Diurnal Variation of the Effect of an Acute Stressor on an Anxiety-like Behavior

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Abstract

Prolonged exposure to uncontrollable aversive stimuli has been shown to elicit a range of behavioral alterations in rats that are representative of an anxiety-like phenotype. One of the common anxiety-like behaviors is a reduction in juvenile social interaction following stress exposure, mediated by the sensitized release of serotonin from the dorsal raphe nucleus onto the basolateral amygdala. Serotonin production and regulation has been shown to be under circadian control, therefore we sought out to examine whether diurnal variations in stressor susceptibility exist. Rats were exposed to a series of inescapable tail-shocks during either their active or inactive phase, then juvenile social interaction behavior was assessed revealing that the expected reduction in juvenile social interaction following stress did not occur in animals stressed during their active phase. mRNA expression of tryptophan hydroxylase (TPH2), the rate-limiting enzyme in serotonin production, and expression of the serotonin inhibitory autoreceptor (5-HT1AR) gene peaked during the inactive phase. Corticosterone concentrations following stress did not differ based on time of day, however stress during the inactive phase relative to the active phase resulted in significantly greater activation of serotonergic neurons in the middle-dorsal raphe nucleus, but the same level of generalized activation of the basolateral amygdala. While these results indicate that an inescapable stress-induced anxiety-like behavior is time of day dependent, they do not supply a clear mechanism for the diurnal variation in stress susceptibility. Additional experiments are needed to assess cell-type specific activation within the basolateral amygdala and diurnal variations in other dimensions of the learned helplessness paradigm.

Introduction

Exposure to prior stress is common in the etiology of several psychiatric disorders including post-traumatic stress disorder (PTSD), depression, and anxiety (DSM-V). Additionally, the degree of control that an individual has over a stressor can modify the behavioral outcomes resulting from the stressor (Southwick et al., 2005). Uncontrollable stressors have been shown to result in a number of human adaptation failures, including depression, poor work performance, illness, or even early death (See Peterson et al., 1993 for review). Therefore it is important to understand the neurological underpinnings that contribute to stressor susceptibility. Large portions of the United States workforce, about 15 million people, are considered 'shift workers' who perform their job at night (Bureau of Labor Statistics, 2014). Considering several factors can contribute to individual stress resilience, it is important to understand the implications of exposure to stress at different times of day.

In rodents, it has been shown that prolonged exposure to uncontrollable stressors can result in a wide range of behavioral alterations, often termed 'learned helplessness' (Maier & Watkins, 2005). These behavioral changes are the result of robust activation of the dorsal raphe nucleus (DRN) (Grahn et al., 1999), a deep brain-stem nuclei responsible for producing serotonin (5-HT) in the brain. The robust activation of the DRN results in sensitization of its activity (Christianson et al., 2010), causing future stress exposure to result in exaggerated activation of this circuit, promoting anxiety like behavior (Amat et al., 1998). Diurnal fluctuations in 5-HT production and release in rats have been observed (Cagampang et al., 1993), and diurnal variations in DRN activation have been

demonstrated in other rodents (Janušonis & Fite, 2001), therefore diurnal variations in stressor susceptibility may exist.

The present study was designed to determine whether the inescapable shock (IS)-induced reduction of juvenile social interaction, an indicator of anxiety, was dependent on the time of day during which the stress occurred. The expression pattern of genes involved in the synthesis and regulation of 5-HT activity was assessed and stress induced activation of the DRN to basolateral amygdala (BLA) pathway mediating this behavior was also analyzed using Fos expression in each region. Furthermore, since corticosterone has been shown to regulate TPH2 rhythmicity and stress responses, corticosterone levels before and after stress at different times of day were also examined.

Background

Learned Helplessness

The phenomena of 'helplessness' was first experimentally demonstrated in animals when researchers at the University of Pennsylvania restrained dogs in a harness and delivered a series of electrical shocks to their paws. They expected that the shocks would induce fear, which would manifest in a faster escape from subsequent electrical shock in a shuttlebox apparatus, however to their dismay, many of the dogs did not try to escape and passively accepted the subsequent electrical shock. This serendipitous finding intrigued other researchers who thought that the passivity observed after shock was a phenomenon within itself (Maier & Seligman, 2016). The shocks delivered in the harness apparatus were by definition inescapable. By testing a variety of parameters (e.g. duration, intensity, number of trials), a following study demonstrated that this behavioral

result of passivity, accepting the electrical shocks without resistance, was a general phenomenon that was the result of exposure to inescapable shock (Overmier & Seligman, 1967).

Although the performance in the shuttlebox was the only quantitative measure collected after shock exposure in these early studies, the researchers noticed what they called 'large qualitative behavioral differences' in the animals that received inescapable shock (Overmier & Seligman 1967). The untreated dogs would vocalize and run frantically until escaping shock in the shuttlebox, however the dogs that were exposed to inescapable shock started off acting the same way during treatment, but then quickly stopped vocalizing and moving agitatedly, starting to passively accept the shock in the shuttlebox. The inescapable shock therefore caused the animals to develop behavior that was maladaptive in nature.

Several theories were put forth to explain what was causing this maladaptive behavior pattern. To dispel the theory that animals lacked sufficient motivation to escape the shuttlebox, researchers increased the intensity of the shock in the shuttlebox, thereby increasing the motivation to escape, and the same general phenomena of passivity was observed (Overmier & Seligman, 1967). To dispel theories that maladaptive motor responses were learned in the harness that translated to a poor escape performance, the animal's motor activity was pharmacologically inhibited while in the harness and the same phenomena was once again observed. Overmier and Seligman therefore theorized that the interference in acquiring an escape response in the shuttlebox was a result of 'learned helplessness'; that all instrumental responses used by the animal in an attempt to escape the shock were ineffective in eliminating or reducing the severity of the shock,

causing the animal to give up on trying any instrumental responses that might aid in escaping future shock.

In the conclusion of their 1967 study, Overmier and Seligman suggested that the degree of control allowed to the animal over the shock might be the determining factor in the behavior following shock. They hypothesized that if the animal was granted control over the shock presentation, that this control might immunize the animal against interference in acquiring escape responses. To test this, three groups of animals were needed; one that received no shock as a baseline comparison, one that was secured in the harness and received the usual inescapable shock (IS), and one that was in the harness and could terminate the electrical shock by pressing their nose against the panel used to secure their head, indicating escapable shock (ES) (Seligman & Maier 1967). It was important that animals experiencing ES and IS received shock of the same duration, intensity, and temporal distribution so that the only differentiating factor was control; therefore animals were 'yoked'. This meant that the onset/offset, duration, and number of trials were identical for animals in both groups, however only the responses of the ES animal terminated the shock. In this paradigm, they observed the expected interference in escape-response in the IS group, however the ES group performed identically in the shuttlebox to the group that didn't receive shock. This led researchers to suggest that differential learning about the control over the adverse stimuli occurred within these two groups, further supporting the theory that the animals that received IS had developed 'learned helplessness'.

As time went on, research shifted from dogs to rats and the same triadic design was employed to investigate the behaviors resulting from IS. In rodents, a series of tail-

shocks were delivered instead of paw-shocks and the ES animals could terminate the shock by turning a wheel. Studies conducted over the following several decades demonstrated that IS, compared to ES, resulted in a wide range of behavioral changes other than just impaired escape response acquisition. These included reduced juvenile social interaction (Short & Maier, 1993, Christianson et al., 2008), potentiated fear conditioning (Baratta et al., 2007), increased freezing (Maier, 1990) and reduced water and food intake (Maier & Watkins, 2005), to name a few. These behaviors were indicative of an anxiety-like phenotype where anxiety is defined as fear that is exaggerated compared to what is appropriate for a given situation.

Since exposure to IS was shown to result in a wide range of behavioral outcomes not seen after ES, researchers began attempting to dissect the neural basis driving these behaviors. Searching for a circuit that was responsible for helplessness was the first step in understanding the phenomena. According to a fifty-year retrospective review on the topic of learned helplessness, Maier and Seligman indicated that they noticed that most of the behavioral sequelae resulting from uncontrollable stress involved either fight/flight responses or fear/anxiety responses (Maier & Seligman, 2016). Using existing research they were able to pinpoint the dorsal periaqueductal grey (dPAG) as the mediator of the fight/flight responses and the regions of the amygdala as the mediators of the fear/anxiety behavior (Graeff et al., 1996). Research indicated that uncontrollable stress resulted in decreased fight/flight responses and exaggerated fear/anxiety responses; therefore they sought out to find a structure that might mediate inhibition of the dPAG and excitation of the amygdala. After extensive analysis, they landed on the dorsal raphe nucleus (DRN) as the mediator. Projections from the DRN inhibit the dPAG and activate regions of the

amygdala (Graeff et al. 1996), therefore future research focused on discerning the role of the DRN in the behavioral outcomes of IS.

Role of the 5-HT system in inescapable stress

5-hydroxytryptamine (5-HT), also known as serotonin, is a monoamine neurotransmitter that influences circuits driving arousal, sensory processing, mood, and some forms of emotion (Nestler et al., 2009). 5-HT is generally viewed as an anxiogenic neurotransmitter since drugs or lesions that reduce 5-HT activity produce an anxiolytic effect whereas pharmacological activation of 5-HT receptors increases response suppression, indicating anxiety (Graeff et al., 1996). 5-HT is synthesized through a biosynthetic pathway that starts with dietary tryptophan, which gets hydroxylated by the enzyme tryptophan hydroxylase (TPH), then decarboxylated by another enzyme to produce 5-HT. TPH is the rate-limiting enzyme in 5-HT production and is transcribed and translated from two different genes; one of which, TPH2, is preferentially expressed in the brain. TPH2 mRNA expression is therefore commonly used as a marker of 5-HT producing cells in the brain (Nestler et al., 2009).

Most of the 5-HT utilized in the brain is produced by only several hundred thousand serotonergic-neurons located within the deep brain-stem raphe nuclei (Nestler et al., 2009). The largest of these nuclei is the dorsal raphe nucleus (DRN), which innervates the cortex, thalamus, striatal regions, amygdala, and the dPAG, among other regions (Vertes, 1991). More than half of the serotonergic neurons in a rat brain are located in the DRN (Jacobs & Azmitia, 1992) and 5-HT fibers project so extensively

throughout the brain that almost every neuron in the brain is innervated by a serotonergic fiber (Nestler et al. 2009).

To show that the DRN is crucial in the learned helplessness circuitry, Grahn et al. demonstrated that IS, not ES preferentially increased the expression of Fos in the 5-HT producing cells of the DRN two hours after stress (Grahn et al., 1999). Fos is a transcription factor synthesized from the immediate-early gene c-fos and is commonly used as an indicator of neuronal activation. When neurons are strongly activated, transcription factors such as Fos interact with DNA to initiate the transcription of other genes that can facilitate the cell's response to activation; therefore expression of Fos in a neuron following some manipulation indicates that that neuron was activated during that event (Grahn et al., 1999). The preferential activation of 5-HT neurons within the DRN during IS relative to ES primarily occurred in the mid-to-caudal regions of the DRN (Grahn et al., 1999), limiting future studies to the examination of these regions. Subsequent studies utilized in vivo microdialysis to measure the levels of 5-HT released from serotonergic neurons in the DRN. These studies demonstrated that IS, not ES produced a transient increase in extracellular 5-HT levels in the DRN (Maswood et al., 1998). Additional studies showed that lesions to the DRN eliminated the escape deficit in a shuttle box (Maier et al., 1993). Therefore exposure to IS causes a pronounced release of 5-HT in the DRN that is necessary to drive the behavioral outcomes of IS.

5-HT is known to modulate anxiety-like behaviors (Graeff et al., 1996) and the proximal brain region that mediates these behaviors is the amygdala (Maier & Watkins, 1998). Utilizing an anterograde tracer, it was shown that the DRN directly projects to the basolateral sub-region of the amygdala (BLA) (Vertez, 1991) and lesions of the BLA

eliminated fear conditioning in the shuttle box (Maier et al., 1993). Microdialysis studies demonstrated that IS increased 5-HT concentrations in the BLA (Amat et al., 1998) as well as other projection regions such as the nucleus accumbens shell (Bland et al., 2003). The BLA expresses 5-HT_{2c} receptors and blockade of these receptors prevents the reduction in juvenile social interaction following IS, while activation of these receptors can mimic anxiety (Christianson et al., 2010). 5-HT acting at 5-HT_{2c} receptors within the BLA therefore mediates the reduction of juvenile social interaction following IS.

The increase in extracellular 5-HT in the projection regions lasted for only a few hours (Amat et al., 1998, Bland et al., 2003), however the behavioral changes as a result of IS lasted much longer. Therefore a new question arose as to how transiently increasing 5-HT release locally in the DRN and within projection regions could drive behavioral changes that persisted well after 5-HT levels returned to baseline (Maier & Seligman, 2016). Amat and his colleagues demonstrated that stimulation of 5-HT neurons, which occurs during behavioral testing 24 hours later, released exaggerated amounts of 5-HT in the BLA after the animal had been exposed to IS (Amat et al., 1998). Therefore even though the extracellular rise in 5-HT as a result of initial stress exposure is transient lasting only a couple of hours, the subsequent activation of projection regions by the DRN is exaggerated for a longer period of time, indicating sensitization of the projection. The mechanism underlying this sustained increase in activity was answered by investigating the different 5-HT receptor subtypes.

5-HT can activate a number of receptor variants, some of which are excitatory and some inhibitory. 5-HT_{2c} G-protein coupled receptors are preferentially expressed in projection regions of the DRN and are generally excitatory, while 5-HT_{1a} inhibitory auto-

receptors are commonly located on the soma and dendrites of serotonergic neurons. 5-HT_{1a} autoreceptors therefore serve to regulate firing of serotonergic neurons, 5-HT release, and the synthesis of 5-HT (Nestler et al., 2009). It was clear from the microdialysis studies that DRN 5-HT neurons were sensitized and the 5-HT_{1a} inhibitory autoreceptor was a likely candidate for causing this sensitization. It was demonstrated that exposure to IS caused a rapid desensitization of the 5-HT_{1a} inhibitory autoreceptors within the DRN which decreased the inhibitory regulation of the DRN, sensitizing the DRN 5-HT cells (Rozeske et al., 2011). This resulted in exaggerated efflux of 5-HT in projection regions upon activation (Amat et al., 1998). Current theories about learned helplessness indicate that it is this sensitization of the DRN that results in the behavioral sequelae following IS (Maier & Seligman, 2016).

At this point, it was clear the presence or absence of control over the stressor resulted in differential activation of the DRN, however there was no known mechanism to describe why IS resulted in DRN activation but ES did not. The original theories put forth regarding learned helplessness predicted that first animals detected the presence of controllability or uncontrollability and that the animals that detected uncontrollability expected future adverse events to again be uncontrollable, preventing any attempts to escape in new situations. Research had demonstrated that excitation and sensitization of DRN neurons resulted in learned helplessness behaviors, however no part of this process indicated a detection of uncontrollability. Learned helplessness appeared to simply be the result of prolonged exposure to aversive stimuli. The idea of detection of control requires integrating information about motor responses and the presence or absence of shock and it was found that this complex integrative process is carried out by the major cortical

input to the DRN, the prelimbic region (PL) of the ventromedial prefrontal cortex (vmPFC) (Amat et al., 2006).

After nearly two decades of extensive research into the pathways between the PFC and the DRN and its projection regions, a newly formulated theory for explaining learned helplessness behavior emerged (Maier & Seligman, 2016). First, prolonged exposure to aversive stimuli results in excitation and sensitization of DRN 5-HT neurons producing increased 5-HT release in projection regions resulting in behaviors such as passivity and heightened anxiety. Second, when the animal has the ability to escape the aversive stimuli, the presence of control is 'detected' by the PL vmPFC, which sends projections to the dorsal medial striatium (DMS) and back. Separate populations of neurons within the PL that project to the DRN are then activated in order to 'act' on the detected control. These neurons activate γ-aminobutyric acid (GABA) inhibitory interneurons in the DRN shutting down overall DRN activation eliminating the behavioral outcomes that normally follow stressor exposure. Activation of the PL to DRN circuit results in the recruitment of plasticity products that strengthen the connection. This plasticity allows the animal to 'expect' control so that when they are exposed to future uncontrollable stressors, they can still react as if they have control, producing immunization (Christianson et al., 2014). This new theory indicates that it is the presence of control, not the absence of control that can be detected, indicating that passivity and anxiety are simply the result of prolonged exposure to uncontrollable aversive stimuli.

Circadian Regulation of 5-HT System

The suprachiasmatic nucleus (SCN) is a collection of the cell bodies in the anterior hypothalamus that is considered the 'master clock' for its circadian control over several physiological and behavioral processes. One of the methods by which the SCN regulates the rhythmicity of physiological responses is through its output to the paraventricular nucleus (PVN) of the hypothalamus, which regulates neuroendocrine activity. The PVN serves as the starting point for the hypothalamic-pituitary-adrenal (HPA) axis, which is widely involved in stress responses (Nelson, 2011). Stimulation of the adrenal gland by this axis results in the release of glucocorticoids, such as corticosterone in rats and cortisol in humans, into the blood stream, which can then modulate the activity of regions throughout the brain and body via activation of glucocorticoid receptors (GR). The circadian rhythmicity of SCN activity therefore drives circadian rhymticity of circulating corticosterone levels (Nelson, 2011).

GRs are expressed on serotonergic neurons in the DRN (Harfstrand et al. 1986) and diurnal fluctuations in levels of circulating glucocorticoids such as corticosterone have been shown to regulate TPH2 rhythmic expression (Malek et al. 2007). Rats normally exhibit a circadian corticosterone concentration profile with increasing levels around the light/dark transition, and adrenalectomizing rats removes this rhythmic concentration pattern. By supplementing adrenalectomized rats with corticosterone at the onset of the dark phase to mimic endogenous rhythms, the corticosterone rhythmicity is restored. This is achieved because rats do most of their drinking at the onset of the dark (active) phase, hence the spike in corticosterone seen at this time. In untreated rats, TPH2 mRNA also displays a rhythmic expression profile with increasing expression levels

occurring near the end of the light phase. Adrenalectomy abolishes TPH2 rhythmicity and reestablishing the corticosterone rhythm reestablishes the TPH2 rhythm, indicating a regulatory role of corticosterone over TPH2 expression (Malek et al., 2007).

Extracellular 5-HT within the DRN of rats has been shown to exhibit diurnal rhythmicity under light/dark conditions with a peak in extracellular 5-HT occurring during the early light phase and a trough occurring during the dark phase (Cagampang et al., 1993). When the animals were kept in complete dark conditions, a peak in 5-HT levels was still observed, just out of phase with the peak observed during light/dark conditions. This suggests that an endogenous pacemaker regulates 5-HT rhythmicity. Additionally, animals exposed to constant light after two days of complete darkness showed large increases in 5-HT levels after the lights came on, indicating that light can regulate 5-HT rhythmicity (Cagampang et al., 1993). The corticosterone rhythmicity may serve as the endogenous pacemaker (Malek et al, 2007), while studies have shown that a direct projection from the retina to the DRN exists (Shen & Semba, 1994), which likely serves as a source of photic input for the raphe system allowing light to control the 5-HT rhythm. Although diurnal variations in DRN activation have not been directly demonstrated in rats, significant diurnal Fos expression in the caudal ventral DRN of Mongolian gerbils has been observed (Janušonis & Fite, 2001). These animals also demonstrated diurnal rhythmicity of 5-HT within their DRN (Birkett & Fite, 2005) consistent with observation seen in rats, however Mongolian gerbils are not nocturnal. The combination of 5-HT diurnal rhythmicity seen in rats and the diurnal variation in DRN activation seen in other rodents suggest that the rat DRN may exhibit a diurnal pattern of activation.

Methods

Subjects

Male Sprague Dawley rats (275-400g) were housed in plastic cages with free access to food and water on a 12:12 light:dark cycle (lights on at 7 A.M, off at 7 P.M.). Zeitgeber time 0 (ZT0) refers to lights on. Rats were allowed at least 14 days to acclimate to light and housing conditions before experimental procedures began. All experiments strictly followed the guidelines of the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

Experimental Design

Experiment 1: Is inescapable stress-induced peripheral corticosterone time-of-day dependent?

Rats received IS either in the middle of the light (inactive) phase (ZT6) or in the middle of the dark (active) phase (ZT16). Home cage control (HC) rats were also tested at ZT6 and ZT16. Rats were then sacrificed either immediately or 24 hours after stress termination and trunk blood was collected to determine serum corticosterone levels (described below)

Experiment 2: Does expression of TPH2 or 5-HT1A mRNA follow a circadian time course?

1 mm^3 micro-punches of rat DRN tissue were collected from animals sacrificed at the beginning of the light phase (ZT0), the middle of the light phase (ZT6), the beginning of the dark phase (ZT12), and the middle of the dark phase (ZT18). Relative mRNA expression of TPH2 and 5HT1AR were measured in homogenized DRN tissue using qPCR.

Experiment 3: Is inescapable stress-induced reduction of juvenile social interaction time-of-day dependent?

Rats received IS either in the middle of the light (inactive) phase (ZT6) or in the middle of the dark (active) phase (ZT16). Home cage control (HC) rats were also tested at ZT6 and ZT16. Baseline juvenile social interaction was assessed 24 hours prior to treatments and then rats were tested again 24 and 36 hours following stress termination.

Experiment 4: Does inescapable stress-induced Fos expression of DRN 5-HT neurons or BLA neurons fluctuate diurnally?

Rats received IS either in the middle of the light (inactive) phase (ZT6) or in the middle of the dark (active) phase (ZT16). Home cage control (HC) rats were also tested at ZT6 and ZT16. Rats were sacrificed 90 minutes after the termination of stress and brain tissue was sectioned to assess the DRN and BLA for signs of neuronal activation.

General Methods

Stress Procedure

Animals in IS groups were placed in long Plexiglas tubes (8cm diameter, 18 cm length) with their tail taped to a rod extending from the back of the tube. Two copper electrodes were affixed approximately 1 in. apart around the animal's tail and covered in electrode gel, which were then connected to the shocking apparatus. A series of 100 tail-shocks was then delivered, with an inter-shock interval of 30-90s. The first 33 shocks were delivered at an intensity of 1.0 mA, the next 33 at 1.3 mA, and the final 34 at 1.6 mA. Home cage animals remained undisturbed in their housing conditions.

Corticosterone Quantification - ELISA

Trunk blood was centrifuged (14,000 x g for 10 min) and serum was collected. An enzyme immunoassay for corticosterone (Assay Designs Inc., Ann Arbor, MI) was run following manufacture guidelines. Serum samples were treated with a steroid displacement reagent before being diluted 1:40 using assay buffer. The lower and upper detection limits were 0.128 μg/dL and 80 μg/dL respectively and all samples fell within the range of detectability.

Quantitative real-time PCR (qPCR)

Primers included β-Actin (F: TTCCTTCCTGGGTATGGAAT and R: GAGGAGCAATGATCTTGATC), TPH2 (F: AAATATGGCCAGCCCATTCC and R: GCATGAGTGGGGTAGAGTTT), and 5HT1AR (F: ACGTTACTAGCATCTCCGAC and R: CTTGTTGAGCACCTGGTACA). RNA was extracted from DRN homogenates with TRIZOL reagent and 2 μg of RNA was isolated and reverse transcribed to cDNA using SuperScript III CellsDirect cDNA Synthesis System (Life Technologies). PCR

amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA) with a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Hercules, CA). Gene expression was analyzed in triplicate and expressed relative to β-Actin.

Tissue Preparation

In order to assess c-fos expression, rats were injected with an overdose of sodium pentobarbital. Once reflexes were no longer present (assessed with tail and paw pinch), rats were transcardially perfused with ice-cold 0.9% saline for 3 minutes followed by 4% paraformaldehyde for an additional 3 minutes. Brains were then extracted and post-fixed for 24 hours in 4% paraformaldehyde at 4ºC then cryoprotected in 30% sucrose for 48 hours. Brains were then flash-frozen at -35ºC for 90 seconds in isopentane. Coronal sections 18 μm thick were collected using a cryostat at -22ºC and were mounted on Superfrost Plus microscope slides and stored at -20ºC until immunohistochemical detection.

Juvenile Social Investigation

Baseline JSI tests were conducted 24 hours before stress procedures. Test animals were placed in a small plastic cage containing bedding and a wire lid. Experimental subjects were allowed to acclimate to the cage for 1 hour prior to introducing a juvenile male rat (21-35 d old) to the cage. An observer blind to the experimental conditions recorded the amount of time the experimental rat engaged in social investigative behaviors over 3 minutes. Behaviors that were scored include allogrooming, sniffing,

following, and pinning. JSI test were conducted 24 hours following stress for both the ZT6 and ZT16 groups and an additional test was conducted 36 hours later for the ZT6 group.

Immunohistochemistry – Fos Expression in 5-HT cells of DRN

Assessment of Fos expression was carried out with a Fos/5-HT double labeling immunofluorescence protocol. Sections representing the entirety of the dorsal raphe nucleus were selected for analysis. Tissue was thoroughly washed in phosphate buffered saline (PBS) and incubated overnight in 0.1M PBS containing 0.2% Triton-X with two primary antibodies; a rabbit polyclonal c-fos antibody (Santa Cruz Biotechnology, sc-52) and a sheep tryptophan hydroxylase antibody (EMD Millipore, AB1541, 1:200). Tissue was then washed in PBS with Triton-X before incubating in two secondary antibodies for three hours: a goat anti-rabbit Alexafluor 596 (1:500, Jackson ImmunoResearch), and a donkey anti-sheep Alexafluor 488 (1:500, Jackson ImmunoResearch). Slides were washed three times in 0.1M PBS and then coverslipped using a Vectashield mounting medium. The slides were then dehydrated using a series of increasing concentration ethanol baths and finally defatted with citrisolv before being cover-slipped with permount.

Immunohistochemistry – Fos Expression in BLA

Assessment of Fos expression was carried out with a standard DAB single labeling protocol. Sections representing the entire rostro-caudal BLA were sectioned for analysis. Tissue was thoroughly washed in phosphate buffered saline (PBS) and then

incubated for 90 minutes in a H_2O_2 solution prepared by diluting 30% H_2O_2 1:40 in 0.01M PBS containing 0.2% Triton-X. Tissue was then washed again in PBS before incubating overnight at RT in a PBS blocking solution containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.25% Triton-X and a rabbit polyclonal primary antibody (1:7500; Santa Cruz Biotechnology). After a series of PBS washes, tissue was incubated at RT for 2 hours in a biotinylated goat anti-rabbit IgG secondary antibody (1:200; AffiniPure). After another series of PBS washes, tissue was incubated for 1 hour at RT in the avidin-biotin complex (ABC) solution. Tissue was then washed thoroughly in 0.1M PB before carrying out the DAB reaction. The DAB solution was prepared by adding nickelous ammonium sulfate, cobalt chloride, and ammonium chloride as stain intensifiers as well as the DAB tablet, glucose oxidase, and beta-D-glucose. This reaction was allowed to proceed for 8 minutes until staining was evident, and then the reaction was stopped with a series of PBS washes. The slides were then dehydrated, defatted, and cover-slipped with permount.

Image Analysis - DRN

Images of the entire rostral to caudal extent of the DRN were taken using a bright-field microscope (Olympus BX61, Olympus America) and analyzed using CellSense Dimension software. All images were taken with a 10X objective. Separate images of rostral, middle, and caudal DRN were taken according to Grahn et al., 1999 at -1.36 mm from interaural for rostral, -1.00 mm for middle, and -0.70 mm for caudal. Fos-positive cells were identified as red roundish spots and 5-HT containing cells were identified as green roundish spots. Fos-positive 5-HT cells were therefore identified as red spots

within the green spots. Images were quantified using the program Fiji. Regions of interest for cell counting as well as lighting and contrast were kept consistent for every section.

Image Analysis – BLA

Images of the left and right BLA of each subject were collected with the same microscope and objective lens as before at -2.80 mm from bregma. The BLA region of interest was generated by affixing an inward facing oval to the ventral peak of the corpus callosum and all cells within this region were counted. Fos positive cells appeared as black roundish dots. Images were quantified using Fiji. The counts from the left and right BLA were averaged and used for statistical analysis.

Statistical Analysis

All data results are represented as mean ± standard error of the mean. Data analysis was carried out using Prism5 GraphPad Software using analysis of variance (ANOVA). F values for each ANOVA were reported and the threshold for statistical significance was set at $p<0.05$.

Results

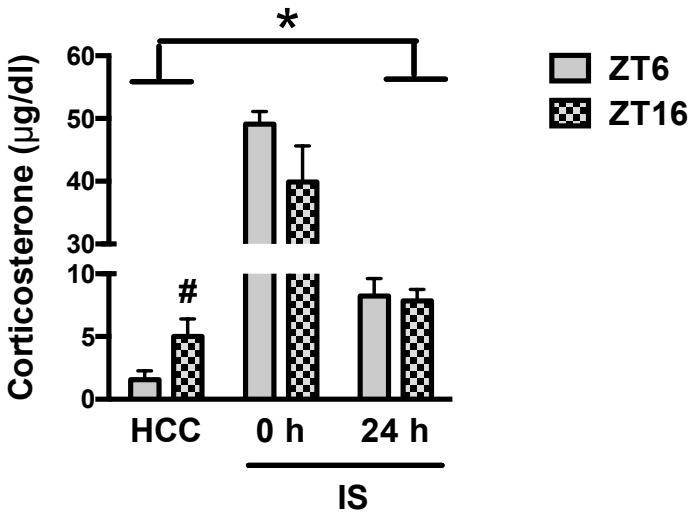

Figure 1. Inescapable stress-induced peripheral corticosterone is not time of day dependent. Interaction (Treatment x ZT): F (2, 31) = 3.790, $p = 0.0337$; Main effect (Treatment): F (2, 31) = 185.4 , $p < 0.0001$; Main effect (ZT) F (1, 31) = 1.196, $p = 0.2825$

Corticosterone concentrations in trunk blood was measured in HC animals at ZT6 and ZT16 and in IS animals immediately and 24 hours following exposure to IS at either ZT6 or ZT16. Basal corticosterone levels in HC animals differed significantly when measured at ZT6 versus ZT16 ($F(1,31)=1.196$, $p<0.0001$). An ANOVA indicated that immediately following IS, corticosterone levels did not differ significantly between ZT6 and ZT16 animals and that 24 hours following treatment, corticosterone levels did not differ between ZT6 and ZT16 groups. but were still significantly elevated with respect to HC ($F(2,31)=3.790$, $p=0.0337$).

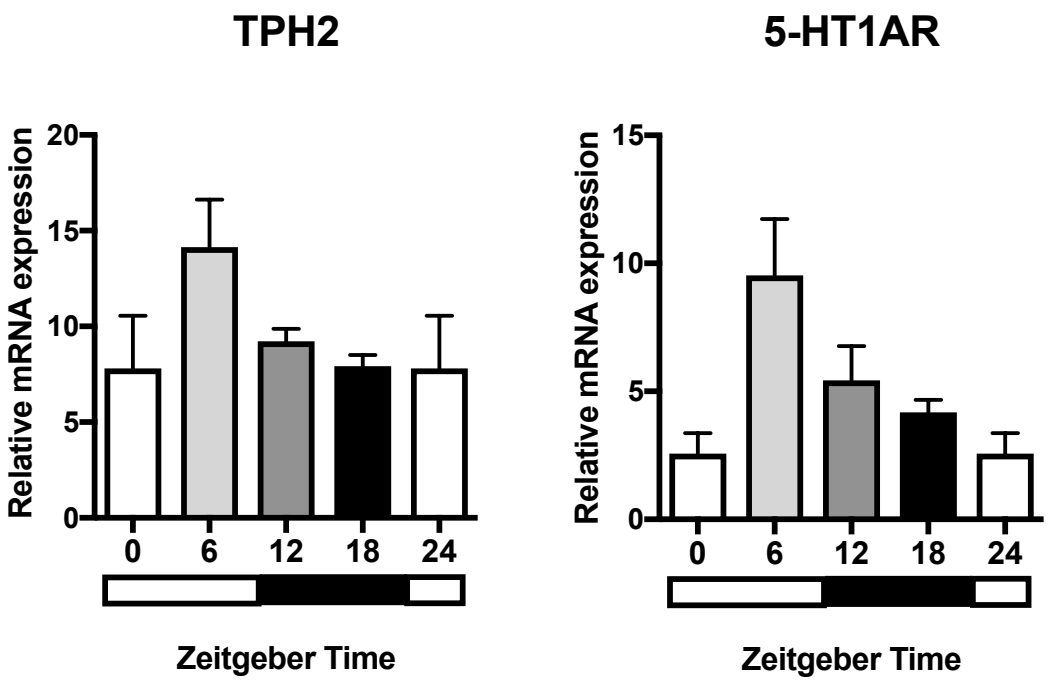

Figure 2. Circadian time course of TPH2 and 5HT1AR gene expression in the DRN. TPH2 ($F(3, 26) = 2.281$, $p = 0.1028$); 5HT1AR ($F(3, 26) = 4.337$, $p = 0.0132$)

TPH2 mRNA exhibited peak expression occuring during the middle of the light phase (ZT6). 5-HT1AR mRNA also displayed peak expression also occuring during the middle of the light phase (ZT6).

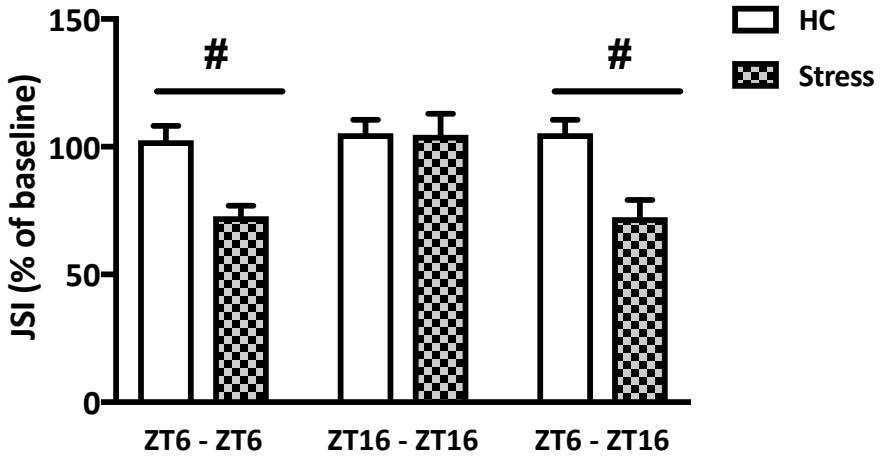

Figure 3. Inescapable stress-induced reduction in social investigation behavior is time of day dependent. Interaction (Treatment x ZT): F (1, 39) = 4.972, p = 0.0316; Main effect (Treatment): F (1, 39) = 14.54, p = 0.0005; Main effect (ZT): F (1, 39) = 0.7866, p = 0.3806)

IS occuring during the middle of the light phase (ZT6) resulted in a significant reduction of juvenile social interaction behavior compared to HC (p=0.0005), however IS occuring during the middle of the dark phase (ZT16) did not result in a significant change in juvenile social interaction relative to HC subjects. Animals exposed to IS at ZT6 also displayed significantly reduced social interaction 36 hours later.

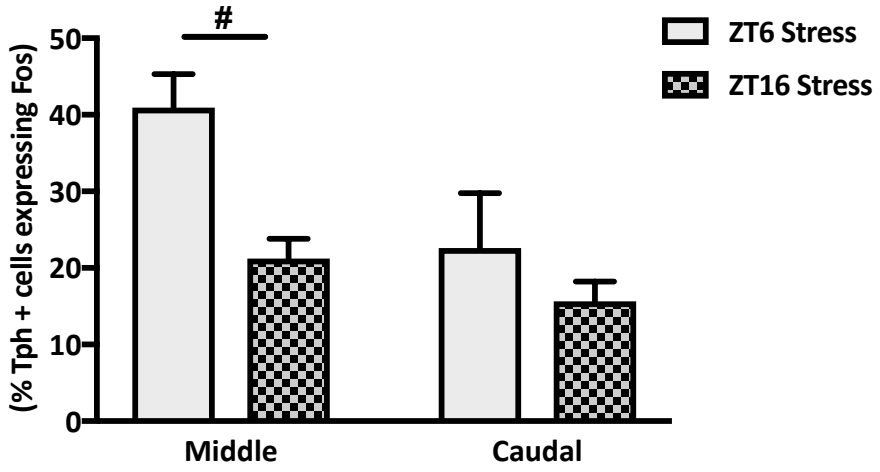

Figure 4. Inescapable stress-induced fos immunoreactivity of 5-HT producing cells in the DRN is time of day dependent. Significantly greater activation seen at ZT6 relative to ZT16 in middle-DRN. P=0.0016

Exposure to IS at ZT16 resulted in significantly less Fos immunoreactive 5-HT cells than did IS occuring at ZT6 in the middle DRN, demonstrated with a two-tailed t-test (p=0.0016). No significant difference in caudal DRN was observed (p=0.117)

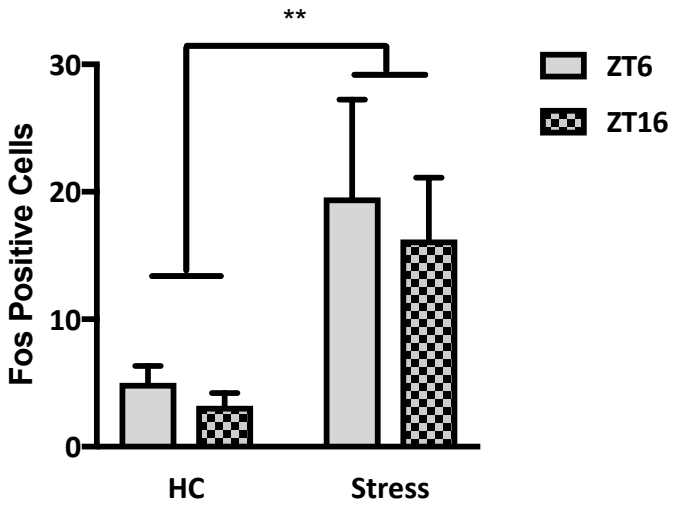

Figure 5. Inescapable stress induced Fos expression in the basolateral amygdala is not time of day dependent. Treatment ($F(1,27)=8.343$, $p=0.0075$), effect ZT ($F(1.27)=0.2847$, $p=0.5980$), Interaction ($F(1,27)=0.02553$, $p=0.8742$)

ANOVA revealed that exposure to IS at both ZT6 and ZT16 resulted in signicanlty increased Fos immunoreactivity in the BLA ($F(1,27)=8.343$, $p=0.0075$) however Fos expression did not differ significantly between animals tested at ZT6 versus ZT16.

Discussion

To determine whether a behavioral outcome of IS is time of day dependent, animals were tested in the juvenile social interaction (JSI) test 24 hours following exposure to IS at ZT6 and ZT16. Previous studies have demonstrated that exposure to IS results in an increase in anxiety-like behavior manifesting in decreased juvenile social interaction (Short & Maier, 1993, Christianson et al., 2008). Animals that were exposed to IS at ZT6, during their inactive phase, did indeed show the expected decrease in social interaction relative to baseline, however animals that were exposed to IS at ZT16, during their active phase, did not show any decrease in social interaction (Fig 3.). To ensure that

the decrease in social interaction was not simply a function of the time of testing, the ZT6 animals were again tested in the JSE test 36 hours following IS at the same time of day ZT16 animals would have been tested. These animals still displayed a reduction in social interaction 36 hours after the stressor (Fig 3.), indicating that IS-induced reduction in social interaction is time-of-day dependent.

In order to ensure that diurnal rhythmicity of corticosterone concentration did not influence the animal's response to the stressor, serum corticosterone concentrations were measured immediately and 24 hours following exposure to IS at both ZT6 and ZT16. Analysis of HC animals confirmed previous data that plasma corticosterone concentrations are higher in the dark phase, at ZT16, than in the light phase at ZT6 (Malek et al. 2007). Although basal corticosterone concentrations differed for each group at the time of IS, there were no significant differences in corticosterone concentrations immediately following exposure to IS at ZT6 versus ZT16, nor were there differences in corticosterone levels 24 hours later (Fig 1.). These results indicate that IS-induced corticosterone is not time-of-day dependent, and therefore is not likely to mitigate time-of-day differences in the behavioral outcomes of IS. These results are consistent with a recent review paper that summarized numerous studies showing that the maximum amount of stress-induced corticosterone is constant, regardless of stressor parameters (Spencer & Deak, 2016).

In an attempt to understand why the timing of the stressor might modulate the animal's behavioral response to the stressor, neuronal activation of components of the circuit that is involved in anxiety behavior was analyzed. It has been demonstrated that IS sensitizes neurons within the DRN through desensitization of 5-HT_{1a} inhibitory

autoreceptors and that this sensitization drives the passivity and anxiety following exposure to IS (Rozeske et al., 2011). This sensitization allows the DRN to release exaggerated amounts of 5-HT into its projection regions such as the BLA upon activation (Amat et al., 1998). Excessive 5-HT release into the BLA induces anxiety-like behavior, decreasing interaction during a JSI test. Therefore Fos expression in the BLA and in 5-HT producing neurons of the DRN were analyzed 90 minutes following IS at both time-points.

Exposure to IS resulted in a significant increase in Fos expression, and thus neural activation, in 5-HT producing cells in the middle DRN at both ZT6 and ZT16 relative to HC, however significantly more 5-HT neurons expressed Fos after IS at ZT6 than IS at ZT16 (Fig 4.). This indicates that IS more robustly activates serotonergic-neurons within the middle-DRN when the stress is experienced during the animal's inactive phase.

While IS resulted in significantly increased Fos expression in the BLA compared to HC at both time-points, the number of Fos-positive nuclei in the BLA did not depend on the time-of-day at which the IS was experienced (Fig 5.). It is difficult to draw any conclusions from the BLA results, however, because only generalized activation of the BLA and not cell-type specific activation was assessed. The BLA is comprised of large glutamatergic neurons and small GABAergic neurons that have a diversity of inputs and projections (McDonald, 1982) Therefore it cannot be determined whether there is time of day differences in IS-induced activation of BLA neurons driving anxiety responses without analyzing activation in the specific cell type responsible for this activity. Future studies will use double-labeling protocols to examine Fos expression in GABAergic neurons of the BLA, however even these analyses will become complicated as the

GABAergic neurons of the BLA come in a wide variety based on functional, neurochemical, and anatomical differences (Capogna, 2014). Other potential methods include examining Fos expression solely in neurons that express the 5-HT_{2c} receptor, which has been shown to be necessary for anxiety responses following IS (Christianson et al., 2010), or using in vivo microdialysis to measure extracellular 5-HT in the BLA during stress at both time-points.

The daily pattern of mRNA expression for the genes that encode TPH2, the rate-limiting enzyme in 5-HT production, and the 5-HT_{1a} inhibitory autoreceptor were analyzed in DRN tissue using qPCR with measurements conducted at ZT0, 6, 12, and 18 (Fig 2a). These measurements indicate that TPH2 mRNA expression peaks at ZT6, consistent with previous data demonstrating that TPH2 expression was highest during the light phase (Malek et al., 2007). The mRNA expression of 5-HT1AR also peaked at ZT6 in DRN tissue (Fig 2b). These results indicate that the production of 5-HT and the self-regulation of 5-HT neural activity are most active during the animal's inactive phase, which occurs during the day since rodents are nocturnal. It is possible that the heightened 5-HT1AR expression at ZT6 allows for substantially greater sensitization of the DRN, which would explain the differential IS-induced activation of DRN neurons. These results are not conclusive, however, because mRNA is not functional. There is a time-delay between the expression of mRNA and the actual translation of the corresponding protein, therefore the role of TPH2 and 5-HT1AR in regulating diurnal responses is ambiguous and protein expression levels need to be analyzed in future experiments.

Interestingly, animals stressed during their subjective active phase (ZT16) did not show reductions in social interaction indicative of anxiety, however they did display

significantly increased activation of serotonergic neurons within the DRN and neurons in the BLA compared to HC animals. This suggests one of two things: either there is a threshold of DRN activation after stress required to induce anxiety behaviors that is not achieved while animals are awake, or there is some other diurnal factor mitigating the observed behavioral resilience. It is possible that complete sensitization only occurs when 5-HT_{1a} receptors are saturated with 5-HT, and that saturation is not achieved at ZT16. Future studies could examine whether diurnal variations exist with respect to sensitization of the DRN 5-HT neurons, or within the PL to DRN circuitry driving expectation of control, to see if cognitive expectation of control over stressors varies diurnally.

All of these results indicate that animals might display more resilience to prolonged exposure to aversive stimuli while they are awake rather than asleep. For decades, learned helplessness paradigms have been carried out during the subjective day, which is when rodents are normally asleep, however these results suggest that this phenomena could be restricted to stress exposure during the inactive phase. However in order to claim that a manipulation, such as time-of-day, prevents learned helplessness, a demonstration that the manipulation reverses more than just one outcome of learned helplessness is needed (Maier & Watkins, 2005). To determine whether the entire phenomenon of learned helplessness is time of day dependent, more of the anxiety-like behaviors (such as fear conditioning) and more of the fight/flight related behaviors (such as food and water intake) would need to be analyzed, as well as activation of other parts of the circuitry involved in learned helplessness. Further analysis of the full behavioral

sequelae resulting from IS and more of the underlying circuitry is needed to determine if there are more diurnal variations in resilience to exposure to aversive stimuli.

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