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Acute Tryptophan Depletion Increases Translational Indices of Anxiety but not Fear: Serotonergic Modulation of the Bed Nucleus of the Stria Terminalis?

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Serotonin is strongly implicated in the mammalian stress response, but surprisingly little is known about its mode of action. Recent data suggest that serotonin can inhibit aversive responding in humans, but this remains underspecified. In particular, data in rodents suggest that global serotonin depletion may specifically increase long-duration bed nucleus of the stria terminalis (BNST)-mediated aversive responses (ie, anxiety), but not short-duration BNST-independent responses (ie, fear). Here, we extend these findings to humans. In a balanced, placebo-controlled crossover design, healthy volunteers ($n = 20$) received a controlled diet with and without the serotonin precursor tryptophan (acute tryptophan depletion; ATD). Aversive states were indexed by translational acoustic startle measures. Fear and anxiety were operationally defined as the increase in startle reactivity during short- and long-duration threat periods evoked by predictable shock (fear-potentiated startle) and by the context in which the shocks were administered (anxiety-potentiated startle), respectively. ATD significantly increased long-duration anxiety-potentiated startle but had no effect on short-duration fear-potentiated startle. These results suggest that serotonin depletion in humans selectively increases anxiety but not fear. Current translational frameworks support the proposition that ATD thus *disinhibits* dorsal raphé-originating serotonergic control of corticotropin-releasing hormone-mediated excitation of the BNST. This generates a candidate neuropharmacological mechanism by which depleted serotonin may increase response to sustained threats, alongside clear implications for our understanding of the manifestation and treatment of mood and anxiety disorders.

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INTRODUCTION

Serotonin is strongly implicated in mammalian stress responses, both healthy and pathological, but its precise neurobiological mode of action is surprisingly unclear. Neurocognitive and computational research suggests that serotonin inhibits aversive responses (Cools *et al*, 2008b; Crockett *et al*, 2009; Dayan and Huys, 2008, 2009; Robinson *et al*, 2011), but aversive responses are heterogeneous (Cassella and Davis, 1985; Davis *et al*, 1988; Joordens *et al*, 1996; Silva *et al*, 2004) and identifying the *specific* responses impacted by serotonin is critical. Particular questions exist concerning short- and long-duration aversive states, as they relate to pathological forms of fear and anxiety, respectively (Davis *et al*, 2010; Grillon, 2008b). Phobias, for example, are characterized by phasic responses to acute threats; such acute responses have been described with the term '*fear*'. Generalized anxiety disorder, in contrast, involves more sustained responses to uncertain threats (Grillon, 2008b); such sustained responses have been described with the term '*anxiety*'. In humans, the unique, differential role of serotonin in *fear* and *anxiety* remains minimally explored.

We used a well-established human startle paradigm to examine the impact of a serotonin manipulation on responses to phasic fear to an explicit threat cue (fear-potentiate startle) and anxiety to more sustained threatening *contexts* (anxiety-potentiated startle) (Davis *et al*, 2010; Grillon *et al*, 2011a). In both human and rodent research, explicit threat cues consist of cues that predict shocks, whereas threatening 'contexts' are conditions in which shocks are administered (Grillon *et al*, 2006a; Milad *et al*, 2007; Otto and Poon, 2006; Vansteenwegen *et al*, 2008). Explicit threat cues evoke *phasic* 'fear' responses because the associated threat is imminent and of short duration, while threatening contexts elicit more *sustained* 'anxious'

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responses. Sustained anxiety responses are, moreover, greatest in contexts associated with unpredictable compared with predictable shocks (Grillon *et al*, 2006a, 2004, 2011a).

This paradigm has two notable strengths. First, it has been well validated in humans. Anxiety-potentiated startle, but not fear-potentiated startle is increased in panic disorder and posttraumatic stress disorder (Grillon, 2008a; Grillon *et al*, 2009). Second, the task is translational by design and the procedures and responses have directly comparable rodent counterparts (Davis *et al*, 2010; Grillon, 2008b). Such work in rodents shows that (1) the bed nucleus of the stria terminalis (BNST) supports 'anxiety-potentiated-startle', while the medial central nucleus of the amygdala drives 'fear-potentiated startle' and (2) pharmacologically, anxiety but not fear is mediated by BNST corticotropin-releasing hormone (CRH) (Davis *et al*, 2010). Assessing the impact of serotonergic manipulations on human 'fear-' and 'anxiety'-related startle responding thus uniquely bridges basic and clinical research on the anxiety-stress relationship; allowing us to dissociate the impact of serotonin on fear and anxiety while placing observed effects within the context of a well-defined neuropharmacological framework.

Previous work with this paradigm in humans showed that elevating serotonin via semichronic administration of the selective serotonin reuptake inhibitor (SSRI) citalopram (Grillon *et al*, 2008a) reduces anxiety-, but not fear-potentiated startle, which is consistent with work in the rodent on the effects of serotonin augmentation (Cassella and Davis, 1985; Davis *et al*, 1988; Inoue *et al*, 2011; Joordens *et al*, 1996; Silva *et al*, 2004). Of note, work in the rodent also suggests that *reductions* in serotonin *increase* sustained anxiety responses (Burghardt *et al*, 2004; Davis *et al*, 2010; Klemenhagen *et al*, 2005; Miles *et al*, 2011). However, this work has not been extended to humans. Thus, translational findings point to a plausible, well-characterized mechanism whereby reduced serotonin might promote anxiety responses (Bhagwagar *et al*, 2002; Harmer, 2008; Murrough *et al*, 2011; Soubrié, 1986), but the same effect in humans remains to be demonstrated.

To rectify this, we used acute tryptophan depletion (ATD) to globally reduce serotonin function (Booij *et al*, 2003; Crockett *et al*, 2012; Young *et al*, 1985) and examined its impact on fear- and anxiety-potentiated startle (Schmitz and Grillon, 2012). We predicted that, in contrast with the effects of chronic SSRIs in humans (Grillon *et al*, 2008a), but consistent with the effects of serotonin reductions in rodents (Burghardt *et al*, 2004; Cassella and Davis, 1985; Davis *et al*, 1988, 2010; Joordens *et al*, 1996; Miles *et al*, 2011; Silva *et al*, 2004), ATD would selectively increase long-duration anxiety while having no effect on short-duration cue-specific fear.

MATERIALS AND METHODS

Participants

Participants were paid healthy volunteers who gave written informed consent approved by the NIMH Human Investigation Review Board and were free to withdraw from the study without penalty. Inclusion criteria included (1) no past or current psychiatric disorders as per Structured Clinical Interview for DSM-IV (SCID; First *et al*, 2002), (2) no history of a psychiatric disorder in any first-degree relatives; (3) no medical condition that interfered with the objectives of the study as established by a physician, and (4) no use of illicit drugs or psychoactive medications as per history and confirmed by a negative urine screen. Participants met with a psychiatrist before providing consent. Twenty-four subjects participated in the study but two did not return for the second session and two had large numbers of trials with no startle response and were excluded from the study. The final group consisted of 20 subjects (13 males) with a mean age of 25.1 years (SD = 5.6).

Procedure

The procedure was similar to that of our previous psychopharmacology studies examining responses to short- and long-duration threat of shocks (Grillon *et al*, 2004, 2008b, 2009) and is described in detail elsewhere (Schmitz and Grillon, 2012). Subjects participated in two identical testing sessions separated by at least 1 week, with order of sessions being randomized. Before arrival, subjects were asked to follow a low-protein diet (they were given detailed instructions on how to consume 10–15 g of protein) during the day before arrival. They then arrived at the research center between 0830 and 1030 h. A blood sample was taken, and subjects consumed the amino-acid tablets and the meal prepared by the metabolic kitchen. They were then allowed to consume water and were given a low-protein lunch. After a resting period of ~3.5 h to ensure low tryptophan levels (Carpenter *et al*, 1998), subjects moved to the psychophysiology laboratory where the equipment was set up (an ~45-min process) and they completed the startle paradigm. After this, a second blood sample was taken. It is conceivable that further depletion occurred during the testing period and that the depletion at this blood sample time point is somewhat greater than that observed mid experiment, but technical constraints meant that it was not possible to take a blood sample during the startle procedure.

Acute Tryptophan Depletion

A double-blind crossover design was implemented with each subject being exposed to each treatment—placebo and ATD—on separate sessions. The order of treatment was counterbalanced across subjects. On both visits subjects consumed 70 tablets and a meal (apple juice, apple sauce, vanilla pudding, decaf coffee, sugar, non-dairy creamer). On the depletion day, the tablets contained balanced amino acids minus tryptophan (4.2 g L-isoleucine, 6.6 g L-leucine, 4.8 g L-lysine, 1.5 g L-methionine, 6.6 g L-phenylalanine, 3.0 g L-threonine, 4.8 g L-valine), whereas on the placebo day the tablets contained 31.5 g lactose, and the food was mixed with Nestle Nutrition Beneprotein Whey Powder (Nestle, Vevey, Switzerland), which included 2.25 g tryptophan (see Supplementary information). On both sessions, subjects consumed lunch before completion of the startle paradigm (mashed potato with butter and non-dairy creamer, green beans with butter, salad (green leaf lettuce, celery, olive oil, and balsamic vinegar), apple sauce, vanilla pudding, cherry Italian ice, diet lemonade, salt, pepper), with Nestle Nutrition Beneprotein Whey Powder added on the placebo

day only. The two meals were given to avoid the effects of hunger, which are frequently observed during tryptophan-depletion studies and commercial nutritional supplement was provided on the placebo day (in contrast to some recent procedures; eg, Cools *et al*, 2008b; Robinson *et al*, 2011; and Robinson and Sahakian, 2009) because it successfully avoided the tryptophan and tryptophan ratio increase which is frequently observed on the placebo day.

Startle Paradigm

The electrodes to record the eyeblink/startle reflex were first attached and a startle habituation procedure consisting of nine startle stimuli (every 18–25 s) was conducted to reduce initial startle reactivity. The shock electrodes were then attached on the forearm and a shock workup procedure was initiated to set the shock intensity at a highly annoying level. Immediately after this, the threat experiment was started. It comprising three types of 150 s conditions, a no-shock condition (N), a predictable-shock condition (P), and an unpredictable-shock condition (U). In each condition, an 8-s cue was presented four times. The cues consisted of different geometric colored shapes for the different conditions. The cues were signals for a shock only in the P condition but had no signal value in the N and U conditions (see Figure 1 for a schematic).

Participants received precise verbal and written instructions regarding risk of shock in each condition, including the contingency between shocks and cues in P and U. Specifically, they were told that in the P condition shocks could be administered only in the presence of the threat cue and that in the U condition shocks could be administered at any time. Subjects were also informed that they could receive up to 12 shocks. This verbal instruction avoided potential interpretation confounds which would be associated with a non-instructed conditioning design. Instructions were also showed on a computer monitor throughout the experiment displaying the following information: 'no-shock' (N), 'shock only during shape' (P), or 'shock at any time' (U). In each N, P, and U condition, six acoustic startle stimuli were delivered: (1) three during intertrial intervals (ITIs; ie, in the absence of cues), one at times 15–52 s, a second at time 53–96 s, and a third at time 97–140 s following the beginning of a condition and (2) one during three of the four cues, 5–7 s following cue onset.

The threat experiment consisted of two series with a 5–10 min rest between series. Each series started with the delivery of four startle stimuli (pre-threat startle) and consisted of three N, two P, and two U in one of the following two orders: PNUNUNP or UNPNPNU. Each participant received both orders, with half the participants starting with P and the other half starting with U. One shock was administered in each individual P and U condition for a total of eight shocks. In each P, the shock was randomly associated with one of the four threat cue, being administered 7.5 s following the onset of that cue. The shock was given either 7 or 10 s following the termination of a cue in the unpredictable condition. No startle stimuli followed a shock by <10 s.

The Spielberger's state portion of the state-trait anxiety inventory questionnaire (Spielberger, 1996) was administered three times: (1) arrival (pre-treatment), (2) $\sim$3 h after ingestion of placebo/ATD, and (3) $\sim$1 h later, upon arrival in the psychophysiology laboratory. In addition, after each series, subjects retrospectively rated their anxiety level in the presence of the cues and in their absence (ITI) in each condition (N, P, U) on an analog scale ranging from 0 (not-at-all anxious) to 10 (extremely anxious). Immediately after the last recording, subjects were also asked to retrospectively rate the level of shock pain experienced during testing on an analog scale ranging from 0 (not-at-all painful) to 10 (extremely painful).

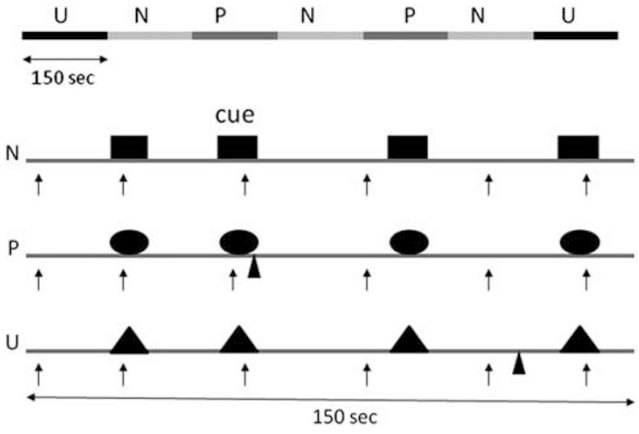

Figure 1 Schematic of the experiment. There were three conditions: no shock (N), predictable shock (P), and unpredictable shock (U). Each subject was presented with two series, each including three N, two P, and two U in each of the two orders (UNPNPNU as shown or PNUNUNP). Each N, P, and U condition contained four 8-s cues of different colors and geometric shapes (for illustration purposes, the cues are squares in N, circles in P, and triangles in U). In each P condition, a shock (indicated by ▲) was randomly associated with one of the four threat cues; it was administered 7.5 s after its onset. In each U condition, a shock was administered randomly in the absence of the cues. In the N condition, no shock was administered. Startle stimuli (indicated by ↑) were delivered in the presence and in the absence of the cue (ie, during intertrial intervals) (Grillon *et al*, 2011b) (reprinted by permission of Biological Psychiatry, 2011).

Stimuli and Physiological Responses

Stimulation and recording were controlled by a commercial system (Contact Precision Instruments, London, England). The acoustic startle stimulus was a 40-ms duration, 103-dB (A) burst of white noise presented through headphones. The eyeblink reflex was recorded with two electrodes placed under the left eye. The electromyographic (EMG) signal was amplified with bandwidth set to 30–500 Hz and digitized at a rate of 1000 Hz. The shock was administered on the left wrist.

Level of Amino Acids

Two blood samples were taken; one just as the subject arrived (T0) and a second immediately following the psychophysiological testing (T1; $\sim$5 h later). Plasma was spun out from the sample and frozen before analysis for free tryptophan and large neutral amino-acid (ΣLNAA) composition. Depletion was confirmed by comparing the change in the ratio between these two measures from the first to the second time point. Due to difficulties with blood drawing, these measures were unavailable for the placebo visit of one subject.

Data Analysis

The EMG eyeblink was rectified and smoothed using a 10-point moving average. Peak magnitude of the startle/blink reflex was determined in the 20–100 ms time frame following the stimulus onset relative to a 50-ms pre-stimulus baseline. The magnitude scores were (1) standardized into T-scores based on data across sessions within each participant, (2) averaged within each condition, and (3) analyzed with analyses of variance (ANOVAs) with repeated measures. Consistent with our previous studies, fear-potentiated startle and anxiety-potentiated startle were analyzed separately (Grillon *et al*, 2004, 2008b, 2009). Fear-potentiated startle was defined as the increase in startle magnitudes from ITI to the threat cue in the P condition. Anxiety-potentiated startle was defined as the increase in ITI startle reactivity from P to U and from N to U. Fear-potentiated startle during P was analyzed in a Stimulus Treatment (Placebo, ATD) × Stimulus Type (ITI, cue) ANOVA. Anxiety-potentiated startle was analyzed in a Treatment (Placebo, ATD) × Condition (N_{ITI}, P_{ITI}, U_{ITI}) ANOVA. Prior work has demonstrated that the U_{ITI} condition is considerably more anxiogenic than the P_{ITI} condition (Grillon *et al*, 2006a; Mineka and Hendersen, 1985). The variable 'order of treatment' was initially added to the models but because this variable did not significantly affect the results (no interaction effect with treatment) it was dropped from the analyses. Alpha was set at 0.05 for all statistical tests.

RESULTS

Depletion Check

A significant two-way ATD by time interaction was seen in the TRP/ΣLNAA ratio ($F(1,18) = 51.1$, $p < 0.0001$) driven by an 81.9% decrease in the TRP/ΣLNAA ratio between T0 (0.16) and T1 (0.03) on the ATD visit ($F(1,18) = 254$, $p < 0.0001$) but no significant change in the TRP/ΣNAA ratio between T0 (0.15) and T1 (0.19) on the BAL visit ($F(1,18) = 2.4$, $p = 0.14$). In addition, the T0 ratios were the same on both visits ($F(1,18) = 0.34$, $p = 0.57$), but T1 ratios were significantly decreased on the depletion *vs* placebo visit ($F(1,18) = 63$, $p < 0.0001$). All psychophysiological differences can thus be attributed to a tryptophan decrease on the depletion day.

Startle Magnitude

Table 1 shows the mean startle magnitude during the habituation, pre-threat, and during ITI and the cues in each condition.

Baseline startle. To examine the effect of treatment on baseline startle, the mean habituation and mean pre-threat startle magnitudes were compared between treatments in a Treatment (Placebo, ATD) × Phase (habituation, Pre-threat) ANOVA with repeated measures. There was a trend for baseline startle (Table 1) to be increased by ATD, but this effect failed to reach significance ($F(1,19) = 3.6$, $p = 0.07$).

Cued fear-potentiated startle. Fear-potentiated startle was defined as the increase in startle from ITI to the threat cue in the P condition (Table 1). Fear-potentiated startle was not affected by ATD. As expected (Grillon *et al*, 2006b), there was a Stimulus Type main effect ($F(1,19) = 43.5$, $p < 0.0009$), reflecting larger startle during the threat cue relative to ITI in P. This effect was not affected by ATD (Treatment × Stimulus Type interaction, $F(1,19) = 0.09$, $p = 0.76$).

Anxiety-potentiated startle. Anxiety-potentiated startle was defined as the increase in ITI startle magnitude from N_{ITI} to P_{ITI} and from N_{ITI} to U_{ITI}. Anxiety-potentiated startle was increased by ATD (Table 1; Figure 2). As expected (Grillon *et al*, 2006b), there was a Condition main effect ($F(2,38) = 31.5$, $p < 0.0009$), reflecting larger startle magnitude during P_{ITI} ($F(1,19) = 36.0$, $p < 0.0009$) and U_{ITI} ($F(1,19) = 51.5$, $p < 0.0009$) compared with N_{ITI} as well as larger U_{ITI} startle magnitude compared with P_{ITI} ($F(1,19) = 9.0$, $p < 0.007$). Importantly, there was a significant Treatment × Condition interaction ($F(2,38) = 4.5$, $p < 0.02$) that confirmed that anxiety-potentiated startle was impacted by ATD. Follow-up analyses showed that ATD increased anxiety-potentiated startle in both the P ($F(1,19) = 5.1$, $p < 0.03$) and the U ($F(1,19) = 7.0$, $p < 0.02$) conditions.

Subjective Anxiety, State Anxiety, and Pain

The retrospective ratings of anxiety are shown in Table 1 and the state anxiety and pain ratings are shown in Table 2. The state anxiety scores were analyzed with a Treatment

Table 1 Mean (SEM) Startle Magnitude (T Scores) During Startle Habituation and Pre-Threat

	Baseline		Neutral		Predictable		Unpredictable	
	Hab	Pre-threat	ITI	Cue	ITI	Cue	ITI	Cue
T scores								
Placebo	51.4 (2.3)	52.0 (1.9)	44.6 (0.8)	44.8 (0.8)	47.2 (0.9)	55.0 (1.5)	48.9 (1.1)	51.7 (1.2)
ATD	56.4 (2.1)	54.4 (1.9)	44.0 (0.8)	44.6 (0.9)	49.4 (1.2)	57.7 (1.5)	52.5 (1.1)	55.2 (1.5)
Retrospective subjective report								
Placebo	—	—	1.71 (0.2)	2.02 (0.3)	4.52 (0.6)	6.42 (0.4)	6.52 (0.5)	6.13 (0.5)
ATD	—	—	2.00 (0.3)	1.68 (0.3)	4.82 (0.6)	7.31 (0.4)	6.92 (0.5)	6.68 (0.5)

Mean (SEM) startle magnitude and retrospective subjective reports of fear and anxiety during the cue and ITI in each condition.

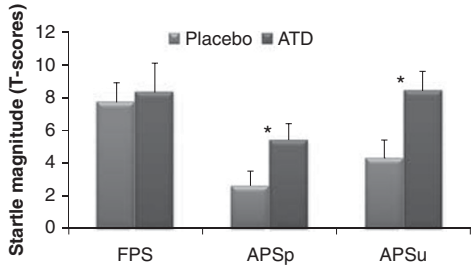

Figure 2 Fear-potentiated startle (FPS) and anxiety-potentiated startle scores on each of two within-subject treatment visits (placebo and ATD). FPS is the difference score between startle magnitude during the threat cue and during ITI in the predictable condition. Anxiety-potentiated startle scores were calculated for the predictable and unpredictable condition as the difference score between ITI startle magnitude in the predictable condition and the neutral condition (APSp) and the unpredictable condition and the no-shock condition (APSu), respectively. Anxiety-potentiated startle, but not fear-potentiated startle, was significantly increased by ATD compared with placebo. *Indicates a significant ($p<0.05$) effect. Error bars represent SEM.

(placebo, ATD) × Phase (baseline, post-treatment, laboratory) ANOVA. None of the main effects or interactions were significant (all $p>0.1$). The retrospective anxiety ratings were analyzed using similar ANOVAs as the startle data, first by examining fear in the P condition and then anxiety across N, P, and U. In the P condition, the Treatment (placebo, ATD) × Stimulus Type (ITI, CS) ANOVA revealed only a significant Stimulus Type main effect ($F(1,18)=32.7$, $p<0.0009$) with no significant differential effect of Treatment on fear. Retrospective anxiety during ITI in the N, P, and U conditions was analyzed using a Treatment (placebo, ATD) × Condition (N_{ITI}, P_{ITI}, U_{ITI}) ANOVA. The Condition main effect was significant ($F(2,36)=62.6$, $p<0.0009$), due to greater subjective anxiety during P_{ITI} *vs* N_{ITI} ($F(1,18)=32.6$, $p<0.0009$), U_{ITI} *vs* N_{ITI} ($F(1,18)=151.7$, $p<0.0009$), and U_{ITI} *vs* P_{ITI} ($F(1,18)=24.2$, $p<0.0009$). None of these effects were affected by ATD (all $p>0.1$).

DISCUSSION

Consistent with hypotheses, we found that reducing serotonin via ATD increased eyeblink startle-reflex potentiation to long-duration contextual threat but not short-duration explicit threat cues. This has key implications for our understanding of serotonin, which is strongly involved in the expression and treatment of mood and anxiety disorders, but which still has a largely unknown method of action. The present startle paradigm is a well-established translational paradigm recruiting defined neural and pharmacological substrates (which are largely beyond the temporal and spatial resolution of current functional imaging techniques in humans) and thus provides a clear framework in which to understand the impact of serotonin reduction on anxiety responding.

Our primary finding is that ATD significantly increased the sustained potentiation of startle (anxiety-potentiated startle) in the P and U conditions (P_{ITI} and U_{ITI}). This concurs with, and provides translational validity to, rodent findings (Burghardt *et al*, 2004; Davis *et al*, 2010; Klemenhagen *et al*, 2005; Miles *et al*, 2011), which, in turn, provide clues as to the neuropharmacological underpinnings of this effect. In particular, the anxiety-potentiated startle response is driven by a well-defined circuit between the BNST and the amygdala (Davis *et al*, 2010; Grillon, 2008b). As such, the findings indicate that a global reduction in serotonin brought about by ATD (Crockett *et al*, 2012) increases activity within this circuit. The precise mechanism by which this occurs is unclear, but one possibility is that it is driven by an interaction between serotonin and CRH, a stress hormone that has a well-defined role in modulating the BNST-linked startle circuit (Davis *et al*, 2010). In particular, during anxiety responses, the lateral central nucleus of the amygdala is thought to activate the BNST via excitation of amygdala-BNST CRH neurons, which then drive increased BNST-mediated startle responses (Davis *et al*, 2010). However, these BNST-terminating CRH neurons receive innervation from dorsal raphe-originating serotonergic neurons (Phelix *et al*, 1992), which modulate this CRH response. In particular, serotonin reduction via ATD increases CRH (Tyrka *et al*, 2004) but increasing serotonin via chronic SSRI administration reduces CRH (de Bellis *et al*, 1993). Administration of hydrocortisone, which increases BNST CRH levels (Schulkin *et al*, 2005) also has an identical effect to the ATD effect seen here (Grillon *et al*, 2011b). As such, one possibility is that serotonin *inhibits* CRH release in BNST, which then prevents BNST-mediated startle potentiation during anxious responding (Figure 3). Consistent with this, *in-vitro* whole-cell patch-clamp electrophysiological findings have shown that serotonin in the BNST can, via 5-HT1A receptors, inhibit BNST activation (Levita *et al*, 2004), *especially* in the presence of CRH (Hammack *et al*, 2009). Indeed, CRH is thought to flip serotonin's role from a 5-HT2A, 5-HT2C, and 5-HT7-mediated excitatory role in the BNST to a 5-HT1A-mediated inhibitory role (Hammack *et al*, 2009). As such it is neither serotonin, nor CRH alone which mediates this effect, but the interaction between them. The reduction in anxiety-potentiated startle following an *increase* in serotonin (via chronic SSRI administration) (Grillon *et al*, 2008a) may therefore be driven by increased serotonin function inhibiting CRH release in the BNST, thereby reducing startle magnitude to sustained aversive contexts (Grillon *et al*, 2008a). The present effect of ATD, by contrast, may be the flipside of the same coin: reduced serotonin *disinhibiting* CRH-mediated anxiety responses. This neuropharmacological inhibitory mechanism concurs remarkably with recent neurocognitive and computational data which suggest that the role of serotonin is in the *inhibition* of responses toward aversive stimuli

Table 2 Mean Spielberger (1996) State Anxiety Ratings at each Time Point, and Shock Ratings (SEM)

	State anxiety			Subjective pain
	Baseline	Post-treatment	Psycho-physiology laboratory	
Placebo	26.7 (1.4)	27.6 (1.5)	27.6 (1.4)	5.8 (0.5)
ATD	28.0 (1.2)	28.2 (1.3)	30.0 (1.4)	6.2 (0.5)

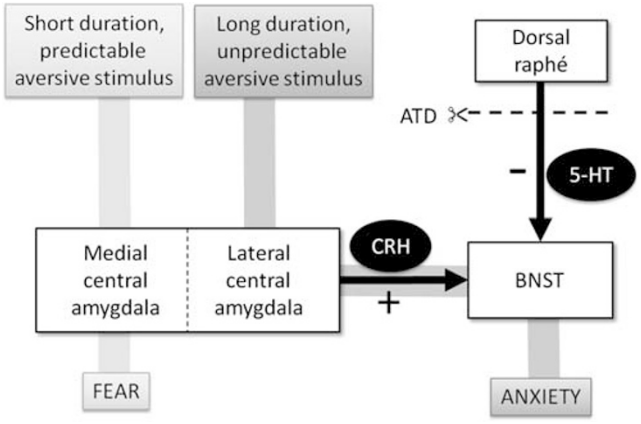

Figure 3 Proposed model of serotonergic action on anxiety. Corticotropin-releasing hormone (CRH)-induced excitation of the bed nucleus of the stria terminalis BNST (and subsequent anxiety responding) is inhibited by serotonergic (5-HT) neurons from the dorsal raphé nucleus (Hammack *et al*, 2009). Acute tryptophan depletion (ATD) removes this inhibition and allows for increased CRH-induced, BNST-driven anxiety responding, while having no effect on fear responding. Disruption of this serotonergic inhibitory mechanism may be what leads to the increased aversive responding in anxiety disorders and depression (Cools *et al*, 2008a; Dayan and Huys, 2009; Grillon, 2008b). '+', excitatory connection and '−', inhibitory connection.

(Cools *et al*, 2008b; Crockett *et al*, 2009; Dayan and Huys, 2009; Robinson *et al*, 2011). However, these psychological findings did not stratify punishment by the nature of the threat (ie, short *vs* long duration) or adopt such directly translational paradigms. The present findings thus extend these psychological findings to encompass a specific subset of *sustained duration* aversive responding and, via translation, a specific neural circuit which may be disinhibited by serotonin reduction (Robinson *et al*, 2011). In addition, these findings may be related to the dorsal raphe-dependent serotonergic 'inhibitory avoidance' mechanism proposed by Graeff and colleagues (Zangrossi Jr *et al*, 2001).

By contrast, serotonin reduction has no effect upon fear responding as measured by short-duration startle potentiation to the explicit threat cue. This lack of significant impact on fear-potentiated startle demonstrates that the effect of serotonin reduction is not a generic increase in startle potentiation. Rather, the disinhibitory effect of ATD on anxiety-potentiated startle is restricted to the BNST and CRH-mediated sustained anxiety circuit. Thus, serotonin may have no effect on the *medial* central nucleus of the amygdala-mediated fear-potentiated startle response (Davis *et al*, 2010; Figure 3). Critically, although there are CRH receptors in the amygdala, this circuit has also been shown to *not* depend upon CRH (de Jongh *et al*, 2003; Toufexis *et al*, 2004). This therefore provides additional support for the proposition that serotonin exerts its influence over anxiety responding by mediating CRH in the BNST. Of course, more work is necessary to fully clarify this mechanism, but the well-established nature of the present psychophysiological paradigm provides us with a head start with regards to the underlying neuropsychopharmacology (Davis *et al*, 2010; Grillon, 2008b). Indeed, the size of structures like the BNST and amygdala subunits, alongside the temporal differences between fear and anxiety responses, puts them largely beyond the current resolution of functional magnetic resonance imaging. As such, the *only* way to assess these questions at present may be via the use of such translational paradigms.

Taken together, these findings have implications for our understanding of psychiatric disorders. In particular, the present dissociation may explain why serotonergic medications are more likely to benefit individuals demonstrating symptoms of anxiety rather than fear (ie, generalized anxiety disorders rather than phobia, for which the first-line treatments are SSRIs and cognitive behavioral therapy, respectively; McNaughton and Corr, 2004). Moreover, these findings have implications for our understanding of resilience to mood and anxiety disorders. Specifically, serotonin is thought to promote resilience to such disorders through its role in the inhibition of aversive responses (Robinson, 2011; Robinson *et al*, 2011). The present data suggest that one way in which this protective role is exerted is via the inhibition of CRH-mediated BNST responses to uncertain aversive stimuli. In healthy individuals, this serotonergic inhibition of anxious responses may be achieved via top-down control of raphe nuclei by the prefrontal cortex (Amat *et al*, 2005; Passamonti *et al*, 2012), which results in serotonergic inhibition of CRH anxiogenic action in the BNST. From a psychological perspective, this putative serotonergic circuit could plausibly afford a means to avoid worrying about sustained threats that one cannot predict or control (indeed the Amat *et al* (2005) reference argues that this top-down inhibition may depend upon controllability of the aversive stimulus, which is strongly intertwined with predictability; Mineka and Hendersen, 1985). As such, one hypothesis might be that when serotonin is depleted in depression (Bhagwagar *et al*, 2002; Harmer, 2008) or in anxiety (Murrough *et al*, 2011; Soubrié, 1986), the lack of such protection leads to long-duration ruminatory aversive thoughts (Cooney *et al*, 2010; Nolen-Hoeksema, 2000) and responses (Grillon, 2008b). Thus, this serotonergic prefrontal-raphe-BNST circuit may prove a more precise target for the treatment of mood and anxiety disorders (Insel *et al*, 2010; Sanislow *et al*, 2010), but this will of course require further study.

As a potential caveat, it is important to note that previous work with this task showed that *acute* SSRI administration increased some anxiety responses (Grillon *et al*, 2006c) but also increased fear responding. This is distinct from the effects of chronic SSRI administration (Grillon *et al*, 2008a) that reduced anxiety responses. This discrepancy is partially consistent with the proposition that SSRIs have, somewhat paradoxically, opposite effects when administered acutely and chronically (Bhagwagar *et al*, 2002). It has been argued that this may be because acute SSRIs actually serve to *reduce* serotonin levels via autoreceptor feedback effects (Chamberlain *et al*, 2006). However, this effect is far from clear and acute SSRIs can, in fact, immediately induce some of the same neurocognitive effects as chronic SSRIs (for a review, see Harmer, 2008). As such, the anxiogenic effects of acute SSRIs may not necessarily be serotonergically mediated. This would explain why, unlike ATD, acute SSRI administration also increased fear responding, and why a closer reciprocity was seen between ATD and chronic SSRI treatment (which both have relatively more defined effects

on serotonin). Further research is, however, necessary to understand the neurochemical effects of acute SRRIs, especially on distinct 5-HT receptor subtypes (Zangrossi Jr *et al*, 2001), before we can draw too many conclusions. On a related note, the impact of ATD upon serotonin has recently been drawn into question (van Donkelaar *et al*, 2011; but see Crockett *et al*, 2012) so this close reciprocity between the effects of ATD in the present study and chronic SSRIs in our previous study provides additional support for the serotonergic basis of ATD.

As a final caveat, it should be noted that our depletion technique was somewhat novel, and although it achieved its primary aim of depleting tryptophan ratio on the depletion day (while having no significant effect upon the placebo day), the possibility that subjects were able to subjectively distinguish the sessions, or that the depletion was not at its maximal point during testing cannot be fully ruled out. This seems unlikely, given the correspondence between the amino-acid analysis and the reflexive (ie, non-subjective) startle measure, the lack of an effect of treatment on subjective measures, and the clear correspondence between the present effects and those of chronic SSRIs, but it is worth considering.

Conclusions

The present study extends prior rodent work by providing the first evidence that a global reduction in the serotonin precursor tryptophan can increase psychophysiological concomitants of anxiety but not fear in humans. This clarifies that the role of serotonin is not only in the inhibition of aversive responses, but also in the inhibition of responses to *sustained* aversive stimuli. As such, we argue that the reduced serotonin in disorders like depression and anxiety may exert their pathological effects via the disinhibition of serotonergic dorsal raphé control of CRH-mediated BNST-driven anxiety responses (Figure 3). This further finesses our understanding of the role of serotonin in emotional processing and provides a more precise target for treatment and detection of mood and anxiety disorders. The mechanism by which one of the most ubiquitous neurotransmitters contributes to the treatment and manifestation of mood and anxiety disorders is still largely mysterious and given the enormous emotional, social, and financial cost of these disorders such clarification is of clear value.

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