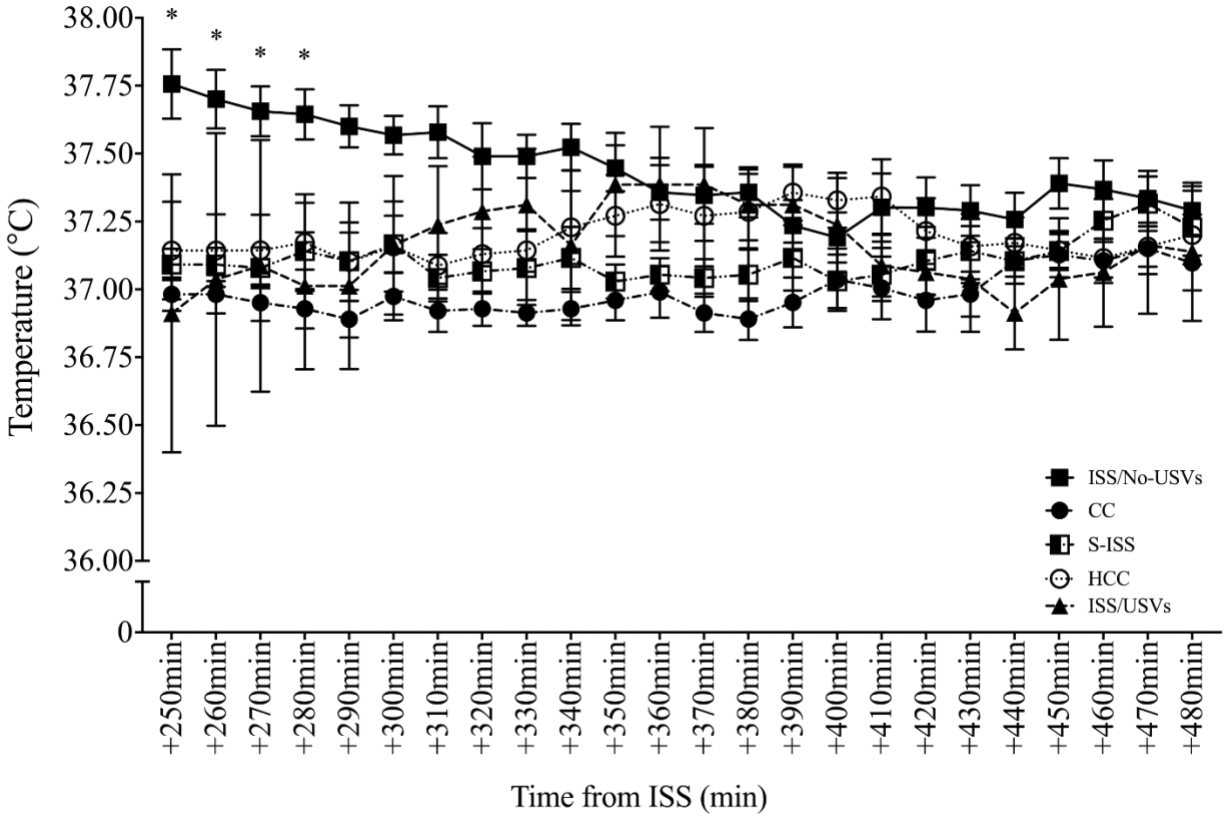

Figure 7. Mean (± SEM) core body temperature 250min to 480min following ISS, CC, S-ISS, HCC. *indicates significantly different from ISS/USVs ($p < 0.05$).

Discussion

Experiment 1 investigated the reliability of USVs to predict stress resilience and further explored the behavioral strategy employed by vocalizing rats during ISS that may contribute to resilience. Experiment 1 replicated the previous behavioral findings from our laboratory (Stafford et al., 2015) that rats not emitting USVs exhibited social anxiety indicated by a

reduction in social exploration baseline, while the USV-emitting rats exhibited an anxiolytic phenotype. Furthermore, this experiment provided initial insight into a preferential coping style for vocalizing rats during ISS. Vocalizing rats engaged in a behavioral strategy during ISS that was counter to hypothesized, and overall consisted of fewer active behaviors than non-vocalizing rats. Statistically significant decrease in core body temperature from baseline (i.e., hypothermia) observed during ISS was greater in non-vocalizing rats, and the reduction in core body temperature may reflect heat dissipation due to activity.

Social Anxiety

The first three experiments from our laboratory investigating the phenomenon of stress resilience associated with USVs utilized both a water based stressor and posttest (Drugan et al., 2009, 2014), and the consistent findings demonstrated the effect is reliable in water-based tests. A subsequent experiment demonstrated the transituational nature of these effects by finding USVs also predicted resilience to ISS-induced deficits in the absence of water during a social anxiety test (Stafford et al., 2015). The present study, by replicating the social anxiety experiment, establishes USVs emitted during ISS are a reliable predictor of resilience to both behavioral depression and social anxiety induced by this particular model of swim stress in rats.

Coping Strategy

Experiment 1 is the first to demonstrate distinctive coping styles during ISS, specifically comparing rats that do and do not emit USVs. We hypothesized that vocalizing rats would engage in similar active behaviors demonstrated by others to protect against the negative consequences of an inescapable stressor. The behavioral results were contrary to our hypothesis as we found vocalizing rats engaged in less active behaviors throughout the stress session compared with non-vocalizing rats. There were no differences in the social exploration pretest

that suggested either group is predisposed to a particular activity phenotype. The changes revealed during the post-stress social exploration were therefore likely a direct result of ISS experience, which appears to be fundamentally different between vocalizing and non-vocalizing rats.

The core body temperature measurements revealed rats that did not emit USVs exhibited greater hypothermia than those that did vocalize. Importantly, there were no obvious group weight differences between vocalizing and non-vocalizing rats to explain body temperature loss. The greater activity of the non-vocalizing rats is likely to have contributed to this hypothermia, while immobility in vocalizing rats preserved body temperature. In a previous experiment, rats identified as resilient to ISS exhibited a nonsignificant mild hypothermia compared with vulnerable rats (Levay et al., 2006), rather than the relative increase in core body temperature above baseline (i.e., hyperthermia) found in the present experiment. It is not clear why this discrepancy occurred, however, rats were classified based on post-ISS instrumental learning and not USV emission during the initial stressor. The arbitrary classification and the high degree of variability observed in learning may not have captured discrete groups as was found in the present experiment, thus the temperature effects did not adequately reflect innate resilient/vulnerable characteristics.

It is possible that a passive strategy is more beneficial to cope with an initial intermittent cold-water swim as a stressor. Reduced activity would preserve core body temperature and serve as an adaptive strategy to avoid succumbing to hypothermia, and prolonged swimming leads to increased immobility as a survival mechanism (Nishimura et al., 1988; Pintér, Domokos, Mergl, Mikics, & Zelena, 2011). The bimodal distribution of active or passive behavioral strategies did not appear immediately, rather differences emerged after the first 10 trials, and persisted for 30

trials. Thus, the change in behavior may reflect a shift in strategy in the vocalizing rats, whereas the non-vocalizing rats maintain a consistent strategy. Shifting from active to passive is considered adaptive in the forced swim test after repeated or prolonged exposure as it reduces energy expenditure, increasing chances of survival in an inescapable situation (Commons et al., 2017; De Kloet & Molendijk, 2016; De Pablo, Parra, Segovia, & Guillamón, 1989; Nishimura et al., 1988).

The passive behavior and core body temperature during ISS is consistent with activity of the mesolimbic cholinergic pathway that produces 22-kHz USVs (Brudzynski, 2013). This pathway innervates the anterior-preoptic region responsible for thermoregulation (Blumberg & Moltz, 1987; Boulant, 2000), and results in passive avoidant and immobile postures due to dorsomedial hypothalamic activation (Brudzynski & Chiu, 1995; Brudzynski, Iku, & Harness neé Savoy, 2011). However, the dorsomedial hypothalamic target of this cholinergic pathway results in avoidant behaviors as an anxiety response (Hakvoort Schwerdtfeger & Menard, 2008; Hassell et al., 2017). The lack of post-ISS anxiety is not consistent with avoidant behaviors produced by dorsomedial hypothalamus. Further investigation is needed to uncover which pathways are activated during ISS, with particular attention to the serotonergic circuitry involved in coping, to produce these active or passive behaviors.

Potential Mechanisms

Serotonergic neurons in dorsal raphe are one possible mechanism that may drive active or passive behaviors during ISS. Three candidate subregions may play a role in generating ISS-induced active or passive behaviors. The mid-rostral dorsal subregion, caudal subregion, and ventrolateral wings of dorsal raphe are implicated in the production of depressive and anxiety-like behaviors, as well as active vs. passive coping strategies during inescapable stress (Amat et

al., 2008, 2006; Commons, Connolley, & Valentino, 2003; Paul et al., 2011; Paul, Johnson, Shekhar, & Lowry, 2014; Roche et al., 2003). Therefore, a follow-up experiment was conducted to investigate the functional activation of serotonergic neurons in dorsal, caudal, and ventrolateral subregions of dorsal raphe nucleus.

IV. EXPERIMENT TWO

Preferential coping strategies employed by an organism when exposed to a stressor may be adaptive or maladaptive. Organisms that engage in active coping strategies to mitigate the negative consequences of stress exposure are considered to confer stress resilience. One of the mechanisms that controls the development of active or passive behaviors is a cortico-limbic serotonergic circuit originating in dorsal raphe nucleus (Puglisi-Allegra & Andolina, 2015). Reduced serotonergic activation is associated with the development of active coping strategies via local inhibitory GABAergic neurons.

The present understanding of the neurobiology associated with ISS is limited to indirect pharmacological manipulation (Christianson, Rabbett, Lyckland, & Drugan, 2008; Drugan, MacOmber, & Warner, 2010; Warner et al., 2013; Warner & Drugan, 2012) and assessment of post-stress dorsal raphe functioning (Stafford et al., *in prep*). For example, post-ISS increases in forced swim immobility are reversed by peripherally administrated noradrenergic reuptake inhibitors (Drugan et al., 2010), while peripheral administration of serotonergic reuptake inhibitors administrated prior to or following ISS, or disruption of serotonin synthesis prior to ISS, have no effect (Christianson, Rabbett, et al., 2008). However, without assessment of centrally administered compounds, it is difficult to interpret the role of dorsal raphe in post-ISS behavioral effects. Unpublished evidence from our laboratory supports that caudal dorsal raphe is activated in post-stress vulnerability to ISS indicated by open field and social anxiety, as well as forced swim immobility (Stafford et al., *in prep*). These previous studies assessed behavior and serotonergic activation 24 h post-ISS and the role of dorsal raphe in active or passive behavioral strategies exhibited during the initial ISS session is unknown. Thus, experiment 2

was conducted to investigate the potential role of dorsal raphe serotonergic and GABAergic neurons in the development of active or passive behavioral strategies during ISS.

Methods

Procedure

A total of 70 adult rats were used in this experiment consisting of ISS (ISS/USVs, n=7; ISS/No-USVs, n=16), CC (n=23), S-ISS (n=12), or HCC (n=12). The timeline for experiment 2 is depicted in Figure 8. Experiment 2 was identical to experiment 1 except that rats were transcardially perfused (see below) 90 min post-ISS or control condition. A subset of rats received datalogger implantation (ISS/USVs, n=4; ISS/No-USVs, n=8; CC, n=13; S-ISS, n=8; HCC, n=8). Temperature data from one HCC rat was lost due to datalogger malfunction.

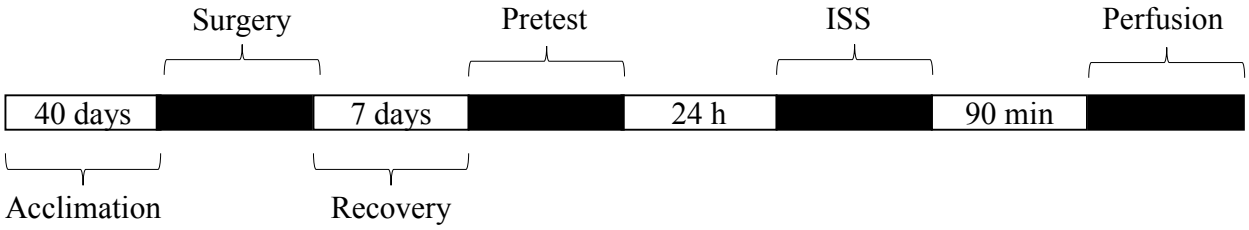

Figure 8. Timeline for experiment 2.

Tissue Extraction.

Rats were anesthetized with a ketamine/xylazine combination (100/10 mg/kg) and transcardially perfused with ~100ml ice cold 0.9% saline followed by ~75ml ice cold 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde (24 h), phosphate buffered saline (24 h), and 30% sucrose (72 h). Tissue was stored at -80°C until sectioned on a cryostat (Leica model 1950, Leica Biosystems, Nußloch, DE) at 30µm.

Antibodies

Immunodetection of the protein product of the immediate early gene cFos was completed using an affinity purified polyclonal rabbit anti-cFos antibody (1:5,000, Cat. No. ABE-457, Lot

No. 2905394, Millipore Sigma, Burlington, MA, USA). For immunodetection of tryptophan hydroxylase (TPH), an affinity isolated polyclonal sheep anti-TPH antibody raised against recombinant rabbit TPH (1:2,500, Cat. No. T-8575, Lot No. SLBN2143V, Millipore Sigma, Burlington, MA, USA). Immunodetection of GABA was achieved using a purified monoclonal antibody against the cytoplasmic 67 kDa isoform of glutamic-acid decarboxylase (GAD67; the biosynthetic enzyme for GABA synthesis from glutamic acid) raised against recombinant GAD67 (1:1,000, Cat. No. MAB5406, Lot No. 2923238, Millipore Sigma, Burlington, MA, USA). Secondary antibodies were as follows: for cFos, biotinylated donkey anti rabbit (1:500, Jackson ImmunoResearch, Cat# 711-065-15); for TPH, a biotinylated sheep anti-rabbit (1:500, Vector ABC kit, Vector Laboratories, Burlingame, CA, USA); and for GAD67, a biotinylated donkey anti-mouse (1:500, Jackson ImmunoResearch, Cat# 711-065-151) were used.

Immunohistochemistry

All immunohistochemistry was conducted on free-floating sections in well plates containing 2 ml of solution. All washes were 15 min in duration, unless otherwise stated. For cFos-TPH double-immunohistochemistry, all washes and incubations occurred at 4°C. Prior to primary antibody incubation, tissue was washed 2x in 0.05M phosphate buffered saline (PBS) followed by 1% H_2O_2 to block for endogenous peroxidases. Tissue was washed again 2x in PBS and followed by PBS with 0.3% Triton X-100 (PBST). Tissue was incubated with primary antibody (24 h for cFos, 72 h for TPH) in PBS with 0.8% Triton X-100 with 4% normal serum and 0.1% bovine serum albumin.

Following primary antibody incubation, tissue was washed 2x in PBST followed by 90 min incubation in secondary antibody in PBST. Tissue was rinsed 2x in PBST and incubated for 90 min in an avidin-biotin complex (Vector ABC kit, Vector Laboratories, Burlingame, CA,

USA) to block for endogenous biotin, followed again by 2x wash in PBST. Finally, a horseradish peroxidase substrate reaction was used to visualize cells. Visualization of cFos was accomplished via horseradish peroxidase chromogen reaction (Vector SG Chromogen kit, Vector Laboratories, Burlingame, CA, USA) to produce a blue-gray colorization of nuclei. TPH was visualized accomplished via reaction with 3, 3 -diaminobenzidine (DAB) horseradish peroxidase (Vector ImmPACT DAB kit, Vector Laboratories, Burlingame, CA, USA) to produce a brown-red colorization of soma. Reactions were terminated by washing the tissue 3x in PBS for 15 min, after which tissue was dehydrated, cleared with xylenes, and mounted onto slides.

For cFos-GAD67 double-immunohistochemistry, the procedure was identical except all incubations and washes were conducted at 20°C and Triton X-100 was excluded, and the concentration of bovine serum albumin was increased to 3% in the GAD67 primary incubation solution. Adjacent sections to those used for cFos-TPH double-immunohistochemistry were used for visualization of GABA activation.

Cell Counting

Representative sections at 180μm intervals from -7.28mm to -9.26mm posterior to bregma were chosen for quantification of cFos and TPH immunoreactivity within dorsal raphe (Figure 9). Rostral-caudal level and subregion were confirmed against the rat stereotaxic atlas (Paxinos & Watson, 1998) and TPH-specific immunostaining atlas (Abrams et al., 2004). The subregions analyzed within dorsal raphe were dorsal and ventral (-7.28mm to -8.18mm; figure 8, A-F), ventrolateral (-7.64mm to -8.18mm; figure 8, D-F) caudal (-8.36mm to -9.26mm; figure 8, G-L), and interfasicular nucleus (-8.36mm to -8.72mm; figure, G-I). Comparable sections for dorsal and ventral (-7.28mm to -8.18mm) and caudal (-8.36mm to -8.90mm) subregions were analyzed for cFos and GAD67 immunoreactivity. All cell types were manually counted under

brightfield microscopy at 100x magnification with confirmation of double immunostaining at 400x magnification (see Figures 10-11 for photomicrographs).

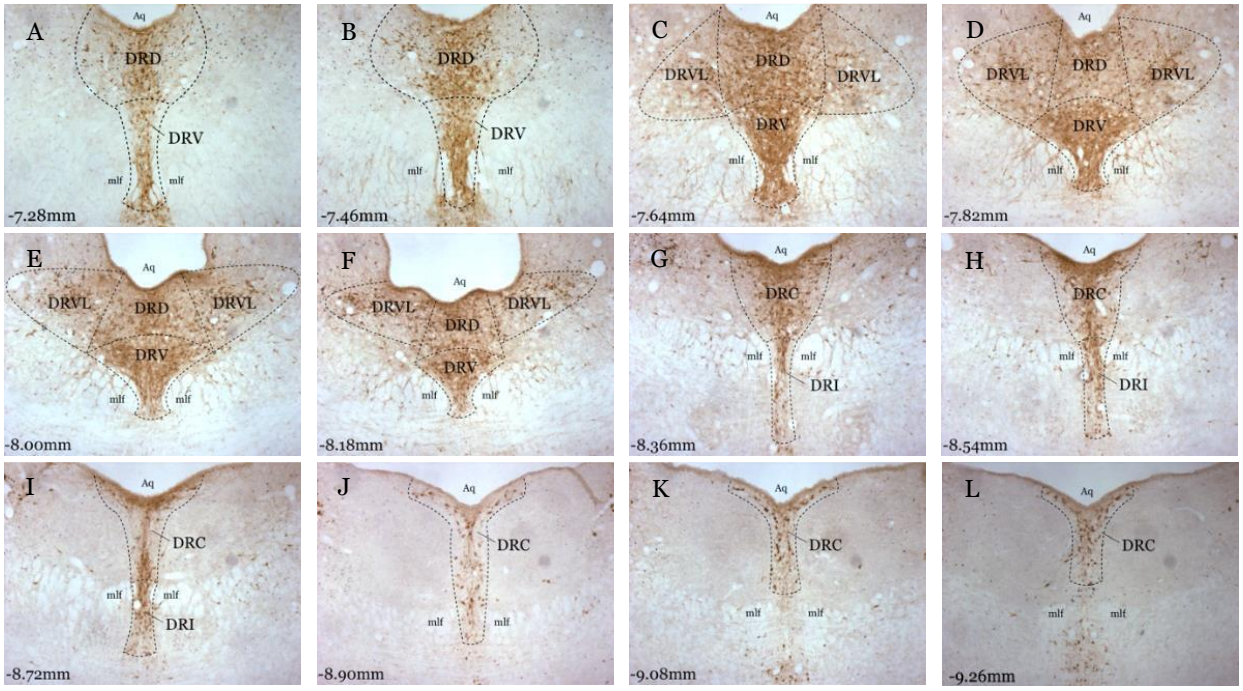

Figure 9. Photomicrographs of cFos-ir/TPH-ir immunostained sections demonstrating the 12 rostrocaudal levels chosen for analyses. Measurements are posterior to bregma. Abbreviations: DRD, dorsal raphe nucleus, dorsal part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral wings; DRC, dorsal raphe nucleus, caudal part; DRI, dorsal raphe nucleus, interfasicular part; mlf, medial longitudinal fasciculus.

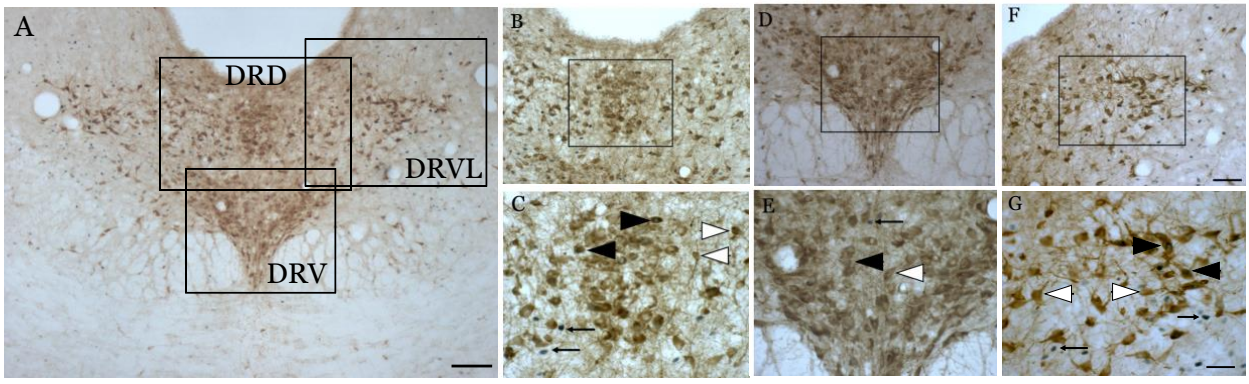

Figure 10. Photomicrographs of cFos/TPH immunostaining in the mid-rostral dorsal raphe representative of primary areas of analyses. Black arrows depict cFos-immunoreactive/TPH-immunonegative cells, white arrowheads depict cFos-immunonegative/TPH-immunoreactive cells, and black arrowheads depict cFos-immunoreactive/TPH-immunoreactive double-labeled cells. A: dorsal raphe -8.00mm posterior to bregma shown at 10x magnification (DRD, dorsal subregion; DRV, ventral subregion; DRVL, ventrolateral subregion). B, D, F: DRD, DRV, and

DRVL shown at 25x magnification. C, E, G: DRD, DRV, and DRVL shown at 50x magnification with representative cell types. Scale bar: 250µm (A), 100 µm (F), and 50µm (G).

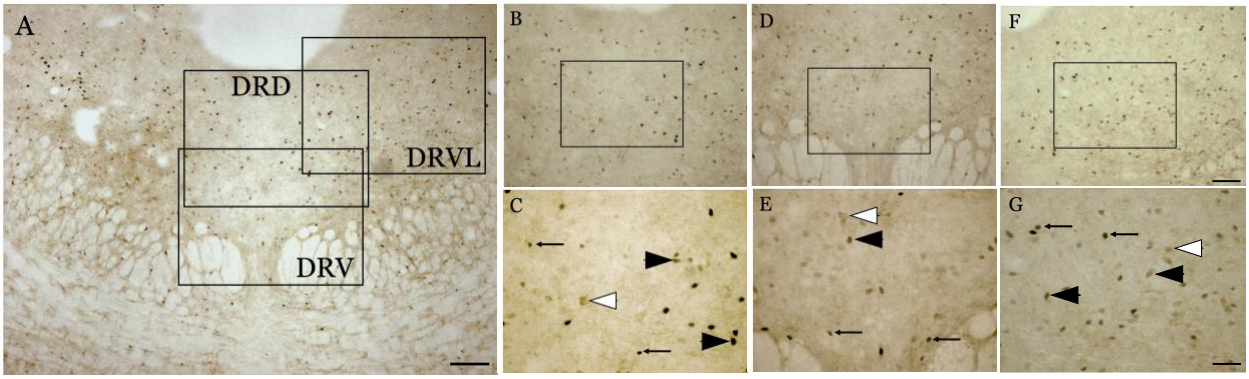

Figure 11. Photomicrographs of cFos/GAD67 immunostaining in the mid-rostral dorsal raphe representative of primary areas of analyses. Black arrows depict cFos-immunoreactive/GAD67-immunonegative cells, white arrowheads depict cFos-immunonegative/GAD67-immunoreactive cells, and black arrowheads depict cFos-immunoreactive/GAD67-immunoreactive double-labeled cells. A: dorsal raphe exemplar of -8.00mm posterior to bregma shown at 10x magnification (DRD, dorsal subregion; DRV, ventral subregion; DRVL, ventrolateral subregion). B, D, F: DRD, DRV, and DRVL shown at 25x magnification. C, E, G: DRD, DRV, and DRVL shown at 50x magnification with representative cell types. Scale bar: 250µm (A), 100 µm (F), and 50µm (G).

Statistical Analyses

Social exploration pretests, core body temperature, and ISS behavior were analyzed identical to experiment 1. Tables presenting pairwise comparisons of means for behavioral data are available in appendix B. Immunohistochemistry data for cFos-TPH, cFos, TPH, cFos-GAD67, and GAD67 at each subregion (dorsal, ventral, ventrolateral, caudal, and interfasicular) were tested separately using linear-mixed effects modeling with unstructured covariance structure. Treatment conditions were entered as fixed effects and stereotaxic level was treated as repeated measures in the model with interaction between treatment and stereotaxic level to test for significant treatment effects across stereotaxic levels sampled. Tables presenting estimated marginal means are available in appendices C and D.

Results

Behavioral Data

There was no effect of treatment (ISS/USVs, ISS/No-USVs, CC, S-ISS, or HCC) on social exploration pretest time [$F(4,68) = 0.6827$, $p = 0.6064$]. During ISS, there was a significant effect of treatment [$F(1,21) = 27.28$, $p < 0.001$], but not trial [$F(7,147) = 1.002$, $p = 0.432$] on active behaviors, while the treatment x trial interaction was significant [$F(7, 147) = 2.645$, $p = 0.013$]. Planned comparisons were conducted to assess differences in active or passive behaviors at each trial block. Vocalizing rats engaged in less active behaviors between blocks 2-7 (trials 11-70; all p's < 0.05). For passive behaviors, there was a significant effect of treatment [$F(1,21) = 25.71$, $p < 0.001$], a nonsignificant effect of trial [$F(7,147) = 0.701$, $p = 0.671$], while the interaction was significant [$F(7,147) = 3.15$, $p = 0.004$]. Vocalizing rats engaged in greater passive behaviors between trials 21-70 compared with non-vocalizing rats (Figure 12) (all p's < 0.05).

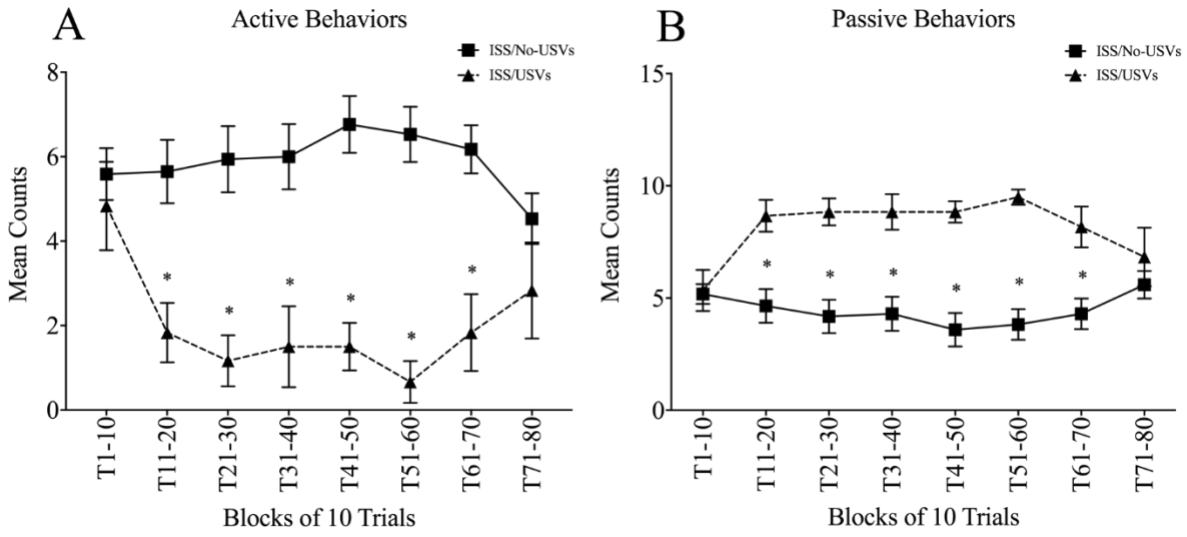

Figure 12. Mean (± SEM) active behaviors (A) and passive behaviors (B) during ISS aggregated into 8 blocks of 10 trials. * indicates significantly different from ISS/No-USVs group ($p < 0.05$).

Core Body Temperature

Baseline measurements indicated all rats had equivalent core body temperature prior to manipulation as there was no effect of treatment [$F(4,39) = 0.159$, $p = 0.958$], however the effect of time was significant [$F(5,195) = 3.16$, $p = 0.009$]. The treatment x time interaction was nonsignificant [$F(20,195) = 0.837$, $p = 0.667$]. There was no effect of treatment on temperature during social exploration pretest [$F(4,39) = 0.234$, $p = 0.917$]. The effect of time was significant [$F(12,468) = 25$, $p < 0.001$], while the interaction of treatment x time was nonsignificant [$F(48,468) = 0.9787$, $p = 0.517$]. The effect of time was furthered explored via within subjects contrasts that revealed hyperthermia after the start of the acclimation period for 30 min and again after the testing for 60 min (all p's < 0.05). For core body temperature during ISS, there was a significant effect of treatment [$F(4,40) = 150.4$, $p < 0.001$], time [$F(22,880) = 126.3$, $p < 0.001$], and treatment x time interaction [$F(88,880) = 107$, $p < 0.001$]. Planned comparisons compared the following: change in temperature between the two ISS conditions and all control conditions, temperature differences between the ISS/USV and ISS/No-USV group, and temperature changes in confined conspecific and sham-ISS relative to each other and the home cage control. Both vocalizing and non-vocalizing rats exhibited significant hypothermia compared to all control groups within 10 minutes, and ISS-induced hypothermia was maintained until the end of sampling immediately prior to perfusions (all p's < 0.05). Non-vocalizing rats exhibited greater hypothermia than vocalizing rats from 30 min after the start of ISS until the end of sampling just prior to perfusion (Figure 13) (all p's < 0.05). There were no differences in weight on the day of ISS between vocalizing and non-vocalizing rats [$t(18) = 0.412$, $p = 0.684$, Mean Diff = 6.029 ± 14.63].

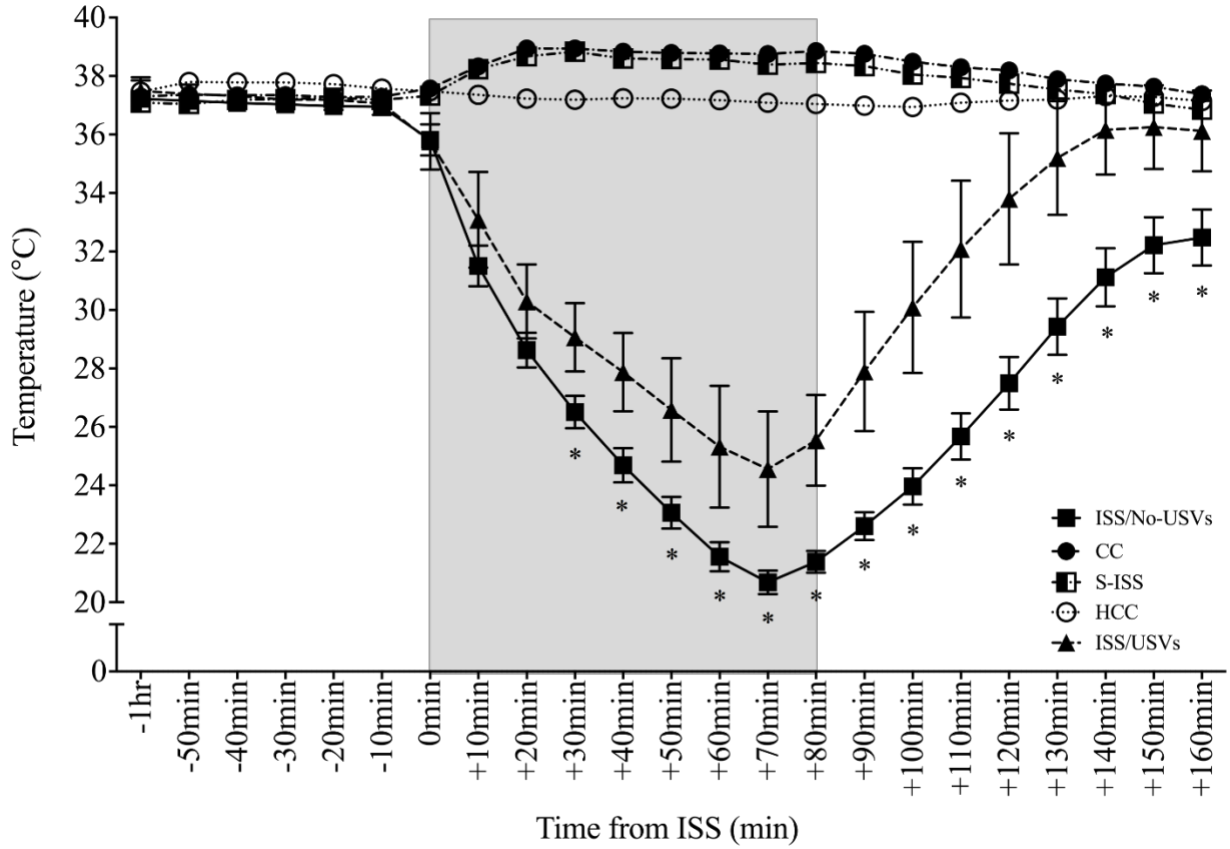

Figure 13. Mean (± SEM) core body temperature 1 hr prior to ISS, CC, S-ISS, or equivalent HCC time point until 160min post-ISS. Gray highlights indicate the period of ISS exposure. *indicates significantly different from ISS/USVs ($p < 0.05$).

Cell Count Data

cFos-immunoreactive/TPH-immunoreactive

Dorsal subregion: For the dorsal subregion, there was a significant effect of treatment [$F(4,57.872) = 19.049$, $p < 0.001$], stereotaxic level [$F(5,43.925) = 11.640$, $p < 0.001$], and treatment x level interaction [$F(20,44.455) = 2.520$, $p = 0.005$]. Pairwise comparisons for the main effect of treatment indicated that overall ISS/USVs had significantly greater double-labeled cells than all other groups ($p < 0.001$). The ISS/No-USVs, confined conspecific, sham-ISS, and home cage controls were equivalent. Pairwise comparisons for the main effect of level indicated

that overall there was variability of serotonergic cell activation across the different levels of bregma sampled as the total number of cells changes across the rostral-caudal axis.

Pairwise comparisons for the treatment x level interaction indicated the effect of treatment varied at specific stereotaxic levels. There were significantly greater double-labeled cells in the ISS/USVs group compared with all other groups at -7.28mm ($p < 0.001$), -7.82mm ($p < 0.001$), -8.00mm ($p < 0.001$), and -8.18mm ($p < 0.001$). There were no other significant differences between groups at any level of bregma, with the exception of increased double-labeled cells in the ISS/USVs group compared with S-ISS ($p = 0.010$) and HCC ($p = 0.002$) conditions at -7.46mm (Figure 14).

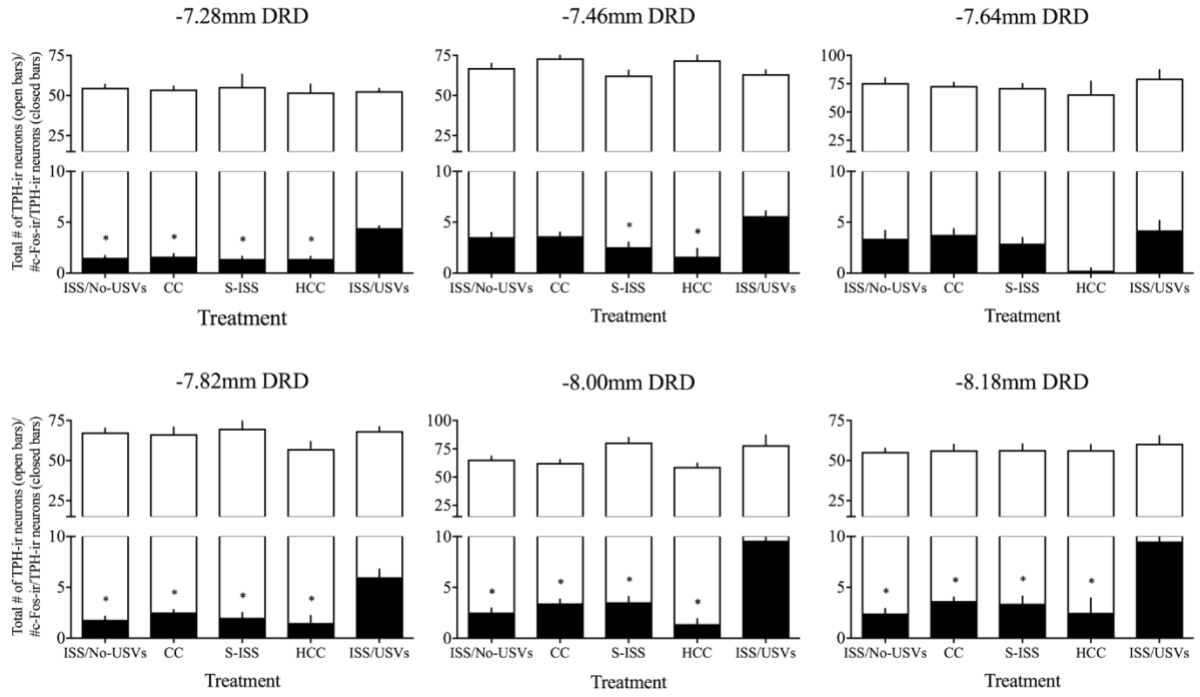

Figure 14. Mean (± SEM) cFos-immunoreactive/TPH-immunoreactive (closed bars) and TPH-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of dorsal subregion (DRD). * indicates significantly different from ISS/USVs group ($p < 0.05$).

Ventral subregion: Within the ventral subregion there was also a significant effect of treatment [$F(4,56.271) = 6.472$, $p < 0.001$], stereotaxic level [$F(5,38.871) = 3.360$, $p = 0.013$], and treatment x level interaction [$F(20,42.344) = 2.061$, $p = 0.024$]. Pairwise comparisons for

the main effect of treatment indicated that like the dorsal region, there were overall significantly greater double-labeled cells in the ISS/USV group than the ISS/No-USV and confined groups (p = 0.003), the sham-ISS group (p = 0.001), and the home cage control group (p <0.001). Pairwise comparisons for the main effect of level indicated again that serotonergic cell activation varied across the different levels of bregma sampled.

Pairwise comparisons for the treatment x level interaction indicated the effect of treatment varied depending upon stereotaxic level. Specifically, there were significantly greater double-labeled cells in the ISS/USVs group compared all other groups at -7.82mm (p < 0.001), -8.00mm (p < 0.001), and -8.18mm (p < 0.001). There were no other significant differences between groups at any level of bregma (Figure 15).

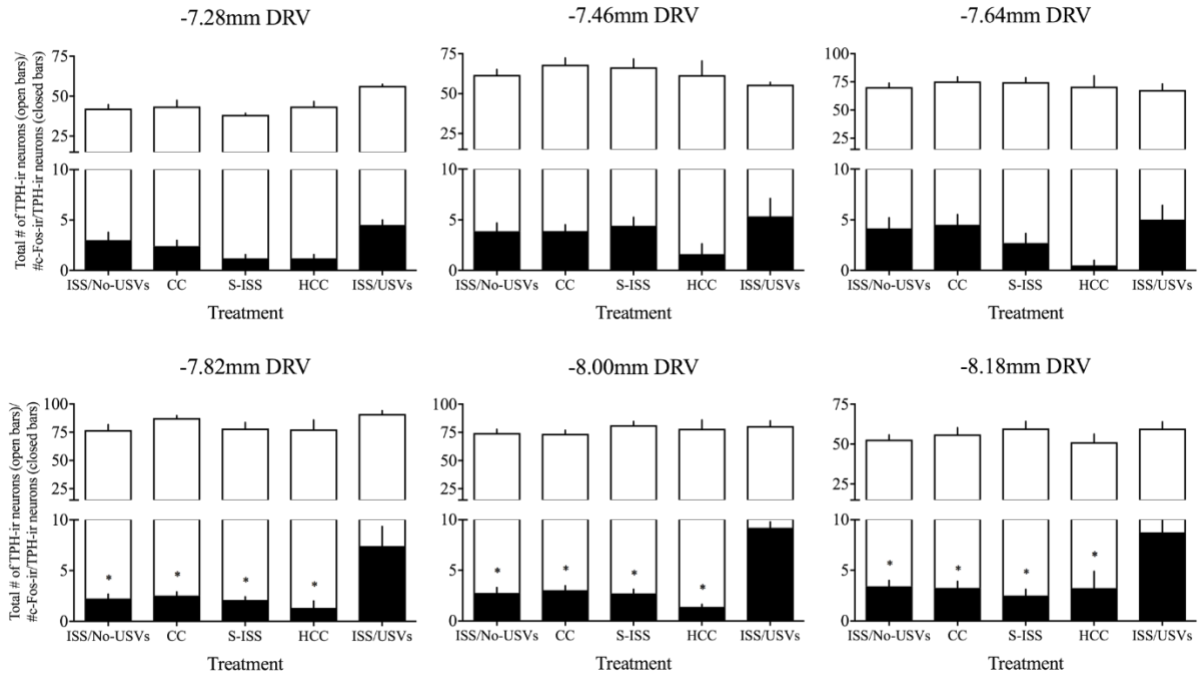

Figure 15. Mean (± SEM) cFos-immunoreactive/TPH-immunoreactive (closed bars) and TPH-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of ventral subregion (DRV). * indicates significantly different from ISS/USVs group (p < 0.05).

Ventrolateral subregion: At the ventrolateral "wings" a significant effect of treatment [$F(4,54.837) = 14.265$, $p < 0.001$] was observed, while the effect of stereotaxic level was nonsignificant [$F(3,46.103) = 2.105$, $p = 0.113$]. The treatment x level interaction was significant [$F(12,51.654) = 1.975$, $p = 0.005$]. Pairwise comparisons for the main effect of treatment indicated like dorsal and ventral subregions there were overall significantly greater double-labeled cells in the ISS/USV group than all other groups ($p < 0.001$).

Pairwise comparisons for the treatment x level interaction indicated the effect of treatment varied depending upon stereotaxic level. At -7.64mm, there were significantly greater double-labeled cells in the ISS/USVs group compared with confined conspecifics ($p < 0.001$), sham-ISS ($p = 0.001$), and home cage controls ($p = 0.001$). At -7.82mm, only the ISS/USVs group had greater double-labeled activity than home cage controls ($p = 0.036$). Furthermore, the ISS/USVs group had significantly greater activation than all other groups at -8.00mm ($p < 0.001$) and -8.18mm ($p < 0.001$), while all other groups were equivalent (Figure 16).

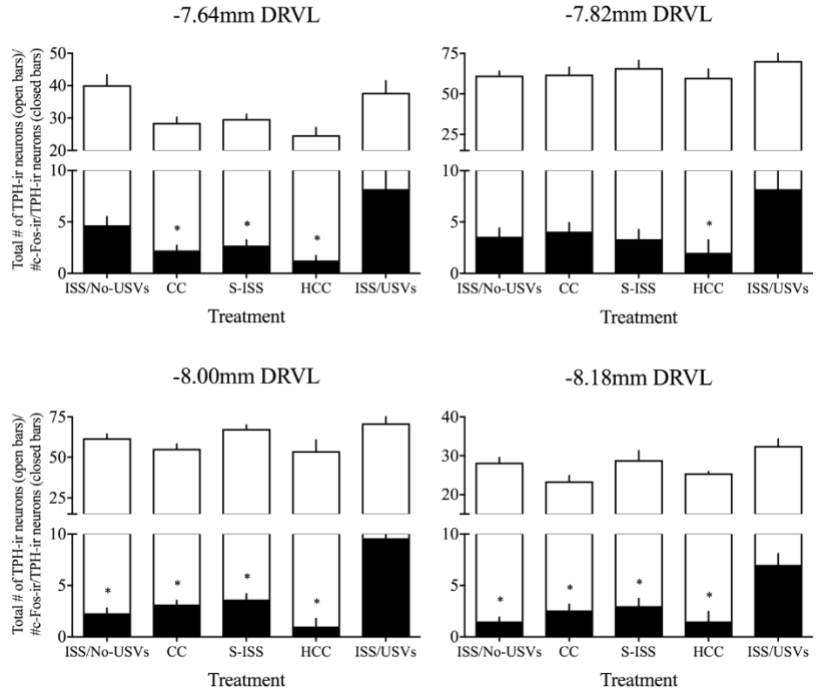

Figure 16. Mean (± SEM) cFos-immunoreactive/TPH-immunoreactive (closed bars) and TPH-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of ventrolateral subregion (DRVL). * indicates significantly different from ISS/USVs ($p < 0.05$).

Caudal subregion: Within caudal subregions, there was a significant effect of treatment [$F(4,53.033) = 5.037$, $p = 0.002$], stereotaxic level [$F(5,47.141) = 25.392$, $p < 0.001$], and treatment x level interaction [$F(20,49.238) = 3.790$, $p < 0.001$]. Pairwise comparisons for the main effect of treatment indicated that overall ISS/USVs had significantly greater double-labeled cells than HCC ($p = 0.001$). There were no differences between ISS/USVs and ISS/No-USVs, confined conspecifics, or sham-ISS. Pairwise comparisons for the main effect of level indicated that overall there was variability of serotonergic cell activation across the different levels of bregma sampled.

Pairwise comparisons for the treatment x level interaction indicated several levels with differences between ISS/No-USVs and ISS/USVs compared with control groups. At -8.36mm there were significantly greater double-labeled cells in the ISS/No-USVs compared with confined conspecifics ($p = 0.011$) and home cage controls ($p = 0.001$) groups; and, there were greater double-labeled cells in the ISS/USVs group compared with the confined conspecifics ($p = 0.008$), sham-ISS ($p = 0.025$), and home cage controls ($p = 0.001$). At -8.54mm there were significantly greater double-labeled cells in the ISS/No-USVs group compared with the confined conspecifics ($p = 0.001$), S-ISS ($p = 0.036$), and home cage controls groups ($p = 0.020$); and, there were greater double-labeled cells in the ISS/USVs group compared with the confined conspecifics ($p = 0.001$), sham-ISS ($p = 0.015$), and home cage controls ($p = 0.007$) (Figure 17).

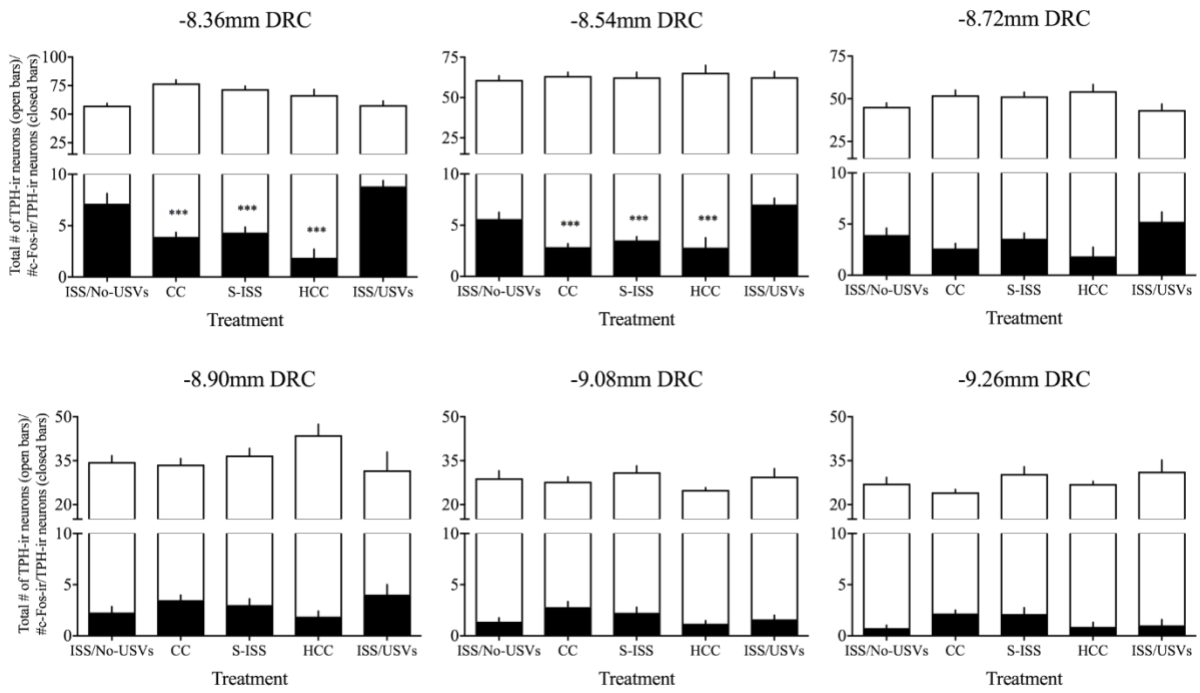

Figure 17. Mean (± SEM) cFos-immunoreactive/TPH-immunoreactive (closed bars) and TPH-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of caudal subregion (DRC). *** indicates significantly different from both ISS groups ($p < 0.05$).

Interfasicular nucleus: There was a significant effect of treatment [F(4,39.470)=3.884, p = 0.009], the effect of level was nonsignificant [F(2,47.012)=0.114, p = 0.892, while the treatment x level interaction was significant [F(8,47.178)=3.100, p 0.007]. Specifically, pairwise comparisons for the main effect of treatment indicated that overall the confined conspecifics group had greater double-labeled cells than the ISS/No-USV group (p = 0.006). Pairwise comparisons for the treatment x level interaction revealed the ISS/USV group had greater double-labeled cells at -8.36mm compared with ISS/No-USVs (p = 0.001) and the home cage controls group (p = 0.002). Furthermore, the confined conspecific group had significantly fewer double-labeled cells than the ISS/No-USVs (p = 0.003) and the ISS/USVs group (p = 0.033) at -8.72mm (Figure 18).

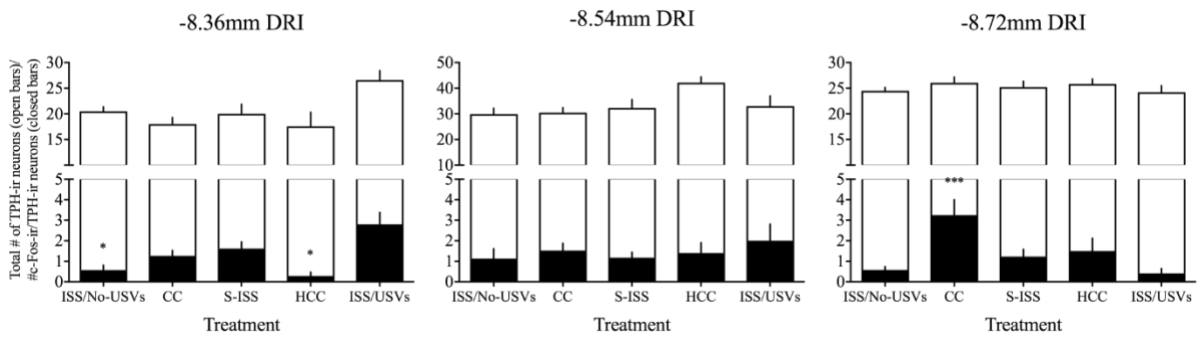

Figure 18. Mean (± SEM) cFos-immunoreactive/TPH-immunoreactive (closed bars) and TPH-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of interfasicular nucleus (DRI). * indicates significantly different from ISS/USVs group ($p < 0.05$), *** indicates significantly different from both ISS groups ($p < 0.05$).

cFos-immunoreactive/TPH-immunonegative

Dorsal subregion: There was a significant effect on cFos expression in nonserotonergic cells of treatment [$F(4,58.124)=17.736$, $p < 0.001$], stereotaxic level [$F(5,40.936)=31.182$, $p < 0.001$], and treatment x level interaction [$F(20,41.854)=3.195$, $p < 0.001$] on cFos expression in non-serotonergic cells within the dorsal subregion. Pairwise comparisons for the main effect of treatment indicated that overall ISS/USVs had greater cFos immunoreactive cells throughout the region than confined conspecifics, sham-ISS, and home cage controls ($p < 0.001$), while no different than ISS/No-USVs ($p = 0.998$). Furthermore, the control groups were equivalent to each other. Pairwise comparisons for the main effect of level indicated that overall there was variability of cFos immunoreactivity across the different levels of bregma sampled. Pairwise comparisons for the treatment x level interaction indicated that ISS/No-USVs had significantly greater cFos positive cells compared to confined conspecifics, sham-ISS, and home cage control groups at -7.28mm, -7.46mm, and -7.82 (all p's < 0.05). ISS/No-USVs had greater cFos immunoreactive cells at -8.00mm compared with sham-ISS and home cage control groups (all p's < 0.05). The ISS/USVs group had significantly greater cFos positive cells compared to

home cage controls at -7.28mm and confined conspecifics and home cage controls at -7.64mm (all p's < 0.05, Table 1).

Table 1. *cFos-immunoreactive cells within dorsal subregion.*

	Group									
	ISS/No-USVs		CC		S-ISS		HCC		ISS/USVs	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
-7.28mm	23.42	3.42	11.00**	1.40	9.00**	2.10	4.80***	1.16	22.50	1.94
-7.46mm	23.29	2.43	9.65**	1.00	11.33**	0.96	6.20**	1.56	16.60	2.98
-7.64mm	10.54	2.06	6.22*	0.67	6.20	0.96	4.25	1.55	13.00	2.07
-7.82mm	7.69	1.03	4.00**	0.68	3.22**	0.60	1.67**	0.49	6.60	1.21
-8.00mm	6.29	0.96	4.00	0.70	2.56**	0.44	1.40**	0.40	5.60	0.93
-8.18mm	6.67	1.52	3.71	0.78	4.25	0.70	2.25	1.31	8.50	1.55

* indicates significantly different from ISS/USVs within the same stereotaxic level, ** indicates significantly different from ISS/No-USVs within the same stereotaxic level. *** indicates significantly different from both ISS groups within the same stereotaxic level.

Ventral subregion: For the ventral subregion, there was a significant effect on cFos expression in nonserotonergic cells of treatment [$F(4,60.031)=10.462$, $p < 0.001$], and stereotaxic level [$F(5,43.017)=18.122$, $p < 0.001$], while the treatment x level interaction was nonsignificant [$F(20,43.113)=0.743$, $p = 0.760$]. Pairwise comparisons for the main effect of treatment indicated that overall ISS/No-USVs had greater cFos immunoreactive cells throughout the region than confined conspecifics ($p = 0.018$) and home cage controls ($p < 0.001$). ISS/USVs had greater cFos immunoreactivity compared with confined conspecifics ($p = 0.001$), sham-ISS ($p = 0.013$), and home cage controls ($p < 0.001$). Furthermore, the sham-ISS condition resulted in greater cFos positive nonserotonergic cells compared with home cage controls ($p = 0.030$). Pairwise comparisons for the main effect of level indicated that overall there was variability of cFos immunoreactivity across the different levels of bregma sampled (Table 2).

Table 2. *cFos-immunoreactive cells within ventral subregion.*

	Group									
	ISS/No-USVs		CC		S-ISS		HCC		ISS/USVs	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
-7.28mm	4.50	0.65	2.40	0.34	2.00	0.55	1.17	0.17	5.25	0.48
-7.46mm	8.64	1.09	6.19	1.07	8.56	1.21	4.00	0.89	11.00	0.58
-7.64mm	7.15	1.25	3.75	0.58	4.30	0.79	1.25	0.95	7.80	0.86
-7.82mm	3.56	0.67	2.40	0.46	2.00	0.24	1.17	0.48	5.80	1.24
-8.00mm	5.32	0.83	3.61	0.66	3.73	0.73	1.40	0.51	6.40	0.51
-8.18mm	5.38	0.92	3.67	0.76	5.10	0.80	1.00	0.71	6.75	1.38

Note: the treatment x level interaction was nonsignificant.

Ventrolateral subregion: There was a significant effect of treatment [$F(4,21.970) = 23.195$, $p < 0.001$] on cFos expression in nonserotonergic cells within the ventrolateral subregion. There was a nonsignificant effect of stereotaxic level [$F(3,3.351) = 2.714$, $p = 0.202$] and treatment x level interaction [$F(12,3.312) = 2.950$, $p = 0.185$]. Pairwise comparisons for treatment indicated that the ISS/No-USVs group had greater cFos expression than all control groups ($p < 0.001$). The sham-ISS group had significantly more cFos positive cells than the home cage control group ($p = 0.041$). The ISS/USVs group exhibited more cFos cells than confined conspecifics ($p = 0.001$), sham-ISS ($p = 0.01$), and home cage controls ($p < 0.001$) (Table 3).

Table 3. *cFos-immunoreactive cells within ventrolateral subregion.*

	Group									
	ISS/No-USVs		CC		S-ISS		HCC		ISS/USVs	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
-7.64mm	26.62	3.19	17.50	1.37	19.60	1.20	8.25	1.18	25.80	3.54
-7.82mm	28.27	1.77	18.30	1.21	19.82	0.99	10.33	1.23	28.50	3.66
-8.00mm	29.36	3.83	17.78	1.57	18.78	0.91	7.60	1.21	39.00	5.28
-8.18mm	29.40	3.43	18.57	2.85	19.67	1.14	15.67	3.28	28.80	2.13

Note: the treatment x level interaction was nonsignificant

Caudal subregion: There was a significant effect on cFos expression in nonserotonergic cells of treatment [F(4,58.312)=13.629, $p < 0.001$], and stereotaxic level [F(5,46.229)=3.859, $p = 0.005$] within the caudal subregion, while the treatment x level interaction was nonsignificant [F(20,47.118)=1.664, $p = 0.076$]. Pairwise comparisons for the main effect of treatment indicated that overall the ISS/No-USVs group had greater cFos immunoreactive cells throughout the region than confined conspecifics ($p < 0.001$), sham-ISS ($p = 0.013$), and home cage controls ($p < 0.001$). The ISS/USVs group had greater cFos immunoreactive cells than confined conspecifics, sham-ISS, and home cage control ($p < 0.001$). Pairwise comparisons for the main effect of level indicated that overall there was variability of cFos immunoreactivity across the different levels of bregma sampled (see Table 4).

Table 4. *cFos-immunoreactive cells within caudal subregion*

	Group									
	ISS/No-USVs		CC		S-ISS		HCC		ISS/USVs	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
-8.36mm	5.19	0.56	2.74	0.36	3.08	0.70	2.00	0.58	8.00	1.30
-8.54mm	7.80	1.50	2.17	0.39	3.15	0.66	1.60	0.40	10.00	1.22
-8.72mm	6.86	1.39	3.86	0.76	4.11	0.82	1.17	0.40	9.20	1.53
-8.90mm	7.36	1.38	3.19	0.65	5.13	0.93	3.71	1.06	7.67	1.33
-9.08mm	7.82	1.72	3.29	0.74	3.67	0.82	2.00	0.82	7.80	2.13
-9.26mm	7.64	1.32	3.18	0.68	4.50	0.82	4.00	0.62	12.33	3.18

Note: the treatment x level interaction was nonsignificant.

Interfasicular nucleus: There were no significaint effects of treatment [F(4,53.310)=1.015, $p = 0.408$], level [F(2,46.445)=0.724, $p = 0.490$], or treatment x level interaction [F(8,50.726)=0.807, $p = 0.600$] within the interfasicular nucleus (Table 5).

Table 5. *cFos-immunoreactive cells within interfasicular subregion*

	Group									
	ISS/No-USVs		CC		S-ISS		HCC		ISS/USVs	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
-8.36mm	1.44	0.43	1.33	0.27	2.46	0.55	0.86	0.26	3.60	1.08
-8.54mm	2.40	0.62	1.94	0.48	2.67	0.75	3.00	1.00	2.00	0.82
-8.72mm	1.64	0.53	2.13	0.46	2.09	0.51	1.83	0.54	3.40	0.51

Note: all main effects were nonsignificant.

TPH-immunoreactive/cFos-immunonegative

Dorsal subregion: For dorsal subregion, the effect of treatment was nonsignificant [$F(4,47.322)=0.168$, $p = 0.954$], while there was a significant effect of level [$F(5,43.464)=13.594$, $p < 0.001$] and interaction of treatment x level [$F(20,45.253)=2.004$, $p = 0.027$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of TPH immunoreactivity across the different levels of bregma sampled. Although there was an interaction effect, there were no significant differences between specific groups at a specific stereotaxic level after controlling for family-wise error (see Figure 14).

Ventral subregion: The effect of treatment was nonsignificant [$F(4,50.969) = 0.524$, $p = 0.718$], while there was a significant effect of level [$F(5,31.694) = 42.$, $p < 0.001$] and interaction of treatment x level was nonsignificant [$F(20,35.303) = 1.171$, $p = 0.332$] within the ventral subregion. Pairwise comparisons of the effect of level simply revealed a change in the number of cells depending upon stereotaxic level sampled (see Figure 15).

Ventrolateral subregion: There was a significant effect of treatment [$F(4,53.360) = 2778$, $p = 0.036$] and level [$F(3,50.156) = 131.817$, $p < 0.001$], while the interaction of treatment x level was nonsignificant [$F(12,49.854) = 0.894$, $p = 0.559$]. After correction for family wise error, there were no significant differences between treatment conditions, and pairwise

comparisons for the effect of level indicated that total serotonin cells varied across stereotaxic level sampled (see Figure 16).

Caudal subregion: The effect of treatment was nonsignificant [$F(4,51.428) = 2.483$, $p = 0.055$], while there was a significant effect of level [$F(5,39.081) = 105.722$, $p < 0.001$] and interaction of treatment x level [$F(20,42.851) = 4.520$, $p < 0.001$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of TPH immunoreactivity across the different levels of bregma sampled. Pairwise comparisons for the interaction effect revealed the number of TPH immunoreactive cells at -8.36mm were fewer in the ISS/No-USVs group compared with the CC ($p < 0.001$) and the S-ISS group ($p = 0.001$) (Figure 17.

Interfasicular nucleus: The effect of treatment was nonsignificant [$F(4,53.254) = 1.895$, $p = 0.125$], while the effect of level was significant [$F(2,53.233) = 31.646$, $p < 0.001$], and the treatment x level interaction was nonsignificant [$F(8,53.448) = 1.618$, $p = 0.142$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of TPH immunoreactivity across the different levels of bregma sampled (Figure 18).

cFos-immunoreactive/GAD67-immunoreactive

Dorsal subregion: There was no significant effect of treatment [$F(4,42.980) = 0.400$, $p = 0.807$], while the effect of stereotaxic level was significant [$F(5,42.380) = 13.195$, $p < 0.001$]. The treatment x level interaction was nonsignificant [$F(20,44.951) = 0.672$, $p = 0.832$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 19).

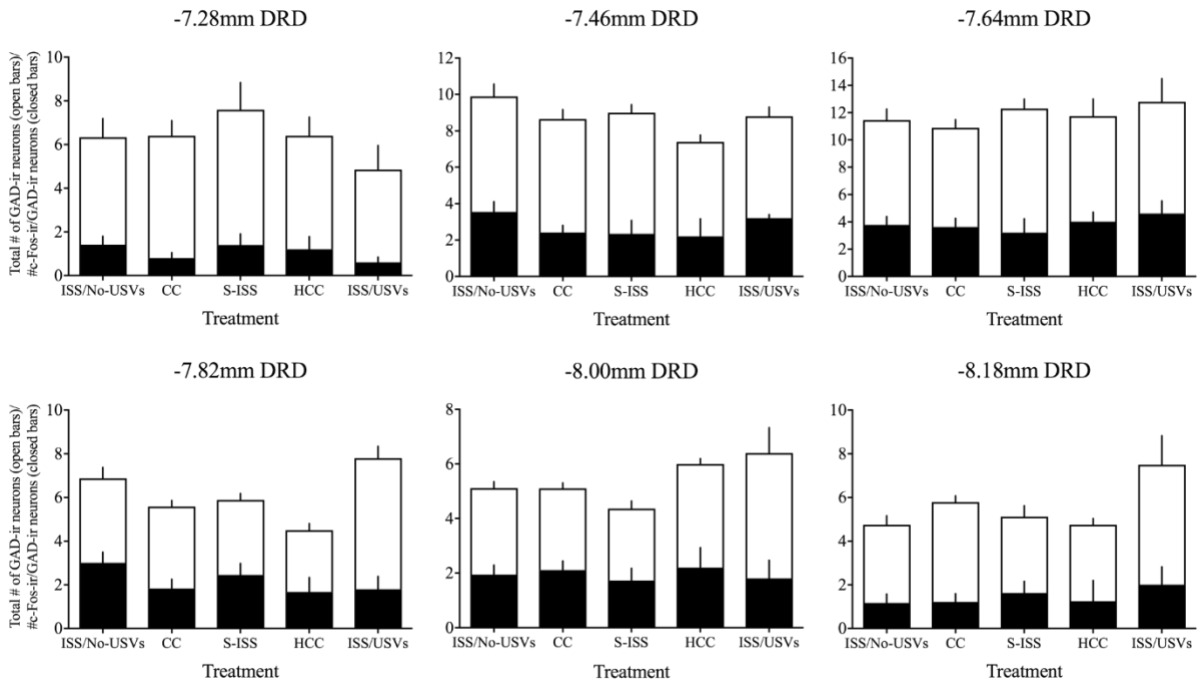

Figure 19: Mean (± SEM) cFos-immunoreactive/GAD67-immunoreactive (closed bars) and GAD67-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of dorsal subregion (DRD). There were no significant differences between groups.

Ventral subregion: There was no significant effect of treatment [$F(4,51.411) = 1.578$, $p = 0.194$], while the effect of stereotaxic level was significant [$F(5,46.810) = 5.294$, $p = 0.001$]. The treatment x level interaction was nonsignificant [$F(20,52.225) = 1.094$, $p = 0.383$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 20).

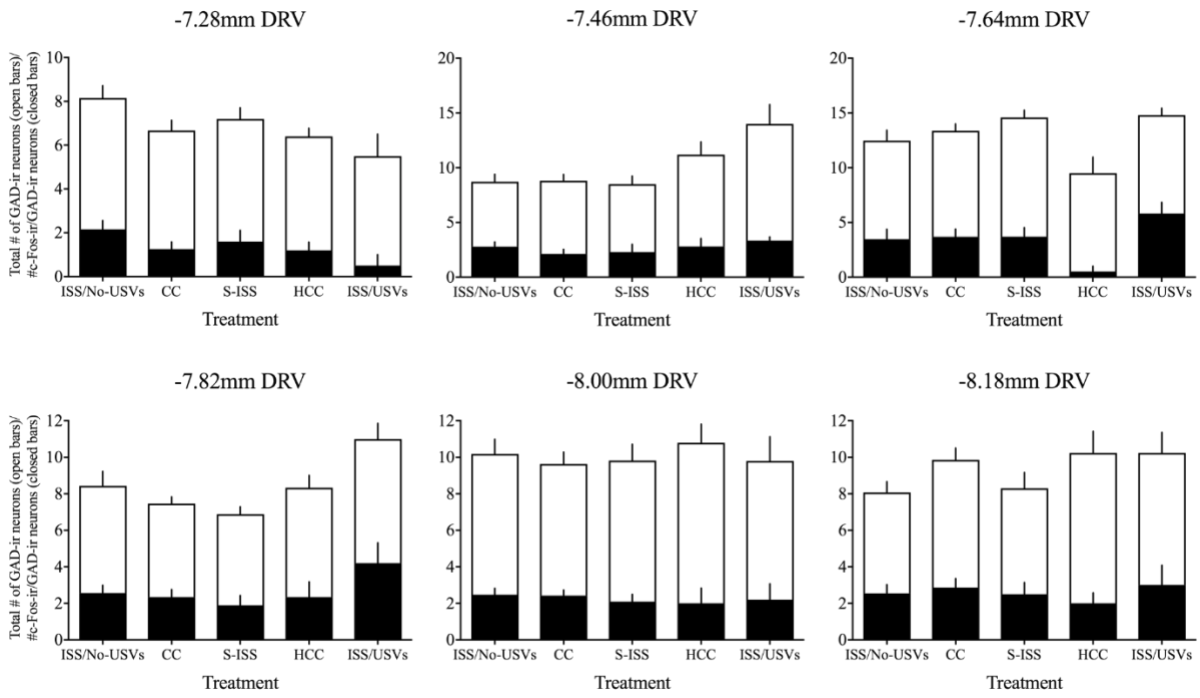

Figure 20: Mean (± SEM) cFos-immunoreactive/GAD67-immunoreactive (closed bars) and GAD67-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of ventral subregion (DRV). There were no significant differences between groups.

Ventrolateral subregion: There was a significant effect of treatment [$F(4,51.992) = 27.093$, $p < 0.001$], stereotaxic level [$F(3,49.042) = 11.443$, $p < 0.001$], and treatment x level interaction [$F(12,49.532) = 2.815$, $p = 0.005$]. Pairwise comparisons for the main effect of treatment indicated that the ISS/No-USVs group had significantly greater double-labeled cells than confined conspecifics, sham-ISS, and home cage controls ($p < 0.001$). Confined conspecifics also had greater double-labeled cells than home cage controls ($p = 0.001$). The ISS/USVs group had greater GABA activity than all control groups ($p < 0.001$) (Figure 21).

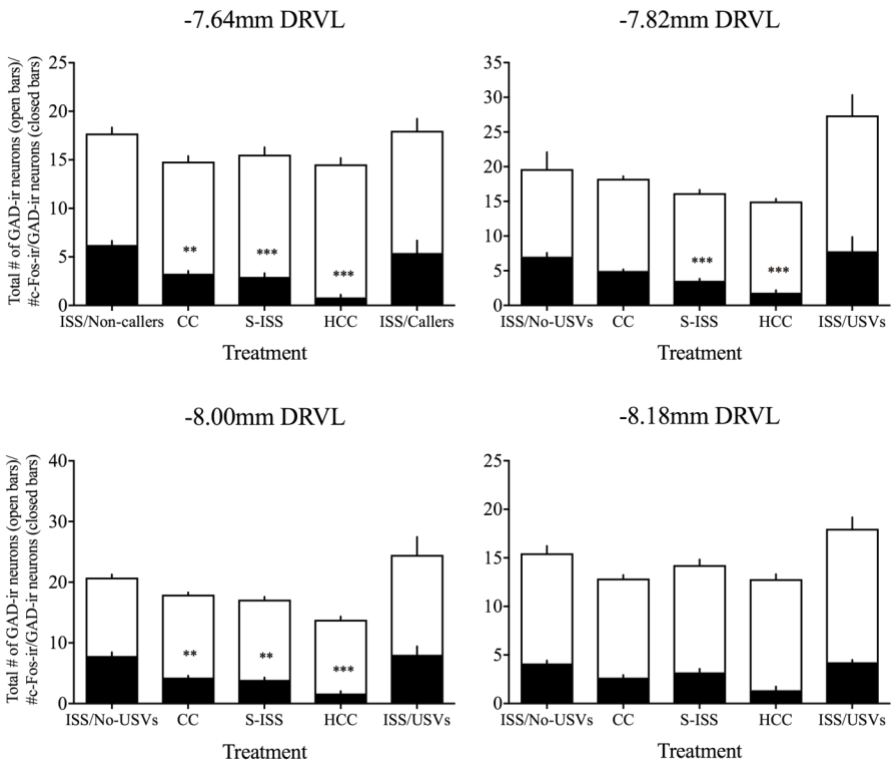

Figure 21: Mean (± SEM) cFos-immunoreactive/GAD67-immunoreactive (closed bars) and GAD67-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of ventrolateral subregion (DRVL). There were no significant differences between groups. ** indicates significantly different from HCC ($p < 0.05$), *** indicates significantly different from both ISS groups ($p < 0.05$)

Caudal subregion: There was a significant effect of treatment [$F(4,51.970) = 3.583$, $p = 0.012$] and stereotaxic level [$F(3,47.963) = 9.105$, $p < 0.001$], while the treatment x level interaction was nonsignificant [$F(12,49.007) = 1.157$, $p = 0.339$]. Pairwise comparisons for the main effect of treatment indicated that overall the ISS/No-USVs group had significantly greater double-labeled cells than the HCC group ($p = 0.006$). Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 22).

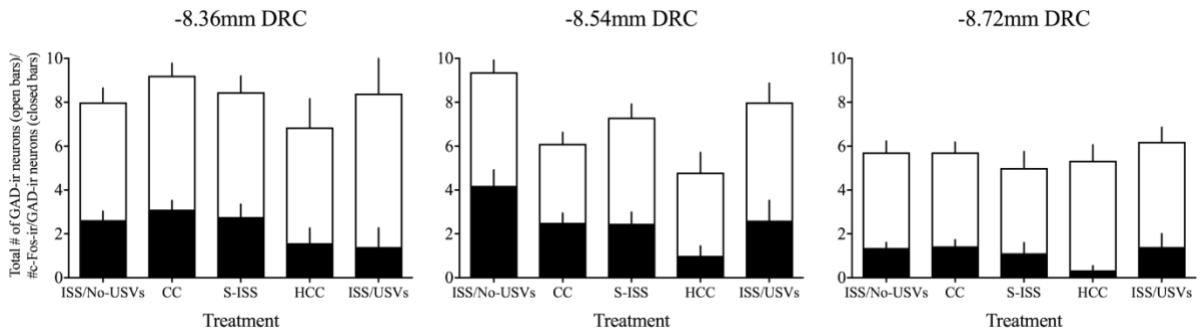

Figure 22: Mean (± SEM) cFos-immunoreactive/GAD67-immunoreactive (closed bars) and GAD67-immunoreactive/cFos-immunonegative (open bars) within each stereotaxic level sampled of caudal subregion (DRC). Note: the treatment x level interaction was nonsignificant.

GAD67-immunoreactive/cFos-immunonegative

Dorsal subregion: The effect of treatment was nonsignificant [$F(4,53.207) = 0.852$, $p = 0.499$], while there was a significant effect of stereotaxic level was significant [$F(5,44.693) = 33.076$, $p < 0.001$] and treatment x level interaction [$F(20,51.804) = 1.824$, $p = 0.043$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 19).

Ventral subregion: There was no significant effect of treatment [$F(4,52.049) = 1.843$, $p = 0.135$], while the effect of stereotaxic level was significant [$F(5,49.233) = 22.270$, $p < 0.001$]. The treatment x level interaction was nonsignificant [$F(20,49.386) = 1.689$, $p = 0.069$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 20).

Ventrolateral subregion: There was a significant effect of treatment [$F(4,52.264) = 4.152$, $p = 0.005$] and stereotaxic level [$F(3,48.749) = 6.172$, $p = 0.001$], while the treatment x level interaction was nonsignificant [$F(12,48.944) = 1.787$, $p = 0.077$]. Pairwise comparisons for the main effect of treatment indicated that there were more GABAergic cell bodies in the ISS-USVs

group compared with the ISS/No-USVs group ($p = 0.007$), confined conspecifics ($p = 0.006$), and sham-ISS ($p = 0.009$) (Figure 21).

Caudal subregion: There was a nonsignificant effect of treatment [$F(4,50.663) = 0.302$, $p = 0.875$], while the effect of stereotaxic level was significant [$F(3,47.106) = 3.967$, $p = 0.013$], and the treatment x level interaction was nonsignificant [$F(12,47.510) = 1.157$, $p = 0.362$]. Pairwise comparisons for the main effect of level indicated that overall there was variability of GABAergic cell activation across the different levels of bregma sampled (Figure 22).

Discussion

Experiment 2 was designed to investigate the functional activation of serotonergic and GABAergic cells in the dorsal raphe nucleus associated with active and passive coping in vocalizing or non-vocalizing rats. Experiment 2 replicated that there were no differences at baseline between groups during the social exploration pretest. Furthermore, experiment 2 replicated that vocalizing rats engaged in less active and greater passive behaviors during ISS. Core body temperature findings from experiment 1 were also replicated as non-vocalizing rats exhibited a greater hypothermic response than vocalizing rats.

Coping Strategy

The replication of behavioral strategies during ISS provided strong support that vocalizing rats represent a different stress-responsive phenotype and respond in an opposing manner to ISS than non-vocalizing rats. Both groups engaged in equivalent active behaviors during the initial 10 trials of ISS and separated into predominantly active or passive behaviors until the final 10 trials. The change in behavior across several trials and eventual asymptote of behavioral responding is consistent with others that reported shifting strategies from active to passive behaviors to conserve energy (Nishimura et al., 1988; Pintér et al., 2011). Core body

temperature results were consistent with behavioral strategies as passive vocalizing rats exhibited reduced hypothermia. It is possible the greater activity of non-vocalizing animals resulted in greater body heat loss than the less active vocalizing rats. The enhanced hypothermia in non-vocalizing rats may have resulted in greater physiological strain (i.e., allostatic load) producing a more stress susceptible organism (Karatsoreos & McEwen, 2011).

Cell Count Data

We hypothesized that active behaviors would be associated with reduced serotonergic activity in dorsal, caudal, and ventrolateral subregions of dorsal raphe nucleus. We found differences contrary to expected with serotonergic activation indicated via cFos expression localized to the mid-rostral dorsal, ventral, and ventrolateral subregions of dorsal raphe as well as the caudal subregion. Activation of rostral (-7.28mm) dorsal subregion, and the mid-rostral (-7.82mm to -8.18mm) dorsal, ventral, and ventrolateral subregions was greatest in vocalizing rats, while non-vocalizing rats were equivalent to all other control groups. Both vocalizing and non-vocalizing rats exhibited increased serotonergic activity at several stereotaxic levels of the caudal (-8.36mm to -8.54mm) subregion compared with controls, and both ISS groups were equivalent to each other. Expression of cFos in nonserotonergic cells was greater in both ISS groups in the most rostral levels, but only the non-vocalizing group was different than controls in mid-rostral levels. At caudal levels, vocalizing and non-vocalizing rats had equivalent cFos expression to each other, which was greater than all other groups. GABAergic activation indicated by cFos expression in GABA cell bodies was equivalent between all groups throughout the dorsal and ventral subregions. GABAergic activation was increased in both swim groups within several levels of ventrolateral and caudal subregions compared with sham-ISS and home-cage controls.

cFos-immunoreactivity in Serotonergic Cells

In the present study, we observed an increase in rostral and several mid-rostral sections of the dorsal subregion in the vocalizing group compared with the non-vocalizing group. Acute (15 min) continuous cold-water (19ºC) swim, tail shock, shock prod, and single social defeat all induce cFos immunoreactivity in serotonergic neurons across the dorsal subregion (Cohen et al., 2017; Grahn et al., 1999; Kelly et al., 2011; Paul et al., 2011). Although we found similar functional activation in our study, the associated behavioral results were inconsistent. We found vocalizing rats that engaged in passive coping exhibited greater functional activation of serotonin neurons in the rostral (-7.28mm, -7.46mm) extent of dorsal subregion. Serotonin neuron activation in this subregion is specifically associated with active behaviors in other studies, such as defensive burying in response to shock probe (Cohen et al., 2017) or upright defensive behavior directed toward an aggressor (Paul et al., 2011).

For the mid-rostral (-7.82mm to -8.18mm) dorsal subregion, we observed increased cFos immunoreactivity in serotonin neurons in vocalizing compared with non-vocalizing rats and all control groups. The mid-rostral dorsal subregion is critical for the generation of vulnerability or resilience following stress. Desensitization of serotonin type-1A inhibitory autoreceptors in dorsal (and caudal) subregions is a consequence of inescapable tail shock stress, and sets the stage for hyperactivity of serotonin neurons that is associated with learned helplessness-like behaviors (Rozeske et al., 2011). The role of this dorsal subregion is clear, as reduced serotonergic activity is associated with learned coping during tail shock (Donner et al., 2018), which suggests it is a potential target mediating the behavioral strategies engaged during ISS. However, again our behavioral results are inconsistent with serotonergic activation. Stress

resilient rats engaged in passive coping during ISS, we would have expected an associated decrease in serotonergic activation.

The ventral subregion contained the majority of mid-rostral cFos positive serotonin neurons in vocalizing rats in the present study. The functional topography of the ventral subregion is not well known, but the present study provides evidence for a potential role in passive coping (or energy conservation) during ISS. The rat ventral subregion may be involved in motoric responses due to sensory exposure as part of the fight or flight response, as it projects to sensorimotor and motor cortices (Waterhouse, Border, Wahl, & Mihailoff, 1993) as well as the rostral pole of locus coeruleus (Kim, Lee, Lee, & Waterhouse, 2004). Further investigation is required as the functional role of ventral subregion is not well characterized. Our data are some of the first to demonstrate a possible role with passive coping behaviors during any model of stress.

In the ventrolateral "wings" in the mid-rostral extent of dorsal raphe, we found increased activation in vocalizing rats compared with non-vocalizing rats and all control groups at two stereotaxic levels (-8.00mm, -8.18mm). Swim stress in ambient (25ºC) or cold (19ºC) water results in a large number of cFos-immunoreactive serotonin cells in this region compared with dorsal or ventral (Drugan, Hibl, et al., 2013; Kelly et al., 2011; Roche et al., 2003), and activation is associated with passive coping strategies during social stress (Paul et al., 2011)

The caudal aspects of ventrolateral wings are contiguous with ventrolateral periaqueductal gray, which is implicated in the generation of passive-avoidant coping behaviors (Keay & Bandler, 2001). The dorsal periaqueductal gray is implicated in producing escape and panic-like responses, and serotonin release in dorsal periaqueductal gray from ventrolateral projections inhibits escape response. Furthermore, serotonergic innervation of dorsomedial

hypothalamus inhibits panic and promotes inhibitory avoidance (Hassell et al., 2017; Paul et al., 2014). The activation of ventrolateral serotonin cells is consistent with the passive behavior exhibited by vocalizing rats and may also provide a mechanism by which to understand the active behavioral strategy in non-vocalizing rats. It is possible that the active behaviors employed by non-vocalizing rats represents a form of maladaptive panic-like behavior, rather than adaptive escape behaviors. However, there are several functional studies that demonstrated opposing activation patterns of serotonergic neurons in dorsal and ventral subregions compared with ventrolateral wings. When anxiogenic stimuli, such as open field or beta-carboline administration, activate dorsal and ventral serotonin neurons, ventrolateral subregion activity is suppressed, and vice versa (Abrams et al., 2004; Bouwknecht et al., 2007). We did not observe the expected opposing activation patterns in the current study, which may partly be due to cold-water exposure or reduction in core body temperature.

The caudal subregion (-8.36mm, -8.54mm) activation reported here is consistent with others that suggests that the region is stress responsive as both ISS groups exhibited increased cFos expression in serotonin cell bodies. Activation is associated with depressive-like behavior, indicated by learned helplessness is observed after acute inescapable tail shock or continuous cold-water swim (Donner et al., 2018; Grahn et al., 1999; Takase et al., 2004). However, when compared with active coping (controllable stress), rats without control that exhibit passive coping demonstrate increased cFos immunoreactivity within caudal serotonin neurons in other studies (Grahn et al., 1999). We did not observe differences between vocalizing and non-vocalizing rats in that respect. The caudal subregion is particularly sensitive to increased post-ISS forced swim immobility, anxiety in the open field, and social anxiety, as we have found cFos

immunoreactivity increased in caudal serotonin neurons associated with those behavioral effects (Stafford et al., *in prep*).

In the interfasicular nucleus, we did not find any differences in serotonergic activation between any groups, with the one exception of increased cFos expression in serotonin cells at the -8.36mm stereotaxic level between vocalizing and non-vocalizing and home cage controls, and the -8.72mm stereotaxic level within the confined conspecific group. The interfasicular nucleus is thermosensitive, particularly to warm ambient temperatures (Hale & Lowry, 2011) and cold-water (19ºC) (Drugan, Hibl, et al., 2013; Kelly et al., 2011). However, activation due to swim stress is differential and future work needs to further elucidate the role of this subregion, as it may also be more sensitive to temperature contrasts in a two-day forced swim procedure with varying water temperatures (Drugan, Hibl, et al., 2013). We should have observed consistent increased activation in swim groups in the current study within the stereotaxic expanse of the subregion, however, it is also possible that a series of swims in cold-water (15ºC) and the hypothermia induced by ISS may not drive serotonin neurons within the interfasicular nucleus in the same manner as continuous swim in relatively warmer temperatures.

cFos-immunoreactivity in Non-serotonergic Cells

Interestingly, cFos expression in non-serotonergic cell bodies was increased in both ISS groups compared with other groups across the rostral-caudal extent of dorsal raphe and all subregions. The increase in cFos expression in non-serotonergic cells was not localized to a particular subregion or stereotaxic level between groups. There were increased numbers of cFos positive cells in the most rostral extent of dorsal subregion compared with the mid-rostral level, and the rostral dorsal subregion and ventrolateral wings contained the greatest number compared with other subregions. We only assessed cFos expression localized around serotonergic neurons,

and the primary candidate for these serotonin-proximal cells are GABAergic (Challis, Beck, & Berton, 2014; McDevitt et al., 2014; Roche et al., 2003) or dopaminergic (Matthews et al., 2016). We assessed cFos immunoreactivity in GABA cells in the present study, and activation of dopaminergic cells should be investigated in future studies as an alternative target.

cFos-immunoreactivity in GABAergic Cells

Although the behavioral and serotonergic effects in the present study were opposite to hypothesized, it is possible that the decreased activation in non-vocalizing rats could be due to a GABAergic mechanism. We only found differences in cFos expression within GABAergic neurons in the ventrolateral subregion of dorsal raphe. Specifically, both ISS groups had greater double-labeled GABA neurons in ventrolateral subregion at the most rostral level (-7.64mm), while the ISS/USVs group had more double-labeled GABA cells at the remaining three levels (-7.82mm to -8.18mm) compared with control groups. At the caudal subregion, vocalizing and non-vocalizing rats were not different.

Previous studies have found that following 15 min acute continuous swim in ambient (25º) water that the majority of cFos immunoreactive neurons within dorsal raphe are GABAergic (Roche et al., 2003). Local inhibitory GABAergic neurons receive glutamatergic input from prelimbic cortex and are responsible for "quieting" serotonergic cells during active coping (Puglisi-Allegra & Andolina, 2015). The primary region of this GABAergic activation is within the dorsal and caudal subregions (Rozeske et al., 2011). The lack of differences in the current study do not rule out a GABAergic mechanism. The mechanism of cFos expression in GABA neurons or the time course of measuring cFos immunoreactivity may not capture actualy GABAergic activity, or there may be additional mechanisms related to hypothermia that alter GABergic activity during the ISS session.

Serotonin- or GABA-immunoreactivity Absent of cFos

Importantly, there were no differences in the numbers of total serotonin or GABA cell bodies between groups. To our knowledge, there are no studies that report a difference in serotonin neuron density due to stress or no-stress treatment. Interestingly, there were more GABAergic neurons in the vocalizing group at -7.64mm from bregma, but that did not affect the total number of cFos-immunoreactive GABA cells compared with non-vocalizing group.

Conclusion

Taken together, the specific subregion serotonin activity are generally consistent with other demonstrations of stress-induced serotonergic activity associated with passive coping. However, the implications of this immediate stress-induced activity are not clear given that the behavioral and serotonergic profiles of vocalizing rats should produce a stress vulnerable, not stress resilient, phenotype. One must consider coping strategies and the role of dorsal raphe serotonin in the context of ISS as a novel intermittent, inescapable, cold-water swim stress not previously investigated.

V. GENERAL DISCUSSION

The social anxiety observed in the present study is consistent with our previous report (Stafford et al., 2015) and consistent with others that have found exposure to a traumatic stressor, such as a forced swim (Christianson et al., 2013), tail shock (Christianson, Paul, et al., 2008; Christianson et al., 2009), or social defeat (Paul et al., 2011; Wood et al., 2010) reduced post-stress social interaction. The post-stress avoidance of a conspecific is considered stress vulnerability because rats that learn a coping response (Christianson, Paul, et al., 2008; Christianson et al., 2013) or engage in innate dominant, active behaviors (Cohen et al., 2017; Wood et al., 2010) associated with resilience do not display reduced post-stress anxiety-like avoidance. Thus, engaging in proactive behavioral strategies may protect the organism against anxiety induced by traumatic stress.

We hypothesized vocalizing rats would engage in proactive behaviors during ISS and buffer against the negative consequences of ISS. Others have found rats that emitted 22-kHz USVs during a stress session engaged in proactive behaviors directed toward a conspecific (Portavella et al., 1993), and displayed quicker recovery from an interoceptive stress of fever (Bassi et al., 2012). Due to the convergence of these behavioral findings, it was expected vocalizing rats in the present experiments would exhibit greater active behaviors. However, we found USV-emitting rats engaged in fewer active behaviors, and instead adopted a less active/more passive behavioral strategy throughout the majority of the stress session.

The distinction between active and passive behaviors as adaptive or maladaptive to stress is not clear in the present study. When confronted with an artificial electrical stimulus, aggressive conspecific, or submerged into water, adopting a proactive behavioral strategy is adaptive as part of the fight or flight response to survive the situation (Koolhaas, De Boer,

Buwalda, & Van Reenen, 2007; Korte, Koolhaas, Wingfield, & McEwen, 2005). As an immediate strategy to mitigate a stressor, an active behavior is beneficial with the goal of escaping or terminating the situation. The initial struggling and vigorous activity during acute continuous swim stress in ambient or cold water tempertures is associated with initial greater circulating adrenocorticotropic-releasing-hormone, increased heart rate, and serum lactate reflecting a sympatho-adrenal "fight or flight" state, which decrease during prolonged swimming until the animal is hypothermic (Abel, 1993; Dal-Zotto, Martí, & Armario, 2000; Drugan et al., 2005; Pintér et al., 2011).

In the long-term, or if faced with repeated stressor exposures, it may be more adaptive to engage in a passive, low activity strategy in order to conserve resources. Rats exposed to multiple sessions of forced swims shift from an initial active strategy to a passive behavioral strategy, likely due to an appraisal of the swim as inescapable and adapting to the situation by ceasing continued escape behaviors (De Pablo et al., 1989; Tye et al., 2012; Warden et al., 2012). Bassi and colleagues (2012) found, similar to the present study, that a small proportion of rats emitted 22-kHz USVs in response to an interoceptive stressor (fever), and these USV-emitting rats were more dominant (e.g., active) during a social interaction test. However, the vocalizing rats in their study *recovered* quicker than non-vocalizing rats, suggesting vocalizations in that study may reflect a resilient phenotype (Bassi et al., 2012).

Passive behaviors as an energy conservation mechanism is supported by core body temperature differences found between vocalizing and non-vocalizing rats. The non-vocalizing group of ISS rats exhibited greater hypothermia compared with vocalizing rats, which we attributed as heat loss due to increased struggling and associated heat dissipation. The enhanced hypothermia in non-vocalizing rats may have resulted in greater physiological strain on the

organism. The physiological stress of cold exposure coupled with the psychological component of inescapability may produce an animal more susceptible to the negative consequences of ISS, compared with the vocalizing group that conserved energy.

Coping strategies are associated with opposing serotonergic activity within the dorsal raphe nucleus (Puglisi-Allegra & Andolina, 2015). Patterns of cFos expression within serotonin neurons across the dorsal, ventrolateral, and caudal subregions were consistent with the passive behavior observed in vocalizing rats. Serotonin cells within the dorsal and caudal subregion are implicated in anxiety as these cells innervate and release serotonin within the basolateral amygdala (Hale et al., 2008). The immobile posture of vocalizing rats is consistent with dorsal serotonergic activity, but not consistent with post-stress reduced anxiety. Therefore, it is possible that the immobility observed in the present experiments is distinct from immobility considered to reflect depressive-like behavior.

Of all the subregions with cFos immunoreactive serotonin cells, the caudal extent sampled of ventrolateral subregion provide insight to interpret the observed behavioral differences. Serotonergic neurons within the ventrolateral wings innervate the dorsal periaqueductal gray and dorsomedial hypothalamus (Hassell et al., 2017). The release of serotonin in dorsal periaqueductal gray reduces escape behaviors. The reduction of escape behaviors is considered panicolytic as serotonin inhibits panic-like behaviors via serotonin type-1A receptors within both regions (Paul et al., 2014). Ventrolateral serotonergic activity suggests that passive strategies engaged by vocalizing rats and active behaviors of non-vocalizing rats are reflective of panicolytic and panicogenic states. However, non-vocalizing rats also exhibited serotonergic activation that was no different than control groups, which creates an additional layer of complexity to interpret differences between vocalizing and non-vocalizing rats. An

alternative interpretation of "resilience" and "vulnerability," must also be considered in the context of the current study. It is possible that vocalizing rats are indeed hypersensitive to the initial ISS session, such as an enhanced initial sympathetic response or enhanced subsequent parasympathetic compensatory response. Subsequent events such as a short duration social exploration with a juvenile, short duration swim in ambient water, or escape response learning is by contrast with ISS a significantly less stressful experience.

Efferent targets of the dorsal raphe implicated in anxiety and that modulate active or passive coping behaviors were not examined. The basolateral amygdala receives substantial efferent projections from dorsal and caudal subregions, and increased serotonergic output from dorsal raphe into basolateral nucleus is associated with development of social anxiety (Christianson et al., 2013) and coping behaviors (Puglisi-Allegra & Andolina, 2015). Serotonergic projections from dorsal raphe to dorsomedial hypothalamus and activation of serotonin type-1A receptors within the dorsomedial hypothalamus is associated with reduced panic and increased inhibitory avoidance. The interaction between amygdala and hypothalamic nuclei may be a possible mechanism through which passive behaviors during ISS are orchestrated.

It is also likely an intermittent swim model such as ISS affects neurobiological systems in a fundamentally different manner than continuous swim stress. A series of brief (5 s) intermittent tail shock results in opioid mediated analgesia, while continuous shock is non-opioid mediated (Terman, Lewis, & Liebeskind, 1983). Similar results are found between intermittent vs. continuous cold-water swim stress (Rochford & Henry, 1988). The mechanism of analgesia also changes across time in response to the number of trials of inescapable tail shock, suggesting patterns of sensitization and habituation to intermittent stress exposure (Drugan et al., 1985).

Interestingly, we did not find stress-induced analgesia after controllable ISS (Brown et al., 2001) or inescapable ISS (Stafford et al., *in prep)*. The lack of stress-induced analgesia and the serotonergic/GABAergic differences observed in the present study may be due to the context of our model. The ISS is a tonic-phasic stressor, whereas continuous swim is a tonic stressor and intermittent tail shock is phasic stressor. The intermittent swims force the animal to engage in active or passive behaviors during a consistent 5 s period (phasic), however these swims are repeatedly interrupted by inter-trial-intervals in which the rat remains cold and continues to develop hypothermia (tonic).

Water temperature and core body temprature may be significant factors driving the behavioral and neurobiological effects in the present experiments. In prior experiments comparing short duration (5-15 min) ambient (25ºC) or cold (19ºC) water continuous swim, colder water temperatures and increased time increased the total time spent in passive postures (immobility) (Drugan, Hibl, et al., 2013; Rabasa, Delgado-Morales, Gomez-Roman, Nadal, & Armario, 2013). Furthermore, ISS-induced immobility during a subsequent forced swim is temperature dependent. Warmer water (30ºC) increases latency to learn and instrumental swim escape response during controllable ISS, but does not result in different behavioral strategies during a subsequent forced swim test in ambient water (25ºC) as is observed in cold water (20ºC or less) (Drugan et al., 2005). Therefore, direct comparisons to continuous, cold or ambient water swim stress, ambient temperature ISS, or ambient (i.e., room) temperature non-swim based stressors may not be appropriate as fundamentally different stress dependent neurobiological systems may be involved.

Limitations and Future Directions

The foregoing studies demonstrated a particular stress-resilient phenotype associated with ISS. In light of the present data compared with other models of resilience, additional experiments are needed to replicate, validate, and extend the understanding of 22-kHz USVs and resilience to swim stress. These studies should, assess the transituational validity of resilience in other models of stress, further test behavioral coping during ISS, conduct a time course of these behavioral effects, and determine if additional pre-stress markers predict vocalizing or non-vocalizing group membership. Furthermore, there are several additional neurobiological pathways and neuroendocrine markers that may elucidate the results of the current experiments.

One of the primary limitations of the current studies is the uniqueness of the present findings to ISS. The ISS as a model of inescapable and intermittent stress is fundamentally different from other models, and translational validity to these other models may be limited due to factors such as prolonged hypothermia post-ISS. Furthermore, one necessary comparison is a subsequent ISS re-exposure to test if vocalizing or non-vocalizing rats shift coping strategies as others have found with repeated continuous forced swim exposures. Additional pre-stress measures, such as USVs emitted during social exploration or aggressive behaviors during social exploration may predict the resilient or vulnerable phenotype. There is also an additional developmental limitation wherein the pre-weaning and transport experience of these rats is unknown. There is evidence that transportation may be a significant source of stress on a naïve rat with long-term consequences (Balcombe, Barnard, & Sandusky, 2004; Grandin, 1997), and laboratory procedures may be especially stressful on weanling rats (Balcombe et al., 2004). Juvenile stress potentiates 22-kHz USV emission in adulthood during fear conditioning (Yee,

Schwarting, Fuchs, & Wöhr, 2012), therefore, future studies should replicate the current findings with rats bred onsite to provide an additional measure of reliability.

There are several potential dorsal raphe mechanisms which future experiments should investigate. First, the current experiments did not investigate changes in dorsal raphe functional activation on the day of social exploration. We have demonstrated the caudal subregion is associated with increased behavioral depression and anxiety post-ISS (Stafford et al., *in prep*), but not in the context of ultrasonic vocalizations. The extent to which activity in the dorsal raphe maintains post-ISS may provide critical information to interpret behavioral activity in the social exploration test. We have also demonstrated ISS-induced deficits likely have a noradrenergic mechanism (Drugan et al., 2010; Warner & Drugan, 2012). It is possible that ISS-induced deficits are produced via locus coeruleus-mediated noradrenergic activity, rather than serotonergic activity. Thus, future experiments should examine functional activation of locus coeruleus between vocalizing and non-vocalizing rats. Others have demonstrated several neuropeptides (galanin, neuropeptide Y) are functionally associated with resilience and noradrenergic function (Holmes, 2014; Sciolino et al., 2015). Furthermore, the involvement of corticotropin releasing factor type-I and type-II receptors would provide valuable insight into the mechanism leading to activation of serotonin neurons within dorsal raphe.

Dorsal raphe dopaminergic and activation of periaqueductal gray may elucidate the mechanisms resulting in social anxiety and active/passive coping. Dorsal raphe dopamine may have regulated the post-stress social anxiety independently from serotonin activity observed during immediately post-ISS. Dorsal raphe dopamine neurons are considered a caudal extent of ventral tegmental area (Stratford & Wirtshafter, 1990) and involved in arousal (Dougalis et al., 2012), motivation, and social approach behaviors (Matthews et al., 2016). Active or passive

coping behaviors are regulated by opposing parallel tracts through periaqueductal gray. The dorsolateral column induces active coping, while activation of the ventrolateral column evokes passive coping (Keay & Bandler, 2001). Future studies should compare how activation of these columns differs vocalizing or non-vocalizing rats. The serotonergic activation observed in the present study, particularly the increase in ventrolateral wings should be considered within the context of active or passive behaviors generated by pathways innervating the periaqueductal gray.

Implications

The present experiments demonstrated that during intermittent swim stress, rats that emitted 22-kHz USVs adopted a distinct behavioral strategy from rats that did not vocalize during extended exposure to a novel cold-water environment unique to ISS. The small proportion of vocalizing rats that exhibited a distinct phenotype from non-vocalizing rats may serve as a promising novel model of innate stress resilience. The ethological implication of ISS-induced USVs are at present not clear and will required further testing, although the current studies do suggest a novel interpretation of 22-kHz vocalizations. Furthermore, the present experiments demonstrated a particular model in which passive behaviors are an adaptive stress coping response to buffer against the development of anxiety-like behaviors due to inescapable intermittent cold-water swim exposure. The emission of these USVs and associated passive coping may serve as a novel model of stress reactivity to a psychological (inescapable, unpredictable) and physiological (cold-water, hypothermic) stressor, and may reflect a novel interpretation of stress resilience.

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APPENDIX A: IACUC APPROVAL

S+S 6/16/17

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

15-Jun-2017

Drugan, Robert C
Psychology
McConnell Hall Rm 434
Durham, NH 03824-2602

IACUC #: 170502
Project: Individual Differences in Stress Reactivity and Anxiety: Relation Between Ultrasonic Vocalizations, Proactive Behavior, and GABA Activity
Approval Date: 18-May-2017

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category E on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are not used.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File

APPENDIX B: ISS BEHAVIOR PAIRWISE COMPARISONS TABLES

Table i: Experiment 1 Active Behaviors

Trial	ISS/USVs	ISS/No-USVs	*Mean Diff.*	*SEM*	*t*	*df*	*p*
T1-10	5.5	6.143	-0.6429	1.389	0.4628	144	0.9997
T11-20	1.167	5.429	-4.262	1.389	3.068	144	0.0204*
T21-30	0.6667	4.714	-4.048	1.389	2.914	144	0.0327*
T31-40	1.667	6.214	-4.548	1.389	3.274	144	0.0106*
T41-50	2.5	6.5	-4	1.389	2.879	144	0.0362*
T51-60	3.167	5.714	-2.548	1.389	1.834	144	0.4343
T61-70	2	5.571	-3.571	1.389	2.571	144	0.0859
T71-80	3.333	4.214	-0.881	1.389	0.6341	144	0.9975

Note: * indicates significantly different $p < 0.05$

Table ii: Experiment 1 Passive Behaviors

Trial	ISS/USVs	ISS/No-USVs	*Mean Diff.*	*SEM*	*t*	*df*	*p*
T1-10	4.833	4.214	0.619	1.354	0.4574	144	0.9998
T11-20	8.833	5.357	3.476	1.354	2.568	144	0.0864
T21-30	9.333	5.643	3.69	1.354	2.727	144	0.0561
T31-40	8.333	4.214	4.119	1.354	3.043	144	0.022*
T41-50	7.5	3.786	3.714	1.354	2.744	144	0.0534
T51-60	7.167	4.571	2.595	1.354	1.917	144	0.3756
T61-70	8.167	4.786	3.381	1.354	2.498	144	0.1039
T71-80	6.833	5.786	1.048	1.354	0.774	144	0.9904

Note: * indicates significantly different $p < 0.05$

Tabel iii: Experiment 2 Active Behaviors

Trial	ISS/USVs	ISS/No-USVs	*Mean Diff.*	*SEM*	*t*	*df*	*p*
T1-10	5.5	6.143	-0.6429	1.389	0.4628	144	0.7763
T11-20	1.167	5.429	-4.262	1.389	3.068	144	0.0179*
T21-30	0.6667	4.714	-4.048	1.389	2.914	144	0.0246*
T31-40	1.667	6.214	-4.548	1.389	3.274	144	0.0106*
T41-50	2.5	6.5	-4	1.389	2.879	144	0.0246*
T51-60	3.167	5.714	-2.548	1.389	1.834	144	0.1924
T61-70	2	5.571	-3.571	1.389	2.571	144	0.0439*
T71-80	3.333	4.214	-0.881	1.389	0.6341	144	0.7763

Note: * indicates significantly different $p < 0.05$

Table iv: Experiment 2 Passive Behaviors

Trial	ISS/USVs	ISS/No-USVs	*Mean Diff.*	*SEM*	*t*	*df*	*p*
T1-10	5.333	5.176	0.1569	1.258	0.1247	168	>0.9999
T11-20	8.667	4.647	4.02	1.258	3.195	168	0.0133*
T21-30	8.833	4.176	4.657	1.258	3.702	168	0.0023*
T31-40	8.833	4.294	4.539	1.258	3.609	168	0.0032*
T41-50	8.833	3.588	5.245	1.258	4.17	168	0.0004*
T51-60	9.5	3.824	5.676	1.258	4.513	168	<0.001*
T61-70	8.167	4.294	3.873	1.258	3.079	168	0.0193*
T71-80	6.833	5.588	1.245	1.258	0.9898	168	0.9562

Note: * indicates significantly different $p < 0.05$

APPENDIX C: SEROTONIN ANALYSES TABLES

Table i: cFos/TPH Information Criteria

	REML	*AIC*	*BIC*
DRD	968.073	1010.073	1084.275
DRV	1145.692	1187.692	1261.643
DRVL	828.707	848.707	880.637
DRC	1159.776	1201.776	1277.652
DRI	546.819	558.819	576.762

Table ii: cFos Information Criteria

	REML	*AIC*	*BIC*
DRD	1449.072	1491.072	1565.19
DRV	1235.561	1277.561	1351.679
DRVL	1021.619	1041.619	1071.455
DRC	1439.913	1481.913	1557.941
DRI	633.514	645.514	663.577

Table iii: TPH Information Criteria

	REML	*AIC*	*BIC*
DRD	2029.977	2071.977	2146.344
DRV	2093.235	2135.235	2209.436
DRVL	1368.387	1388.387	1420.372
DRC	2005.894	2047.894	2123.922
DRI	981.564	993.564	1011.628

Table iv: DRD cFos/TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	2.51	0.26	56.65	1.98	3.03
CC	3.11	0.24	58.27	2.62	3.60
S-ISS	2.51	0.30	65.42	1.90	3.12
HCC	1.33	0.44	61.40	0.46	2.20
ISS-USVs	6.51	0.47	50.08	5.56	7.45

Table v: DRD cFos/TPH Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	2.04	0.13	31.52	1.77	2.31
7.46	3.34	0.25	45.65	2.83	3.85
7.64	2.93	0.38	45.60	2.16	3.69
7.82	2.75	0.20	53.60	2.35	3.16
8.00	3.89	0.32	54.99	3.26	4.52
8.18	4.20	0.29	45.64	3.62	4.78

Table vi: DRD cFos/TPH Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	1.48	0.21	31.47	1.05	1.91
	7.46	3.40	0.42	46.03	2.56	4.25
	7.64	3.36	0.63	46.04	2.09	4.62
	7.82	1.77	0.33	52.25	1.12	2.43
	8.00	2.54	0.51	51.74	1.52	3.56
	8.18	2.48	0.47	45.44	1.53	3.43
CC	7.28	1.66	0.23	31.59	1.20	2.13
	7.46	3.64	0.39	45.89	2.85	4.43
	7.64	3.71	0.57	45.29	2.55	4.86
	7.82	2.49	0.32	56.25	1.84	3.14
	8.00	3.40	0.48	52.85	2.44	4.35
	8.18	3.75	0.44	45.47	2.87	4.63
S-ISS	7.28	1.38	0.32	31.60	0.72	2.04
	7.46	2.48	0.52	46.20	1.43	3.53
	7.64	2.80	0.72	45.19	1.35	4.26
	7.82	1.98	0.41	58.19	1.16	2.80
	8.00	3.26	0.60	56.24	2.05	4.47
	8.18	3.16	0.57	46.35	2.02	4.31
HCC	7.28	1.42	0.33	31.40	0.75	2.08
	7.46	1.57	0.70	46.14	0.16	2.98
	7.64	0.56	1.12	46.57	-1.70	2.82
	7.82	1.52	0.53	53.74	0.46	2.57
	8.00	0.64	0.87	59.82	-1.10	2.38
	8.18	2.25	0.81	46.40	0.63	3.88
ISS-USVs	7.28	4.27	0.36	31.43	3.53	5.02
	7.46	5.60	0.71	44.16	4.16	7.04
	7.64	4.20	1.04	43.61	2.11	6.29

	7.82	6.00	0.60	50.44	4.80	7.20
	8.00	9.60	0.94	51.59	7.72	11.48
	8.18	9.35	0.83	44.20	7.68	11.03

Table vii: DRD cFos Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	13.11	0.78	55.97	11.55	14.67
CC	6.68	0.73	62.28	5.23	8.14
S-ISS	5.84	0.97	69.84	3.91	7.78
HCC	3.30	1.28	55.53	0.73	5.86
ISS-USVs	11.88	1.42	50.70	9.03	14.73

Table viii: DRD cFos Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	14.43	1.31	37.03	11.78	17.08
7.46	13.34	0.91	52.83	11.51	15.17
7.64	7.77	0.74	51.82	6.30	9.25
7.82	4.56	0.46	51.63	3.65	5.48
8.00	3.89	0.44	51.80	3.00	4.78
8.18	4.98	0.62	41.92	3.72	6.24

Table ix: DRD cFos Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	24.83	2.08	36.53	20.61	29.05
	7.46	23.26	1.51	53.43	20.24	26.28
	7.64	10.33	1.22	52.64	7.88	12.78
	7.82	7.64	0.74	50.76	6.14	9.13
	8.00	6.35	0.69	49.82	4.97	7.73
	8.18	6.25	1.01	42.40	4.20	8.29
CC	7.28	12.22	2.19	37.91	7.78	16.65
	7.46	9.93	1.37	53.58	7.19	12.67
	7.64	5.99	1.06	50.97	3.86	8.12
	7.82	3.94	0.71	52.85	2.52	5.37
	8.00	3.97	0.70	54.04	2.58	5.37
	8.18	4.05	0.94	42.45	2.16	5.95
S-ISS	7.28	8.35	3.08	37.70	2.11	14.58
	7.46	10.79	1.86	54.03	7.06	14.52

	7.64	6.07	1.42	51.21	3.22	8.92
	7.82	2.97	0.97	53.69	1.03	4.91
	8.00	2.53	0.91	53.39	0.71	4.34
	8.18	4.34	1.24	42.46	1.84	6.85
	7.28	4.86	3.27	35.66	-1.78	11.49
	7.46	6.12	2.52	53.89	1.07	11.16
HCC	7.64	3.48	2.17	53.66	-0.87	7.83
	7.82	1.68	1.20	52.27	-0.74	4.09
	8.00	1.01	1.23	52.53	-1.46	3.47
	8.18	2.65	1.75	42.94	-0.88	6.18
	7.28	21.89	3.67	36.03	14.46	29.33
	7.46	16.60	2.62	49.98	11.34	21.86
ISS-USVs	7.64	13.00	2.06	48.58	8.86	17.14
	7.82	6.60	1.35	49.54	3.90	9.30
	8.00	5.60	1.27	49.39	3.04	8.16
	8.18	7.60	1.80	40.05	3.97	11.22

Table x: DRD TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	61.31	1.84	45.86	57.60	65.02
CC	61.35	1.70	47.50	57.93	64.77
S-ISS	60.32	2.24	59.38	55.85	64.79
HCC	58.80	3.01	49.19	52.75	64.84
ISS-USVs	60.74	3.31	39.08	54.04	67.45

Table xi: DRD TPH Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	51.38	1.73	31.42	47.86	54.91
7.46	63.42	1.73	46.25	59.94	66.91
7.64	69.17	2.41	46.63	64.33	74.01
7.82	62.71	2.10	47.66	58.50	66.93
8.00	63.94	2.08	53.18	59.78	68.10
8.18	52.40	1.86	44.68	48.65	56.15

Table xii: DRD TPH Estimated Marginal Means Interaction

Treatment	Bregma	*Mean*	*SEM*	*df*	95% C.I.	
					Lower Bound	Upper Bound
ISS-No-USVs	7.28	53.57	2.73	31.54	48.00	59.13
	7.46	60.19	2.87	46.87	54.41	65.97
	7.64	73.26	4.00	47.02	65.22	81.30
	7.82	65.19	3.42	46.20	58.30	72.07
	8.00	63.10	3.29	50.82	56.50	69.70
	8.18	52.57	3.05	44.31	46.42	58.72
CC	7.28	52.06	2.97	31.79	46.01	58.11
	7.46	69.61	2.61	46.72	64.36	74.87
	7.64	68.23	3.62	46.41	60.94	75.52
	7.82	64.45	3.29	50.60	57.85	71.06
	8.00	60.12	3.08	52.41	53.94	66.30
	8.18	53.63	2.82	44.72	47.95	59.30
S-ISS	7.28	52.65	4.20	31.78	44.09	61.21
	7.46	59.20	3.56	47.43	52.04	66.36
	7.64	64.04	4.59	46.50	54.81	73.28
	7.82	64.26	4.38	50.54	55.47	73.04
	8.00	73.22	4.00	54.28	65.20	81.24
	8.18	48.54	3.74	44.98	41.01	56.06
HCC	7.28	50.72	4.26	30.93	42.03	59.40
	7.46	70.32	4.82	46.20	60.61	80.03
	7.64	64.91	7.07	48.38	50.69	79.12
	7.82	57.28	5.47	48.38	46.28	68.27
	8.00	54.64	5.87	55.69	42.89	66.40
	8.18	54.91	5.15	44.82	44.54	65.29
ISS-USVs	7.28	47.91	4.76	30.41	38.19	57.63
	7.46	57.80	4.89	44.69	47.95	67.65
	7.64	75.40	6.67	43.21	61.95	88.85
	7.82	62.40	6.19	44.93	49.94	74.86
	8.00	68.60	6.07	50.69	56.41	80.79
	8.18	52.36	5.39	43.52	41.50	63.21

Table xiii: DRV cFos/TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	3.08	0.40	50.66	2.28	3.88
CC	3.13	0.37	54.13	2.39	3.87
S-ISS	2.65	0.46	59.62	1.73	3.57
HCC	1.63	0.62	63.22	0.38	2.87
ISS-USVs	6.36	0.74	53.40	4.87	7.86

Table xiv: DRV cFos/TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	2.19	0.39	34.78	1.41	2.97
7.46	3.77	0.46	47.93	2.85	4.68
7.64	3.45	0.59	44.78	2.27	4.63
7.82	3.12	0.29	48.99	2.53	3.71
8.00	3.68	0.28	55.84	3.13	4.23
8.18	4.02	0.42	33.60	3.17	4.86

Table xv: DRV cFos/TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	3.07	0.55	33.76	1.95	4.19
	7.46	3.73	0.71	46.62	2.31	5.15
	7.64	3.73	0.98	44.63	1.77	5.70
	7.82	2.13	0.48	46.62	1.17	3.09
	8.00	2.77	0.44	52.03	1.88	3.65
	8.18	3.07	0.68	33.83	1.69	4.45
CC	7.28	2.29	0.59	34.88	1.10	3.47
	7.46	3.85	0.67	47.50	2.49	5.20
	7.64	4.09	0.88	44.23	2.31	5.87
	7.82	2.31	0.48	51.18	1.35	3.27
	8.00	3.00	0.42	53.25	2.17	3.83
	8.18	3.26	0.61	31.67	2.01	4.50
S-ISS	7.28	1.30	0.82	34.93	-0.37	2.98
	7.46	4.29	0.83	47.42	2.62	5.96
	7.64	2.89	1.12	44.68	0.64	5.13
	7.82	2.36	0.61	53.01	1.15	3.58
	8.00	2.52	0.53	57.68	1.47	3.58
	8.18	2.54	0.83	32.29	0.84	4.23

HCC	7.28	1.08	0.86	33.51	-0.67	2.82
	7.46	1.59	1.16	47.70	-0.75	3.94
	7.64	1.56	1.72	47.85	-1.90	5.01
	7.82	1.38	0.77	49.97	-0.16	2.92
	8.00	0.90	0.76	60.74	-0.62	2.43
ISS-USVs	8.18	3.24	1.17	35.71	0.86	5.61
	7.28	3.22	1.28	34.57	0.62	5.82
	7.46	5.38	1.48	47.98	2.40	8.35
	7.64	5.00	1.63	40.40	1.72	8.29
	7.82	7.40	0.87	45.45	5.66	9.14
	8.00	9.20	0.82	52.03	7.56	10.84
	8.18	7.99	1.19	32.39	5.58	10.41

Table xvi: DRV cFos Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	5.61	0.42	53.02	4.76	6.45
CC	3.72	0.39	59.33	2.93	4.50
S-ISS	4.20	0.49	63.72	3.22	5.18
HCC	1.64	0.67	64.10	0.30	2.98
ISS-USVs	7.38	0.81	58.97	5.76	9.00

Table xvii: DRV cFos Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	3.63	0.31	38.28	3.02	4.25
7.46	7.28	0.67	44.87	5.93	8.62
7.64	4.79	0.49	47.86	3.81	5.78
7.82	2.95	0.31	49.98	2.33	3.58
8.00	4.09	0.44	55.67	3.21	4.96
8.18	4.31	0.50	40.89	3.30	5.31

Table xviii: DRV cFos Treatment x Level Interaction Estimated Marginal Means

Treatment	Bregma	*Mean*	*SEM*	*df*	95% C.I.	
					Lower Bound	Upper Bound
ISS-No-USVs	7.28	4.55	0.44	33.76	3.65	5.44
	7.46	8.05	1.02	43.68	5.98	10.11
	7.64	6.91	0.82	48.03	5.27	8.55
	7.82	3.53	0.50	48.91	2.52	4.54
	8.00	5.47	0.70	53.62	4.08	6.86
	8.18	5.14	0.80	40.68	3.52	6.75
CC	7.28	2.67	0.45	38.58	1.76	3.58
	7.46	6.20	0.95	43.75	4.27	8.12
	7.64	3.95	0.75	46.65	2.44	5.45
	7.82	2.57	0.51	51.79	1.56	3.59
	8.00	3.73	0.65	54.58	2.42	5.04
	8.18	3.20	0.75	40.70	1.70	4.71
S-ISS	7.28	3.31	0.67	37.61	1.95	4.68
	7.46	7.78	1.25	46.32	5.27	10.29
	7.64	3.80	0.94	47.63	1.91	5.69
	7.82	1.76	0.65	51.65	0.45	3.07
	8.00	3.57	0.85	56.49	1.88	5.27
	8.18	4.97	0.93	38.03	3.10	6.85
HCC	7.28	1.11	0.70	33.02	-0.31	2.53
	7.46	4.03	1.67	45.81	0.67	7.39
	7.64	1.51	1.44	49.65	-1.37	4.40
	7.82	1.11	0.81	50.70	-0.51	2.73
	8.00	1.26	1.24	57.47	-1.22	3.74
	8.18	0.82	1.43	41.82	-2.06	3.70
ISS-USVs	7.28	6.54	1.00	38.64	4.53	8.55
	7.46	10.34	2.19	43.52	5.92	14.76
	7.64	7.80	1.36	44.36	5.05	10.55
	7.82	5.80	0.91	47.79	3.98	7.62
	8.00	6.40	1.28	53.62	3.83	8.97
	8.18	7.41	1.45	40.71	4.49	10.33

Table xix: DRV TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	59.168	2.278	45.244	54.582	63.755
CC	63.251	2.109	50.864	59.016	67.486
S-ISS	63.189	2.637	56.913	57.908	68.47
HCC	62.164	3.587	54.139	54.972	69.355
ISS-USVs	62.19	4.268	47.842	53.608	70.772

Table xx: DRV TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	42.366	1.931	31.719	38.43	46.301
7.46	59.644	2.819	44.073	53.962	65.326
7.64	66.515	2.456	46.897	61.574	71.457
7.82	79.25	2.474	49.226	74.278	84.221
8.00	73.339	2.183	51.64	68.959	77.72
8.18	50.84	2.267	39.62	46.257	55.424

Table xxi: DRV TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	39.72	2.84	27.46	33.90	45.54
	7.46	55.00	4.41	41.27	46.10	63.91
	7.64	65.09	4.10	46.73	56.85	73.33
	7.82	74.66	3.99	48.23	66.63	82.68
	8.00	71.77	3.45	49.34	64.83	78.70
	8.18	48.78	3.75	40.27	41.20	56.36
CC	7.28	39.12	2.94	31.41	33.13	45.11
	7.46	64.91	4.10	41.91	56.63	73.19
	7.64	67.56	3.75	44.91	60.00	75.11
	7.82	83.99	4.02	50.64	75.91	92.07
	8.00	70.76	3.24	50.74	64.25	77.27
	8.18	53.17	3.39	39.53	46.33	60.02
S-ISS	7.28	37.95	4.04	32.16	29.72	46.17
	7.46	60.27	5.17	42.76	49.85	70.70
	7.64	69.73	4.70	46.51	60.27	79.20
	7.82	77.24	5.21	50.64	66.78	87.70
	8.00	77.21	4.18	53.45	68.84	85.59
	8.18	56.73	4.17	38.70	48.29	65.18

HCC	7.28	42.81	4.45	27.04	33.67	51.95
	7.46	65.22	7.15	44.74	50.81	79.63
	7.64	67.40	7.13	47.59	53.07	81.74
	7.82	76.56	6.46	49.68	63.60	89.53
	8.00	75.36	6.22	53.32	62.88	87.84
	8.18	45.63	6.47	41.13	32.56	58.70
ISS-USVs	7.28	52.23	6.36	31.89	39.28	65.18
	7.46	52.82	9.20	44.13	34.29	71.35
	7.64	62.80	6.86	42.90	48.96	76.64
	7.82	83.80	7.22	47.20	69.28	98.32
	8.00	71.60	6.37	49.34	58.81	84.39
	8.18	49.89	6.61	37.84	36.51	63.26

Table xxii: DRVL cFos/TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	2.927	0.406	53.14	2.112	3.743
CC	2.784	0.375	56.037	2.033	3.535
S-ISS	3.087	0.494	58.434	2.097	4.076
HCC	1.49	0.65	58.748	0.19	2.791
ISS-USVs	8.285	0.731	49.005	6.817	9.754

Table xxiii: DRVL cFos/TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.64	66.515	2.456	46.897	61.574	71.457
7.82	79.25	2.474	49.226	74.278	84.221
8.00	73.339	2.183	51.64	68.959	77.72
8.18	50.84	2.267	39.62	46.257	55.424

Table xxiv: DRVL cFos/TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.64	4.52	0.64	53.35	3.23	5.81
	7.82	3.50	0.82	48.42	1.86	5.14
	8.00	2.29	0.46	52.97	1.37	3.21
	8.18	1.40	0.55	39.44	0.28	2.51
CC	7.64	2.06	0.56	51.00	0.93	3.20
	7.82	3.59	0.80	53.39	1.98	5.20
	8.00	3.12	0.43	54.16	2.26	3.98

	8.18	2.37	0.51	39.55	1.34	3.39
	7.64	2.73	0.75	51.92	1.23	4.24
S-ISS	7.82	3.18	1.05	51.66	1.08	5.29
	8.00	3.73	0.56	54.87	2.60	4.85
	8.18	2.70	0.68	39.55	1.34	4.07
	7.64	1.50	1.12	56.80	-0.75	3.74
HCC	7.82	2.19	1.31	50.93	-0.43	4.81
	8.00	0.92	0.83	56.18	-0.73	2.57
	8.18	1.36	0.95	39.05	-0.57	3.29
	7.64	8.20	1.10	48.36	5.99	10.41
ISS-USVs	7.82	8.20	1.48	47.62	5.23	11.17
	8.00	9.60	0.85	52.97	7.90	11.30
	8.18	7.14	0.96	39.21	5.21	9.08

Table xxv: DRVL cFos Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	30.219	1.425	22.03	27.263	33.175
CC	16.256	1.392	29.007	13.409	19.102
S-ISS	18.788	1.696	21.758	15.269	22.308
HCC	9.512	2.36	22.762	4.627	14.397
ISS-USVs	30.558	2.53	17.844	25.24	35.876

Table xxvi: DRVL cFos Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.64	19.366	1.159	54.748	17.043	21.689
7.82	21.011	1.202	28.32	18.55	23.472
8.00	21.86	1.526	34.915	18.761	24.958
8.18	22.03	1.477	9.435	18.713	25.347

Table xxvii: DRVL cFos Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
	7.64	25.80	1.89	50.41	22.02	29.59
ISS-No-USVs	7.82	26.70	1.97	32.61	22.69	30.72
	8.00	30.31	2.56	35.12	25.11	35.51
	8.18	38.06	2.42	9.71	32.65	43.47
CC	7.64	17.68	1.67	53.23	14.33	21.04
	7.82	16.74	1.91	40.99	12.88	20.60

	8.00	14.69	3.03	37.03	8.56	20.82
	8.18	15.91	2.27	8.83	10.76	21.06
	7.64	19.45	2.25	51.49	14.93	23.98
S-ISS	7.82	20.03	2.37	21.16	15.11	24.95
	8.00	18.04	2.91	34.00	12.13	23.95
	8.18	17.63	2.96	9.45	10.99	24.26
	7.64	8.09	3.35	58.47	1.38	14.80
HCC	7.82	10.94	3.32	29.42	4.15	17.74
	8.00	7.11	3.88	34.35	-0.77	15.00
	8.18	11.90	4.15	10.33	2.71	21.10
	7.64	25.80	3.30	50.01	19.17	32.43
ISS-USVs	7.82	30.64	3.46	24.27	23.51	37.76
	8.00	39.15	4.36	32.79	30.28	48.01
	8.18	26.65	4.20	8.74	17.11	36.19

Table xxviii: DRVL TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	44.685	1.442	48.666	41.787	47.583
CC	39.289	1.359	55.356	36.566	42.012
S-ISS	44.327	1.784	58.17	40.756	47.898
HCC	39.446	2.454	64.316	34.544	44.348
ISS-USVs	44.637	2.578	44.039	39.442	49.832

Table xxix: DRVL TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.64	27.956	1.368	44.666	25.201	30.711
7.82	59.733	2.199	48.011	55.313	64.154
8.00	57.798	1.811	53.064	54.165	61.431
8.18	24.42	0.904	37.703	22.589	26.25

Table xxx: DRVL TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
	7.64	35.09	2.29	44.98	30.47	39.70
ISS-No-USVs	7.82	57.50	3.55	47.61	50.36	64.64
	8.00	59.53	2.84	52.53	53.83	65.23
	8.18	26.63	1.48	37.67	23.64	29.62
CC	7.64	25.74	1.95	44.41	21.80	29.67

	7.82	57.74	3.52	48.50	50.66	64.82
	8.00	52.98	2.68	53.05	47.61	58.35
	8.18	20.70	1.37	37.72	17.93	23.47
	7.64	26.55	2.62	44.55	21.28	31.83
S-ISS	7.82	62.71	4.70	48.43	53.26	72.15
	8.00	62.03	3.52	53.14	54.98	69.08
	8.18	26.02	1.81	37.57	22.35	29.70
	7.64	22.81	4.12	45.45	14.51	31.10
HCC	7.82	58.52	5.77	48.20	46.92	70.12
	8.00	53.05	5.20	53.48	42.64	63.47
	8.18	23.40	2.55	37.86	18.24	28.57
	7.64	29.60	3.72	43.32	22.09	37.11
ISS-USVs	7.82	62.20	6.36	47.42	49.41	74.99
	8.00	61.40	5.24	52.53	50.89	71.91
	8.18	25.35	2.57	37.52	20.15	30.54

Table xxxi: DRC cFos/TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	3.48	0.29	54.50	2.90	4.07
CC	2.88	0.27	53.95	2.34	3.42
S-ISS	3.17	0.33	56.19	2.51	3.83
HCC	1.72	0.44	51.70	0.83	2.60
ISS-USVs	4.55	0.52	51.91	3.51	5.59

Table xxxii: DRC cFos/TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	5.21	0.40	54.15	4.42	6.00
8.54	4.41	0.29	50.32	3.82	5.00
8.72	3.40	0.33	45.81	2.74	4.06
8.90	2.88	0.31	43.52	2.26	3.49
9.08	1.68	0.26	42.95	1.16	2.20
9.26	1.38	0.22	45.77	0.94	1.81

Table xxxiii: DRC cFos/TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	7.13	0.67	54.04	5.78	8.48
	8.54	5.83	0.49	49.17	4.84	6.81
	8.72	3.94	0.56	45.68	2.81	5.07
	8.90	2.35	0.53	43.71	1.27	3.42
	9.08	0.92	0.48	47.13	-0.04	1.88
	9.26	0.73	0.38	46.00	-0.03	1.50
CC	8.36	3.96	0.63	55.06	2.69	5.22
	8.54	2.90	0.45	48.62	2.00	3.81
	8.72	2.44	0.54	46.32	1.36	3.52
	8.90	3.37	0.46	42.81	2.44	4.30
	9.08	2.43	0.43	45.79	1.57	3.29
	9.26	2.17	0.32	44.63	1.51	2.82
S-ISS	8.36	4.31	0.75	54.04	2.81	5.81
	8.54	3.58	0.55	49.56	2.48	4.68
	8.72	3.64	0.69	46.70	2.25	5.04
	8.90	3.09	0.59	43.62	1.91	4.28
	9.08	2.24	0.48	42.28	1.27	3.20
	9.26	2.14	0.46	47.21	1.22	3.06
HCC	8.36	1.86	1.02	54.04	-0.18	3.90
	8.54	2.73	0.82	54.18	1.09	4.37
	8.72	1.78	0.86	45.91	0.06	3.50
	8.90	1.86	0.69	40.37	0.46	3.25
	9.08	1.22	0.67	42.40	-0.13	2.57
	9.26	0.86	0.51	43.58	-0.17	1.88
ISS-USVs	8.36	8.80	1.21	54.04	6.39	11.22
	8.54	7.00	0.86	47.60	5.27	8.73
	8.72	5.20	0.95	44.85	3.30	7.10
	8.90	3.72	1.01	44.15	1.70	5.75
	9.08	1.60	0.75	40.65	0.09	3.11
	9.26	0.99	0.66	45.96	-0.34	2.32

Table xxxiv: DRC cFos Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	6.74	0.55	59.40	5.64	7.83
CC	3.05	0.50	59.38	2.05	4.04
S-ISS	3.95	0.62	61.70	2.72	5.18
HCC	2.38	0.83	54.89	0.71	4.05
ISS-USVs	9.18	0.99	57.15	7.20	11.17

Table xxxv: DRC cFos Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	3.98	0.35	56.08	3.29	4.68
8.54	4.90	0.52	56.15	3.86	5.94
8.72	5.04	0.55	48.83	3.94	6.14
8.90	5.19	0.54	44.76	4.11	6.28
9.08	4.78	0.59	44.84	3.60	5.96
9.26	6.45	0.59	47.11	5.26	7.65

Table xxxvi: DRC cFos Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	4.09	0.59	56.23	2.90	5.28
	8.54	7.73	0.87	55.76	5.98	9.47
	8.72	6.72	0.92	49.09	4.86	8.58
	8.90	6.45	0.93	45.09	4.57	8.33
	9.08	7.30	1.10	47.44	5.08	9.51
	9.26	8.13	0.94	45.74	6.24	10.02
CC	8.36	2.75	0.54	57.31	1.67	3.84
	8.54	2.09	0.80	55.77	0.49	3.68
	8.72	3.66	0.89	52.59	1.88	5.43
	8.90	3.18	0.79	43.25	1.59	4.78
	9.08	3.35	0.99	46.14	1.36	5.34
	9.26	3.24	0.85	45.44	1.52	4.96
S-ISS	8.36	3.07	0.66	56.43	1.76	4.39
	8.54	3.19	0.95	54.20	1.29	5.10
	8.72	4.44	1.09	54.19	2.26	6.62
	8.90	5.15	1.08	45.83	2.97	7.33
	9.08	3.59	1.09	44.00	1.39	5.78
	9.26	4.26	1.21	48.21	1.82	6.69

HCC	8.36	2.00	0.90	55.77	0.20	3.80
	8.54	1.49	1.45	59.77	-1.40	4.38
	8.72	1.19	1.45	47.48	-1.72	4.10
	8.90	3.71	1.23	39.87	1.22	6.21
	9.08	1.86	1.54	44.38	-1.24	4.96
	9.26	4.00	1.35	43.12	1.28	6.73
ISS-USVs	8.36	8.00	1.07	55.77	5.87	10.13
	8.54	10.00	1.54	53.56	6.92	13.09
	8.72	9.20	1.60	45.89	5.97	12.43
	8.90	7.46	1.76	45.95	3.92	10.99
	9.08	7.80	1.70	43.56	4.38	11.22
	9.26	12.64	1.97	48.26	8.68	16.60

Table xxxvii: DRC TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	39.08	1.39	50.63	36.29	41.88
CC	43.46	1.27	51.55	40.91	46.00
S-ISS	43.95	1.53	56.93	40.88	47.01
HCC	44.72	2.11	49.84	40.48	48.96
ISS-USVs	39.47	2.48	49.19	34.49	44.44

Table xxxviii: DRC TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	61.25	1.63	53.59	57.98	64.53
8.54	58.47	1.62	52.22	55.22	61.72
8.72	45.34	1.55	44.93	42.21	48.48
8.90	33.93	1.42	42.52	31.06	36.79
9.08	26.35	1.07	48.68	24.20	28.50
9.26	27.46	1.02	47.51	25.42	29.51

Table xxxix: DRC TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	4.09	0.59	56.23	2.90	5.28
	8.54	7.73	0.87	55.76	5.98	9.47
	8.72	6.72	0.92	49.09	4.86	8.58
	8.90	6.45	0.93	45.09	4.57	8.33
	9.08	7.30	1.10	47.44	5.08	9.51

	9.26	8.13	0.94	45.74	6.24	10.02
	8.36	2.75	0.54	57.31	1.67	3.84
	8.54	2.09	0.80	55.77	0.49	3.68
CC	8.72	3.66	0.89	52.59	1.88	5.43
	8.90	3.18	0.79	43.25	1.59	4.78
	9.08	3.35	0.99	46.14	1.36	5.34
	9.26	3.24	0.85	45.44	1.52	4.96
	8.36	3.07	0.66	56.43	1.76	4.39
	8.54	3.19	0.95	54.20	1.29	5.10
S-ISS	8.72	4.44	1.09	54.19	2.26	6.62
	8.90	5.15	1.08	45.83	2.97	7.33
	9.08	3.59	1.09	44.00	1.39	5.78
	9.26	4.26	1.21	48.21	1.82	6.69
	8.36	2.00	0.90	55.77	0.20	3.80
	8.54	1.49	1.45	59.77	-1.40	4.38
HCC	8.72	1.19	1.45	47.48	-1.72	4.10
	8.90	3.71	1.23	39.87	1.22	6.21
	9.08	1.86	1.54	44.38	-1.24	4.96
	9.26	4.00	1.35	43.12	1.28	6.73
	8.36	8.00	1.07	55.77	5.87	10.13
	8.54	10.00	1.54	53.56	6.92	13.09
ISS-USVs	8.72	9.20	1.60	45.89	5.97	12.43
	8.90	7.46	1.76	45.95	3.92	10.99
	9.08	7.80	1.70	43.56	4.38	11.22
	9.26	12.64	1.97	48.26	8.68	16.60

Table xl: DRI cFos/TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	0.77	0.26	38.35	0.26	1.29
CC	2.09	0.24	40.97	1.60	2.58
S-ISS	1.35	0.30	41.27	0.74	1.95
HCC	1.03	0.41	38.88	0.21	1.85
ISS-USVs	1.71	0.46	38.22	0.79	2.63

Table xli: DRI cFos/TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	Mean	Std. Error	df	Lower Bound	Upper Bound
8.36	1.32	0.16	54.21	1.01	1.63
8.54	1.43	0.24	50.38	0.94	1.91
8.72	1.43	0.29	43.12	0.84	2.02

Table xlii: DRI cFos/TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	0.56	0.27	54.16	0.03	1.10
	8.54	1.11	0.39	49.40	0.33	1.89
	8.72	0.64	0.50	43.30	-0.36	1.65
CC	8.36	1.33	0.25	54.61	0.83	1.83
	8.54	1.55	0.35	49.30	0.84	2.27
	8.72	3.39	0.48	43.64	2.42	4.35
S-ISS	8.36	1.62	0.30	54.16	1.02	2.21
	8.54	1.22	0.43	49.33	0.35	2.09
	8.72	1.21	0.62	43.79	-0.04	2.45
HCC	8.36	0.29	0.40	54.16	-0.52	1.09
	8.54	1.31	0.66	50.96	-0.02	2.64
	8.72	1.50	0.76	42.72	-0.04	3.03
ISS-USVs	8.36	2.80	0.48	54.16	1.85	3.75
	8.54	1.94	0.74	50.58	0.44	3.43
	8.72	0.40	0.83	42.72	-1.28	2.08

Table xliii: DRI cFos Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	1.82	0.35	50.30	1.11	2.54
CC	1.85	0.32	53.57	1.21	2.50
S-ISS	2.35	0.38	56.78	1.58	3.12
HCC	1.84	0.57	54.27	0.70	2.98
ISS-USVs	3.07	0.64	51.80	1.78	4.36

Table xliv: DRI cFos Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	1.95	0.24	52.77	1.47	2.42
8.54	2.43	0.37	49.98	1.69	3.17
8.72	2.18	0.27	49.21	1.65	2.72

Table xlv: DRI cFos Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	1.44	0.40	52.44	0.63	2.25
	8.54	2.45	0.59	48.33	1.26	3.64
	8.72	1.58	0.46	49.71	0.65	2.51
CC	8.36	1.44	0.38	53.50	0.68	2.20
	8.54	2.10	0.54	48.95	1.02	3.19
	8.72	2.02	0.43	50.68	1.15	2.88
S-ISS	8.36	2.39	0.45	53.79	1.50	3.29
	8.54	2.52	0.66	49.69	1.20	3.84
	8.72	2.14	0.52	51.10	1.10	3.17
HCC	8.36	0.86	0.61	52.44	-0.37	2.08
	8.54	2.87	1.01	50.16	0.84	4.91
	8.72	1.79	0.71	48.76	0.35	3.22
ISS-USVs	8.36	3.60	0.72	52.44	2.15	5.05
	8.54	2.21	1.13	50.54	-0.06	4.48
	8.72	3.40	0.79	47.72	1.82	4.98

Table xlvi: DRI TPH Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	24.19	0.98	50.68	22.23	26.16
CC	23.03	0.90	53.29	21.22	24.84
S-ISS	23.68	1.09	53.70	21.49	25.87
HCC	27.55	1.61	54.90	24.32	30.78
ISS-USVs	26.31	1.82	53.19	22.66	29.96

Table xlvii: DRI TPH Stereotaxic Level Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	19.21	0.87	54.09	17.46	20.95
8.54	31.82	1.39	49.26	29.03	34.60
8.72	23.84	0.55	46.99	22.73	24.95

Table xlviii: DRI TPH Treatment x Level Interaction Estimated Marginal Means

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	19.94	1.48	54.07	16.96	22.91
	8.54	28.75	2.21	49.15	24.30	33.20
	8.72	23.90	0.96	47.06	21.97	25.82
CC	8.36	16.95	1.40	54.16	14.15	19.75
	8.54	28.98	2.02	49.16	24.92	33.04
	8.72	23.15	0.89	47.18	21.36	24.95
S-ISS	8.36	18.06	1.65	54.15	14.76	21.35
	8.54	28.95	2.47	49.19	23.98	33.92
	8.72	24.05	1.08	47.22	21.88	26.22
HCC	8.36	17.29	2.24	54.07	12.79	21.79
	8.54	41.08	3.83	49.30	33.38	48.77
	8.72	24.29	1.46	46.92	21.35	27.23
ISS-USVs	8.36	23.80	2.66	54.07	18.48	29.12
	8.54	31.33	4.28	49.29	22.72	39.93
	8.72	23.80	1.60	46.80	20.58	27.02

APPENDIX D: GABA ANALYSES TABLES

Table i: cFos/GAD67 Information Criteria

	REML	*AIC*	*BIC*
DRD	1043.752	1085.752	1160.365
DRV	1067.856	1109.856	1184.387
DRVL	747.67	767.67	799.488
DRC	764.059	784.059	816.529

Table ii: GAD67 Information Criteria

	REML	*AIC*	*BIC*
DRD	1042.48	1084.48	1159.092
DRV	1216.892	1258.892	1333.423
DRVL	895.279	915.279	947.097
DRC	875.571	895.571	928.041

Table iii: DRD cFos/GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	61.31	1.84	45.86	57.60	65.02
CC	61.35	1.70	47.50	57.93	64.77
S-ISS	60.32	2.24	59.38	55.85	64.79
HCC	58.80	3.01	49.19	52.75	64.84
ISS-USVs	60.74	3.31	39.08	54.04	67.45

Table iv: DRD cFos/GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	51.38	1.73	31.42	47.86	54.91
7.46	63.42	1.73	46.25	59.94	66.91
7.64	69.17	2.41	46.63	64.33	74.01
7.82	62.71	2.10	47.66	58.50	66.93
8.00	63.94	2.08	53.18	59.78	68.10
8.18	52.40	1.86	44.68	48.65	56.15

Table v: DRD cFos/GAD Estimated Marginal Means Interaction

Treatment	Bregma	*Mean*	*SEM*	*df*	95% C.I.	
					Lower Bound	Upper Bound
ISS-No-USVs	7.28	53.57	2.73	31.54	48.00	59.13
	7.46	60.19	2.87	46.87	54.41	65.97
	7.64	73.26	4.00	47.02	65.22	81.30
	7.82	65.19	3.42	46.20	58.30	72.07
	8.00	63.10	3.29	50.82	56.50	69.70
	8.18	52.57	3.05	44.31	46.42	58.72
CC	7.28	52.06	2.97	31.79	46.01	58.11
	7.46	69.61	2.61	46.72	64.36	74.87
	7.64	68.23	3.62	46.41	60.94	75.52
	7.82	64.45	3.29	50.60	57.85	71.06
	8.00	60.12	3.08	52.41	53.94	66.30
	8.18	53.63	2.82	44.72	47.95	59.30
S-ISS	7.28	52.65	4.20	31.78	44.09	61.21
	7.46	59.20	3.56	47.43	52.04	66.36
	7.64	64.04	4.59	46.50	54.81	73.28
	7.82	64.26	4.38	50.54	55.47	73.04
	8.00	73.22	4.00	54.28	65.20	81.24
	8.18	48.54	3.74	44.98	41.01	56.06
HCC	7.28	50.72	4.26	30.93	42.03	59.40
	7.46	70.32	4.82	46.20	60.61	80.03
	7.64	64.91	7.07	48.38	50.69	79.12
	7.82	57.28	5.47	48.38	46.28	68.27
	8.00	54.64	5.87	55.69	42.89	66.40
	8.18	54.91	5.15	44.82	44.54	65.29
ISS-USVs	7.28	47.91	4.76	30.41	38.19	57.63
	7.46	57.80	4.89	44.69	47.95	67.65
	7.64	75.40	6.67	43.21	61.95	88.85
	7.82	62.40	6.19	44.93	49.94	74.86
	8.00	68.60	6.07	50.69	56.41	80.79
	8.18	52.36	5.39	43.52	41.50	63.21

Table vi: DRD GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	4.95	0.24	50.75	4.48	5.43
CC	5.02	0.23	56.12	4.57	5.47
S-ISS	5.28	0.30	65.51	4.68	5.88
HCC	4.68	0.40	53.58	3.87	5.49
ISS-USVs	5.63	0.43	43.62	4.77	6.49

Table vii: DRD GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	5.15	0.44	35.36	4.26	6.05
7.46	6.01	0.32	46.04	5.36	6.66
7.64	8.00	0.44	46.45	7.11	8.89
7.82	3.98	0.22	48.78	3.54	4.41
8.00	3.43	0.16	54.87	3.12	3.74
8.18	4.09	0.24	42.70	3.62	4.57

Table viii: DRD GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	4.69	0.69	35.61	3.29	6.10
	7.46	6.49	0.54	46.25	5.40	7.57
	7.64	7.98	0.73	47.78	6.51	9.46
	7.82	3.88	0.35	48.51	3.18	4.59
	8.00	3.18	0.25	53.35	2.68	3.67
	8.18	3.48	0.38	42.56	2.72	4.25
CC	7.28	5.29	0.74	35.93	3.78	6.80
	7.46	6.21	0.49	46.31	5.23	7.20
	7.64	7.19	0.63	45.69	5.91	8.46
	7.82	3.82	0.34	49.26	3.14	4.50
	8.00	3.02	0.23	54.70	2.55	3.48
	8.18	4.58	0.36	42.56	3.86	5.30
S-ISS	7.28	6.47	1.04	35.75	4.36	8.59
	7.46	6.59	0.67	46.38	5.25	7.94
	7.64	8.99	0.85	46.06	7.28	10.69
	7.82	3.36	0.47	49.28	2.43	4.30
	8.00	2.60	0.30	55.55	1.99	3.21
	8.18	3.64	0.47	43.53	2.69	4.58

HCC	7.28	5.26	1.09	34.85	3.04	7.49
	7.46	5.16	0.91	45.78	3.34	6.99
	7.64	7.65	1.32	48.22	5.00	10.30
	7.82	2.82	0.57	48.83	1.66	3.97
	8.00	3.75	0.45	56.23	2.86	4.65
	8.18	3.45	0.65	42.78	2.13	4.77
ISS-USVs	7.28	4.06	1.25	33.43	1.52	6.60
	7.46	5.60	0.91	45.68	3.78	7.43
	7.64	8.20	1.22	43.70	5.74	10.66
	7.82	6.00	0.63	48.24	4.73	7.27
	8.00	4.60	0.46	53.34	3.68	5.52
	8.18	5.31	0.69	41.17	3.93	6.70

Table ix: DRV cFos/GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	2.66	0.25	44.36	2.16	3.16
CC	2.47	0.23	49.91	2.01	2.94
S-ISS	2.45	0.29	56.52	1.87	3.03
HCC	1.74	0.41	60.82	0.93	2.55
ISS-USVs	3.21	0.46	46.44	2.27	4.14

Table x: DRV cFos/GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	1.28	0.24	34.76	0.79	1.78
7.46	2.73	0.30	45.00	2.12	3.33
7.64	3.35	0.45	45.77	2.45	4.25
7.82	2.64	0.28	46.96	2.08	3.20
8.00	2.27	0.23	52.73	1.81	2.72
8.18	2.76	0.32	39.45	2.11	3.42

Table xi: DRV cFos/GAD Estimated Marginal Means Interaction

Treatment	Bregma	*Mean*	*SEM*	*df*	95% C.I.	
					Lower Bound	Upper Bound
ISS-No-USVs	7.28	2.08	0.34	33.86	1.40	2.76
	7.46	2.69	0.46	43.76	1.76	3.62
	7.64	3.35	0.75	45.76	1.85	4.86
	7.82	2.54	0.45	46.70	1.65	3.44
	8.00	2.47	0.36	50.92	1.75	3.19
	8.18	2.83	0.52	38.66	1.78	3.89
CC	7.28	1.23	0.34	34.79	0.53	1.92
	7.46	2.26	0.43	44.56	1.39	3.12
	7.64	3.66	0.67	45.89	2.30	5.02
	7.82	2.34	0.46	47.47	1.42	3.27
	8.00	2.50	0.34	52.45	1.82	3.17
	8.18	2.86	0.49	39.47	1.88	3.84
S-ISS	7.28	1.69	0.53	34.76	0.61	2.77
	7.46	2.29	0.54	44.42	1.19	3.38
	7.64	3.81	0.85	46.21	2.10	5.51
	7.82	1.80	0.59	47.57	0.61	2.99
	8.00	2.14	0.44	53.49	1.26	3.02
	8.18	2.96	0.59	39.22	1.76	4.16
HCC	7.28	1.02	0.54	33.36	-0.09	2.12
	7.46	2.83	0.77	44.10	1.28	4.38
	7.64	0.13	1.33	46.63	-2.55	2.82
	7.82	2.31	0.73	46.97	0.85	3.78
	8.00	2.03	0.65	54.19	0.73	3.33
	8.18	2.10	0.93	40.58	0.22	3.99
ISS-USVs	7.28	0.41	0.82	34.48	-1.26	2.08
	7.46	3.57	0.98	45.39	1.60	5.54
	7.64	5.80	1.23	43.57	3.32	8.28
	7.82	4.20	0.80	46.29	2.59	5.81
	8.00	2.20	0.66	50.92	0.87	3.53
	8.18	3.05	0.94	38.50	1.15	4.96

Table xii: DRV GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	6.78	0.25	43.07	6.28	7.29
CC	6.82	0.23	49.67	6.35	7.29
S-ISS	6.83	0.31	56.90	6.21	7.44
HCC	7.81	0.44	64.87	6.93	8.69
ISS-USVs	7.70	0.46	46.41	6.78	8.63

Table xiii: DRV GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.28	5.26	0.31	39.25	4.63	5.90
7.46	7.59	0.42	45.22	6.74	8.45
7.64	9.48	0.45	43.11	8.58	10.38
7.82	5.74	0.33	49.57	5.07	6.41
8.00	8.03	0.46	52.86	7.10	8.96
8.18	7.03	0.41	41.93	6.20	7.86

Table xiv: DRV GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.28	6.11	0.44	36.76	5.21	7.00
	7.46	6.08	0.65	44.66	4.77	7.39
	7.64	8.87	0.75	43.34	7.37	10.37
	7.82	6.00	0.54	48.23	4.92	7.08
	8.00	7.71	0.74	49.85	6.22	9.19
	8.18	5.93	0.66	41.44	4.59	7.27
CC	7.28	5.11	0.45	38.90	4.20	6.01
	7.46	6.62	0.61	44.92	5.40	7.85
	7.64	9.72	0.67	42.83	8.36	11.08
	7.82	5.16	0.53	52.41	4.09	6.23
	8.00	7.27	0.69	52.02	5.89	8.65
	8.18	7.06	0.62	41.48	5.82	8.31
S-ISS	7.28	5.28	0.68	39.79	3.91	6.65
	7.46	6.17	0.77	44.28	4.62	7.73
	7.64	10.82	0.85	43.27	9.11	12.54
	7.82	4.68	0.69	52.93	3.30	6.06
	8.00	8.11	0.89	55.20	6.32	9.89
	8.18	5.90	0.76	41.60	4.37	7.43

HCC	7.28	5.18	0.72	36.32	3.72	6.64
	7.46	8.15	1.09	44.38	5.96	10.33
	7.64	8.98	1.34	43.67	6.29	11.68
	7.82	6.06	0.87	49.23	4.31	7.82
	8.00	9.47	1.31	55.49	6.85	12.10
	8.18	9.02	1.18	42.38	6.63	11.40
ISS-USVs	7.28	4.64	1.04	39.22	2.54	6.74
	7.46	10.95	1.38	45.61	8.16	13.74
	7.64	9.00	1.22	41.50	6.55	11.45
	7.82	6.80	0.97	47.00	4.85	8.75
	8.00	7.60	1.36	49.85	4.86	10.34
	8.18	7.24	1.20	41.30	4.81	9.66

Table xv: DRVL cFos/GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	6.23	0.29	49.38	5.66	6.80
CC	3.75	0.27	52.47	3.21	4.29
S-ISS	3.14	0.34	55.81	2.45	3.82
HCC	1.43	0.48	60.23	0.47	2.39
ISS-USVs	6.01	0.51	44.39	4.98	7.03

Table xvi: DRVL cFos/GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.64	3.44	0.29	44.62	2.85	4.03
7.82	4.97	0.32	48.44	4.32	5.61
8.00	4.85	0.32	54.51	4.22	5.48
8.18	3.19	0.23	37.48	2.72	3.66

Table xvii: DRVL cFos/GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.64	6.17	0.49	45.04	5.19	7.15
	7.82	6.86	0.52	47.73	5.82	7.90
	8.00	7.82	0.50	52.97	6.83	8.82
	8.18	4.07	0.38	37.43	3.30	4.83
CC	7.64	2.90	0.44	44.47	2.01	3.79
	7.82	5.19	0.53	49.43	4.14	6.25
	8.00	4.28	0.47	53.06	3.34	5.22
	8.18	2.63	0.35	37.42	1.92	3.33
S-ISS	7.64	2.31	0.56	44.33	1.18	3.43
	7.82	3.22	0.68	49.59	1.86	4.59
	8.00	3.68	0.61	54.93	2.45	4.90
	8.18	3.34	0.46	37.44	2.40	4.27
HCC	7.64	0.41	0.87	45.38	-1.35	2.17
	7.82	1.75	0.84	48.72	0.07	3.44
	8.00	1.67	0.89	56.30	-0.12	3.47
	8.18	1.89	0.65	37.69	0.57	3.21
ISS-USVs	7.64	5.40	0.80	43.29	3.79	7.01
	7.82	7.80	0.93	47.17	5.93	9.67
	8.00	6.80	0.92	52.97	4.96	8.64
	8.18	4.03	0.66	37.19	2.71	5.36

Table xviii: DRVL GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	12.29	0.42	50.23	11.45	13.12
CC	12.30	0.40	53.05	11.51	13.10
S-ISS	12.21	0.50	53.90	11.21	13.22
HCC	13.21	0.71	62.42	11.80	14.62
ISS-USVs	15.37	0.74	44.24	13.89	16.85

Table xix: DRVL GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
7.64	12.73	0.47	43.04	11.78	13.69
7.82	14.45	0.50	49.77	13.45	15.45
8.00	13.57	0.38	55.31	12.82	14.32
8.18	11.56	0.45	37.43	10.66	12.47

Table xx: DRVL GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	7.64	11.40	0.79	43.18	9.81	12.99
	7.82	13.28	0.80	48.81	11.66	14.89
	8.00	12.94	0.60	53.22	11.74	14.14
	8.18	11.54	0.73	37.43	10.06	13.01
CC	7.64	12.12	0.71	43.03	10.69	13.56
	7.82	13.36	0.81	51.02	11.73	14.99
	8.00	13.79	0.57	53.23	12.65	14.92
	8.18	9.95	0.68	37.43	8.58	11.32
S-ISS	7.64	12.84	0.90	42.98	11.03	14.66
	7.82	12.42	1.04	52.04	10.34	14.50
	8.00	12.74	0.73	55.88	11.28	14.20
	8.18	10.85	0.89	37.44	9.04	12.66
HCC	7.64	14.70	1.42	43.21	11.83	17.56
	7.82	13.58	1.30	50.02	10.97	16.19
	8.00	12.78	1.06	57.77	10.66	14.90
	8.18	11.79	1.27	37.42	9.22	14.35
ISS-USVs	7.64	12.60	1.28	42.73	10.03	15.17
	7.82	19.60	1.45	47.86	16.69	22.51
	8.00	15.60	1.10	53.22	13.39	17.81
	8.18	13.69	1.26	37.42	11.13	16.25

Table xxi: DRC cFos/GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	2.56	0.23	50.97	2.09	3.02
CC	2.03	0.21	52.73	1.60	2.46
S-ISS	1.83	0.26	54.59	1.30	2.36
HCC	0.98	0.37	51.85	0.24	1.71
ISS-USVs	1.79	0.42	49.90	0.95	2.63

Table xxii: DRC cFos/GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	2.29	0.27	55.00	1.75	2.83
8.54	2.55	0.34	52.83	1.88	3.23
8.72	1.15	0.18	43.40	0.80	1.51

8.90	1.35	0.24	40.37	0.87	1.83

Table xxiii: DRC cFos/GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	2.63	0.46	55.00	1.70	3.55
	8.54	4.21	0.55	52.65	3.11	5.31
	8.72	1.39	0.30	43.70	0.79	1.99
	8.90	2.00	0.41	40.36	1.17	2.84
CC	8.36	3.10	0.42	55.00	2.25	3.95
	8.54	2.50	0.52	52.68	1.47	3.54
	8.72	1.43	0.30	44.22	0.83	2.02
	8.90	1.09	0.34	40.01	0.39	1.78
S-ISS	8.36	2.77	0.51	55.00	1.75	3.79
	8.54	2.46	0.61	52.35	1.24	3.68
	8.72	1.19	0.37	44.34	0.44	1.93
	8.90	0.90	0.48	40.41	-0.08	1.88
HCC	8.36	1.57	0.70	55.00	0.18	2.97
	8.54	0.99	0.97	53.35	-0.97	2.94
	8.72	0.35	0.46	42.80	-0.58	1.27
	8.90	1.00	0.52	39.48	-0.05	2.05
ISS-USVs	8.36	1.40	0.82	55.00	-0.25	3.05
	8.54	2.60	0.98	52.35	0.63	4.57
	8.72	1.40	0.50	42.66	0.39	2.41
	8.90	1.75	0.79	40.60	0.16	3.34

Table xxiv: DRC GAD Treatment Estimated Marginal Means

				95% C.I.	
Treatment	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	4.59	0.31	49.84	3.96	5.22
CC	4.75	0.29	53.14	4.18	5.33
S-ISS	4.77	0.36	53.86	4.04	5.49
HCC	4.82	0.48	48.28	3.85	5.78
ISS-USVs	5.30	0.56	49.08	4.17	6.43

Table xxv: DRC GAD Estimates Estimated Marginal Means

				95% C.I.	
Stereotaxic Level	*Mean*	*Std. Error*	*df*	Lower Bound	Upper Bound
8.36	5.89	0.41	55.18	5.08	6.71
8.54	4.56	0.33	52.25	3.89	5.23
8.72	4.48	0.30	43.23	3.87	5.08
8.90	4.45	0.31	40.46	3.82	5.08

Table xxxiii: DRC GAD Estimated Marginal Means Interaction

					95% C.I.	
Treatment	Bregma	*Mean*	*SEM*	*df*	Lower Bound	Upper Bound
ISS-No-USVs	8.36	5.38	0.69	55.14	3.99	6.77
	8.54	5.19	0.54	52.10	4.10	6.28
	8.72	4.38	0.51	43.17	3.35	5.40
	8.90	3.43	0.55	40.50	2.33	4.53
CC	8.36	6.11	0.64	55.53	4.83	7.38
	8.54	3.61	0.51	52.18	2.59	4.64
	8.72	4.37	0.51	43.86	3.35	5.39
	8.90	4.92	0.45	40.14	4.01	5.84
S-ISS	8.36	5.69	0.77	55.14	4.15	7.24
	8.54	4.85	0.60	52.09	3.64	6.05
	8.72	3.90	0.63	43.83	2.64	5.17
	8.90	4.62	0.64	40.62	3.33	5.90
HCC	8.36	5.29	1.05	55.14	3.19	7.39
	8.54	3.78	0.97	52.36	1.84	5.71
	8.72	4.93	0.78	43.20	3.37	6.49
	8.90	5.29	0.69	39.39	3.89	6.68
ISS-USVs	8.36	7.00	1.24	55.14	4.51	9.49
	8.54	5.40	0.97	52.09	3.46	7.35
	8.72	4.80	0.85	42.52	3.08	6.52
	8.90	3.99	1.04	40.68	1.89	6.10