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## Elevated *tph2* mRNA Expression in a Rat Model of Chronic Anxiety

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### Abstract

**Background**—Allelic variations in TPH2, the gene encoding tryptophan hydroxylase 2, the rate-limiting enzyme for brain serotonin (5-HT) biosynthesis, may be genetic predictors of panic disorder and panic responses to panicogenic challenges in healthy volunteers. To test the hypothesis that *tph2* mRNA is altered in chronic anxiety states, we measured *tph2* expression in an established rat model of panic disorder.

**Methods**—We implanted 16 adult, male rats with bilateral guide cannulae and then primed them with daily injections of the corticotropin-releasing factor (CRF) receptor agonist, urocortin 1 (UCN1, 6 fmoles/100 nl per side, n = 8) or vehicle (n = 8) into the basolateral amygdaloid complex (BL) for 5 consecutive days. Anxiety-like behavior was assessed, 24 hr prior to and 48 hr following priming, in the social interaction (SI) test. A third group (n = 7) served as undisturbed home cage controls. All rats were killed 3 days after the last intra-BL injection to analyze *tph2* and *slc6a4* (gene encoding the serotonin transporter, SERT) mRNA expression in the dorsal raphe nucleus (DR), the main source of serotonergic projections to anxiety-related brain regions, using in situ hybridization histochemistry.

**Results**—UCN1 priming increased anxiety-related behavior in the SI test compared to vehicle-injected controls and elevated *tph2*, but not *slc6a4*, mRNA expression in DR subregions, including the ventrolateral DR/ventrolateral periaqueductal gray (DRVL/VLPAG), a subregion previously implicated in control of panic-related physiologic responses. *Tph2* mRNA expression in the DRVL/VLPAG was correlated with increased anxiety-related behavior.

**Conclusion**—Our data support the hypothesis that chronic anxiety states are associated with dysregulated *tph2* expression.

## Keywords

anxiety/anxiety disorders; biological markers; mood disorders; panic attacks/agoraphobia; stress

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## Introduction

Anxiety disorders, including social anxiety disorder<sup>[11]</sup> and panic disorder,<sup>[2]</sup> are common emotional disorders, significantly impair overall quality of life, and affect about 19% of the male, and 30% of the female population at some point during life.<sup>[3–5]</sup> The neural mechanisms underlying the manifestation of anxiety disorders are complex. However, two interconnected neural systems commonly dysregulated in patients with anxiety disorders are the extended amygdala<sup>[6, 7]</sup> and midbrain serotonergic systems.<sup>[8, 9]</sup>

Stressful life events are likely a major determinant of vulnerability to affective and anxiety disorders.<sup>[10–12]</sup> Corticotropin-releasing factor (CRF) and CRF-related neuropeptides acting at CRF type 1 (CRF<sub>1</sub>) receptors are thought to play an important role in mediating the effects of perceived stress on anxiety-related systems.<sup>[13–15]</sup> Systemic injections of CRF<sub>1</sub> receptor antagonists are anxiolytic following acute intra-basolateral amygdaloid complex (BL) injections of the CRF<sub>1</sub>/CRF<sub>2</sub> receptor agonist<sup>[16]</sup> urocortin 1 (UCN1) and following repeated restraint, suggesting that CRF<sub>1</sub> receptors mediate UCN1- and stress-induced increases in anxiety-related behavior.<sup>[17]</sup> When injected into the BL, a key region controlling anxiety states,<sup>[18,19]</sup> UCN1 increases anxiety-related behavior in rats.<sup>[20]</sup> Under chronic stress conditions, CRF receptor-mediated plasticity within the BL contributes to the development of chronic anxiety states.<sup>[21–24]</sup> Repeated activation (priming) of CRF receptors within the BL with low-dose UCN1 results in a robust anxiety-like state that persists for more than 30 days in rats,<sup>[25]</sup> as confirmed by reduced social interaction (SI) time in the SI test and panic-like physiologic responses, including tachycardia and increased respiratory rate, after intravenous administration of sodium lactate,<sup>[20,25]</sup> an agent used as a research probe for panic disorder in human studies.<sup>[26–28]</sup> In contrast, a single injection of the same “sub-threshold” UCN1 dose is insufficient to alter the animals' emotional state,<sup>[25]</sup> indicating the need for BL-neuronal plasticity before a behavioral phenotype is evident.

Family and twin studies have consistently found that genetic factors explain approximately 48% of the variance in panic disorder,<sup>[29]</sup> and serotonergic genes are important candidate genes for vulnerability to panic disorder.<sup>[30]</sup> Tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme for brain serotonin (5-HT) synthesis.<sup>[31]</sup> *TPH2* allelic variants appear to be genetic predictors of panic disorder itself,<sup>[32,33]</sup> susceptibility to panic attacks,<sup>[34]</sup> and comorbidity between panic disorder and mood disorders<sup>[35]</sup> (but see <sup>[36,37]</sup>). These studies are consistent with the hypothesis that allelic variation in *TPH2* is associated with increased vulnerability to panic disorder.

Selective serotonin reuptake inhibitors (SSRIs) are common, effective treatments for panic disorder<sup>[38]</sup> that target the serotonin transporter (SERT). A number of studies have found no association between a functional promoter polymorphism (serotonin transporter-linked polymorphic region (5-HTTLPR)) of *SLC6A4* (the gene encoding SERT) and panic disorder.<sup>[39–44]</sup> However, recent studies suggest that allelic variation in the *SLC6A4* 3'

untranslated region may be associated with panic disorder<sup>[45, 46]</sup> whereas, allelic variation in the 5-HTTLPR may be associated with susceptibility to panic attacks in healthy volunteers.<sup>[47]</sup>

Given the functional interactions between the BL and the serotonergic dorsal raphe nucleus (DR),<sup>[48]</sup> evidence for elevated brain serotonin turnover in panic disorder patients<sup>[49]</sup> and for dysfunctional serotonergic systems in a rat model of panic disorder,<sup>[50]</sup> we here test the hypothesis that *tph2* and *slc6a4* expression are dysregulated in a rat model of chronic anxiety with vulnerability to panic-like responses to sodium lactate.<sup>[20, 25]</sup> To test this hypothesis, we conducted sham- or UCN1 priming in the amygdala to generate a chronic anxiety state, and measured *tph2* and *slc6a4* mRNA expression within the DR using *in situ* hybridization histochemistry (ISHH).

## Materials and Methods

### Animals

Adult male Wistar rats (250–300 g in weight) from Harlan Laboratories (Indianapolis, IN) were habituated to the new facility for one week, and housed singly under a 12:12 hr light cycle (lights on at 07:00 h) at 22°C with *ad libitum* access to food and water. Sixteen rats were used for intra-BL priming, 16 age-matched conspecifics were used as partner rats in the SI test, and seven rats were kept undisturbed as home cage controls. All animal procedures were conducted at Indiana University Purdue University at Indianapolis School of Medicine (Indianapolis, IN) following IACUC committee approval, and were consistent with the *Guide for the Care and Use of Laboratory Animals*.<sup>[51]</sup> All efforts were made to minimize animal numbers and suffering.

### Experimental Design

We utilized an established rat model of chronic anxiety, characterized by increased anxiety in the SI test and panic-like physiologic responses following intravenous infusions of sodium lactate.<sup>[20, 25]</sup> The experimental timeline is illustrated in Figure 1. Under deep isoflurane anesthesia (MGX Research Machine; Vetamic, Rossville, IN), 16 rats were implanted with one stainless steel guide cannula per brain hemisphere (26 ga, Cat. No. C315GS-4, Plastics One, Roanoke, VA) above the BL at the following coordinates,<sup>[20]</sup> anterior/posterior: –2.1 mm bregma; lateral from midline (bilateral): ±5.0 mm bregma; depth from skull surface: –8.5 mm bregma (Fig. 2),<sup>[52]</sup> incisor bar at –3.3 mm. After surgery, guide cannulae were sealed with dummy cannulae (Plastics One), and rats were allowed to recover. Six days later, experimental rats were habituated to the SI arena, and tested in the SI test on the following day (Day 0) to measure their baseline anxiety-related behavior.<sup>[53]</sup> Starting one day afterwards, rats received daily, bilateral intra-BL injections of vehicle (1% bovine serum albumin, Cat. No. A2153, Sigma-Aldrich, St. Louis, MO, in sterile 0.9% saline,  $n = 8$ ) or the CRF receptor agonist UCN1 (Cat. No. U6631, Sigma-Aldrich; 6 fmoles UCN1/100 nl vehicle per side,  $n = 8$ ) into the BL at 09:00 h for 5 consecutive days.<sup>[20]</sup> This intra-BL priming paradigm produces long-lasting anxiety-like behavior in the SI test and panic-like physiologic responses following lactate challenge, whereas a single bilateral injection of the same “sub-threshold” dose of UCN1 is not

sufficient to exert anxiogenic actions.<sup>[20, 25]</sup> This increased anxiety-like state with vulnerability to panic-like responses is thought to be dependent on N-methyl-D-aspartic acid (NMDA) receptor-dependent neuronal plasticity in the BL.<sup>[25]</sup> Two days after the last injection, the rats were tested in the SI test again to confirm the development of a chronic anxiety-like state. We did not challenge rats with sodium lactate in this study as our previous studies have shown that sodium lactate,<sup>[50]</sup> but not exposure to the SI test,<sup>[48]</sup> profoundly activates DR serotonergic neurons, and therefore may, by itself, influence serotonergic gene expression. Home cage control rats remained undisturbed during the duration of the experiment ( $n = 7$ ). One day after the postpriming SI test, all rats were killed between 09:00 and 10:00 h via rapid decapitation. Their brains were collected, shipped overnight on dry ice to the University of Colorado Boulder (Boulder, CO), and stored there at  $-80^{\circ}\text{C}$  until analysis of *tph2* and *slc6a4* expression. We intentionally killed all rats 24 hr after the postpriming SI test, and not earlier, because certain behavioral tests using socially interactive paradigms themselves, such as social defeat, have been shown to increase rat *tph2* mRNA expression within 4 hr post-exposure.<sup>[54]</sup>

### SI Test

The SI test was conducted as described previously.<sup>[18, 19, 55, 56]</sup> Each experimental rat was exposed to an unfamiliar, age- and weight-matched, male partner rat for 5 min in a 91.5 cm  $\times$  91.5 cm solid wooden box with 30.5-cm high walls and an open top, under low-light conditions between 09:00 and 13:00 h. A video camera above the arena recorded the behavior for later analysis with “The Observer” (version 5.0, Noldus Information Technologies, Wageningen, The Netherlands). SI time was defined as any behavior initiated or fully participatory by the experimental rat: face-on contact, sniffing, following, grooming, crawling over or under the partner. No experimental rat was exposed to the same partner rat more than once. Decreased SI time is indicative of an anxiogenic treatment effect.<sup>[53]</sup>

### Brain Tissue Sectioning and Verification of Cannulae Placements

Forebrains were cryosectioned coronally at 30  $\mu\text{m}$  between approximately  $-1.40$  and  $-3.60$  mm bregma,<sup>[51]</sup> thaw-mounted onto Vista-Vision HistoBond microscope slides (VWR Scientific, West Chester, PA), stained with cresyl violet,<sup>[52]</sup> and cover-slipped with Entellan mounting medium (Cat. No. 14802, EMS, Hatfield, PA). Cannulae placements (Fig. 2) were identified with reference to a stereotaxic rat brain atlas.<sup>[52]</sup> Only rats with both injection sites within the BL region or just ventral of the BL (in which case UCN1 is expected to reach the BL along the cannula tract) were used for analysis of behavior and gene expression (Fig. 3). No outliers were identified. Hindbrains were cryosectioned at 12  $\mu\text{m}$  between approximately  $-7.58$  and  $-8.67$  mm bregma, and sections were thaw-mounted onto VistaVision HistoBond microscope slides (VWR Scientific).

### *tph2* and *slc6a4* *in situ* Hybridization Histochemistry (ISHH)

A 583-base pair fragment of *tph2* cDNA was transcribed and radiolabeled with [35-S]-uridine-5'-triphosphate (UTP, Cat. No. 5610301, MP Biomedicals, Santa Ana, CA) using T7 RNA-polymerase (Promega, Madison, WI) to create a cRNA probe complementary to bases 761–1343 of rat *tph2* mRNA. The probe was cleaned (RNeasy kit, Cat. No. 74104, Qiagen,

Valencia, CA), and used in an ISHH assay as described previously.<sup>[57]</sup> To detect *slc6a4* mRNA, a synthetic 50-base antisense oligonucleotide probe (5'-ACT GCA GAG TAC CCA TTG GAT ATT TGG CTA GGC TCT GCC CTG TCC GCT GT-3', Integrated DNA Technologies, Coralville, IA) complementary to bases 207–256 of rat *slc6a4* mRNA was used. The probe was labeled at the 3' end with [35-S]-deoxyadenosine-5'-triphosphate (dATP, Cat. No. 5620001, MP Biomedicals, Santa Ana, CA) using terminal deoxynucleotidyl transferase (TdT, 20 U/μl, Cat. No. EP0161, Fermentas, Glen Burnie, MD) for 1 hr at 37° C in a water bath, cleaned using a QIAquick nucleotide removal kit (Cat. No. 28304, Qiagen, Valencia, CA), and used in an ISHH assay as described previously.<sup>[58]</sup> Air-dried slides were apposed to a BioMax MR autoradiography film (Cat. No. 871 5187, Carestream Health, Rochester, NY) for 7 (*tph2*) or 11 (*slc6a4*) days, respectively.

### Semiquantitative Analysis of Gene Expression

Digital autoradiography images were analyzed with ImageJ (NIH, Bethesda, MD) while blinded to the treatment groups to measure gray value × area (equivalent to *tph2* or *slc6a4* mRNA expression) using matrices in the shape of each subdivision of the brainstem DR. Area (mm<sup>2</sup>) was defined as the area (within each matrix) that fell above a gray value threshold that was kept consistent throughout each gene's analysis. Based on Gardner et al.<sup>[54]</sup> and Abrams et al.,<sup>[59]</sup> a total of 14 rostro-caudal sections, designated levels +7 to –6 (Fig. 4), containing seven major subdivisions of the DR were analyzed (rostral aspect of the dorsal raphe nucleus, dorsal part, rDRD, –7.580 to –7.916 mm bregma; rostral aspect of the dorsal raphe nucleus, ventral part, rDRV, –7.580 to –7.916 mm bregma; caudal aspect of the dorsal raphe nucleus, dorsal part, cDRD, –8.000 to –8.336 mm bregma; caudal aspect of the dorsal raphe nucleus, ventral part, cDRV, –8.000 to –8.504 mm bregma; dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region, DRVL/VLPAG, –7.832 to –8.504 mm bregma; dorsal raphe nucleus, caudal part, DRC, –8.420 to –8.672 mm bregma; dorsal raphe nucleus, interfascicular part, DRI, –8.420 to –8.672 mm bregma<sup>[52]</sup>). An average value was computed for the DRVL/VLPAG using values from both the left and right hemisphere. The background of each image, measured in the surrounding midbrain tegmentum, was subtracted from each value. Throughout the 14 rostrocaudal levels, values for each of the seven DR subdivisions were averaged, and average *tph2* and *slc6a4* expression levels in the entire DR were also calculated for each rat.

### Statistical Analysis

Data were analyzed using PASW (version 19.0, SPSS Inc., Chicago, IL) after elimination of statistical outliers.<sup>[60]</sup> SI time was analyzed using a repeated-measures analysis of variance (ANOVA) with *treatment* (vehicle or UCN1) as the between-subjects factor, and *time* (baseline versus postpriming SI test) as the within-subjects factor, and Fisher's protected least significant difference (LSD) tests where appropriate. To determine if vehicle priming altered either *tph2* or *slc6a4* mRNA expression, relative to home cage controls, and if UCN1 priming altered *tph2* or *slc6a4* mRNA expression, relative to vehicle-primed rats, we conducted two separate repeated-measures ANOVAs, using *treatment* as the between-subjects factor and *DR subdivision* as the within-subjects factor. Where appropriate, Fisher's protected LSD tests were applied for each of the seven subdivisions of the DR to reveal subdivision-specific treatment effects on gene expression. To compare treatment effects on

the average *tph2* and *slc6a4* mRNA expression in the entire DR, two separate Student's *t*-tests were used to compare vehicle-primed rats with home cage controls, and UCN1-primed rats with vehicle-primed controls. Significance was accepted at  $P < .05$ . Values are shown as the mean + the standard error of the mean (SEM).

## Results

### Behavior in the SI Test

There was no difference in SI time between vehicle and UCN1 groups on Day 0, prior to priming, but 48 hr after the final intra-BL priming injection, the UCN1-treated rats displayed increased anxiety-like behavior in the SI test, relative to vehicle-treated controls (Fig. 5; *treatment*,  $F_{(1, 14)} = 3.73$ ,  $P = 0.07$ ; *time*,  $F_{(1, 14)} = 3.65$ ,  $P = 0.08$ ; *time*  $\times$  *treatment*,  $F_{(1, 14)} = 12.11$ ,  $P < 0.01$ ). Also, UCN1-primed rats had significantly less SI time on Day 7 compared to the initial test on Day 0 ( $t = 4.02$ ,  $P < 0.01$ ), whereas vehicle-primed control rats displayed no difference in SI time compared to Day 0.

### *tph2* mRNA Expression

There were no differences between *tph2* expression in vehicle-primed rats and home cage control rats (*treatment*,  $F_{(1, 13)} = 1.28$ ,  $P = 0.28$ ; *subdivisions*,  $F_{(6, 8)} = 21.48$ ,  $P < .001$ ; *subdivisions*  $\times$  *treatment*,  $F_{(6, 8)} = 0.36$ ,  $P = 0.90$ ). UCN1 priming had a significant treatment effect on *tph2* mRNA expression compared to vehicle-treated rats (*treatment*,  $F_{(1, 14)} = 41.77$ ,  $P < 0.05$ ; *subdivisions*,  $F_{(6, 9)} = 32.02$ ,  $P < .001$ ; *subdivisions*  $\times$  *treatment*,  $F_{(6, 9)} = 2.42$ ,  $P < 0.05$ , Fig. 6). Fisher's protected LSD post hoc analyses of each of the seven DR subdivisions revealed significantly elevated *tph2* mRNA expression within the caudal DRV (cDRV,  $P < 0.05$ , Fig. 6D) and within the DRV/VLPAG of UCN1-treated rats compared to the vehicle group ( $P < 0.01$ , Fig. 6E). No other subdivision showed significant differences in priming-induced *tph2* expression relative to the vehicle-primed rats. *tph2* mRNA expression in the entire DR (average of all subregions) did not differ significantly between treatment groups ( $F_{(2, 20)} = 2.98$ ,  $P = .074$ ; data not shown).

### *slc6a4* mRNA Expression

No differences in *slc6a4* mRNA expression were detected between the vehicle-primed group and home cage controls (*treatment*,  $F_{(1, 13)} = 0.01$ ,  $P = 0.94$ ; *subdivisions*,  $F_{(6, 8)} = 26.61$ ,  $P < 0.001$ ; *subdivisions*  $\times$  *treatment*,  $F_{(6, 8)} = 0.34$ ,  $P = 0.91$ ; Fig. 7). Compared to vehicle-primed rats, UCN1 priming did not alter the expression of *slc6a4* mRNA in subdivisions of the DR (*treatment*,  $F_{(1, 14)} = 0.05$ ,  $P = 0.82$ ; *subdivisions*,  $F_{(6, 9)} = 37.98$ ,  $P < 0.001$ ; *subdivisions*  $\times$  *treatment*,  $F_{(6, 9)} = 2.42$ ,  $P = 0.90$ ; Fig. 7). *Slc6a4* mRNA expression in the entire DR (average of all subregions) did not differ between treatment groups ( $F_{(2, 20)} = 0.02$ ,  $P = 0.98$ ; data not shown).

### Correlation of Behavior and Gene Expression

To address the question of whether the UCN1-induced change in *tph2* mRNA expression, which presumably already existed during the time of the second behavioral SI test one day prior to the collection of the brains, was linked to the observed chronic anxiety state, correlation analyses of *tph2* mRNA expression in the DRV/VLPAG or cDRV and SI



behavior in the SI test of Day 7 were performed for both the vehicle- and the UCN1-primed rats (Fig. 8). Anxiety-like behavior during the second SI test was strongly correlated with *tph2* mRNA expression in the DRVL/VLPAG region ( $r = 0.73$ ,  $r^2 = 0.53$ ,  $P < 0.01$ ) but not the cDRV region ( $r = 0.46$ ,  $r^2 = 0.21$ ,  $P = 0.07$ ). Anxiety-like behavior during the second SI test was not correlated with *slc6a4* mRNA expression in either the DRVL/VLPAG or cDRV.

## Discussion

Intra-BL priming with UCN1, a previously characterized rat model of chronic anxiety with vulnerability to panic-like physiological responses to sodium lactate challenge (increases in blood pressure, heart rate, and respiratory rate),<sup>[20, 25]</sup> produced a robust anxiety-like state as measured in the SI test. In the same rats, *tph2* expression was significantly elevated 24 h following the SI test in both the cDRV and in the DRVL/VLPAG region. However, only *tph2* expression in the DRVL/VLPAG showed a strong, positive correlation with increased anxiety-like behavior in the post-priming SI test. No other DR subdivision revealed altered *tph2* expression.

Our finding that the priming-induced anxiety-like state is associated with increased *tph2* mRNA expression in the DRVL/VLPAG is consistent with other models of chronic anxiety states that also demonstrated site-specific increases in *tph2* mRNA expression in the DRVL/VLPAG. For example, adverse early-life experiences, such as maternal separation of neonatal rats, lead to a chronic anxiety-like state,<sup>[61]</sup> enhanced vulnerability to social defeat induced increases in *tph2* mRNA expression specifically within the DRVL/VLPAG region during adulthood.<sup>[54]</sup> Likewise, neonatal administration of *Salmonella* endotoxin (containing gram-negative lipopolysaccharide, LPS) to rat pups, which results in an increased anxiety state that lasts into adulthood,<sup>[62, 63]</sup> also elevates *tph2* mRNA expression in the DRVL/VLPAG, but not in other DR subdivision.<sup>[64]</sup> The similarities among these three individual rat models indicate that site-specific increases in *tph2* mRNA expression in the DRVL/VLPAG region may be a common feature of the development or maintenance of stress-induced chronic anxiety states.

Although we were not able to test both SI behavior and panic-like physiological responses in the present study, panic-like physiological responses have been detected when rats of the same intra-BL UCN1-priming paradigm were infused intravenously with sodium lactate<sup>[20,25]</sup>, an agent that is also used to probe for panic disorder in human studies.<sup>[27, 28, 65, 66]</sup> This vulnerability to panic-like responses following amygdala priming may be related to our finding that increased anxiety-like behavior was strongly correlated with elevated *tph2* expression in the DRVL/VLPAG, because several other lines of evidence suggest that DRVL/VLPAG serotonergic neurons play a role in control of panic-like responses. For example, an acute challenge with panicogenic stimuli such as sodium lactate or hypercapnia, or exposure to adverse stimuli such as forced swimming in cold water,<sup>[67, 68]</sup> strongly activates serotonergic neurons in the DRVL/VLPAG region, as indicated by increases in c-Fos expression.<sup>[50, 69–71]</sup> In studies using a different rat model of panic disorder,<sup>[26, 65, 66]</sup> we have shown that sodium lactate increases c-Fos expression in DRVL/VLPAG serotonergic neurons of control rats, but not in rats made panic-prone by

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disinhibition of the medial hypothalamus.<sup>[50, 72]</sup> These data suggest that the DRVL/VLPAG serotonergic neurons may in fact inhibit panic-like responses. The consequences of priming-induced increases in *tph2* mRNA expression in the DRVL/VLPAG for serotonergic neurotransmission are uncertain. Increased *tph2* expression in the DRVL/VLPAG may result in increased TPH2 protein, increased 5-HT synthesis, and increased 5-HT release in the DRVL/VLPAG and targets of DRVL/VLPAG serotonergic neurons. Alternatively, a selective increase in serotonergic neurotransmission in the DRVL/VLPAG itself could preferentially activate autoinhibitory 5-HT<sub>1A</sub> receptors, resulting in overall decreased serotonergic output from the DRVL/VLPAG to distal target sites. Finally, increased *tph2* mRNA expression could be a compensatory response to decreased serotonergic neural activity.

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The DRVL/VLPAG contains large, multipolar 5-HT neurons<sup>[73]</sup> that form part of a sympatho-motor command center, controlling both sympathetic outflow via neuronal signaling to the adrenal medulla and motor activity via innervation of hind limb muscles.<sup>[74]</sup> Consistent with these anatomic findings, the DRVL/VLPAG is thought to promote passive stress coping.<sup>[75, 76]</sup> Stimulation of the VLPAG region results in passive emotional coping, including behavioral quiescence, hyporeactivity to environmental stimuli, hypotension, and bradycardia.<sup>[75, 76]</sup> Therefore, dysfunction of this region could result in dysfunctional emotional coping strategies. One target region of the DRVL/VLPAG is the dorsal periaqueductal gray (DPAG), an area known to facilitate a panic/escape-like fight-or-flight response if disinhibited.<sup>[77]</sup> Activation of 5-HT<sub>1A</sub> receptors in the DPAG inhibits panic/escape-like fight-or-flight responses, and therefore dysregulation of serotonergic signaling in this region may lead to increased vulnerability to panic-like responses. The DRVL/VLPAG serotonergic neurons also project to the rostral ventrolateral medulla (RVLM<sup>[78]</sup>); stimulation of the DRVL/VLPAG region induces hypotension and sympathoinhibition, which can be prevented by activation of 5-HT<sub>1A</sub> receptors in the RVLM.<sup>[79, 80]</sup> The DRVL also projects to the perifornical region (PeF) of the lateral hypothalamus,<sup>[81, 82]</sup> a region that contains arousal-promoting orexin/hypocretin neurons implicated in anxiety disorders.<sup>[83]</sup> Orexin/hypocretin neurons appear to be tonically inhibited by serotonergic input from the DR under normal conditions.<sup>[82, 84]</sup> Thus, serotonergic neurotransmission arising from the DRVL/VLPAG acting in the DPAG, RVLM, and the PeF of the lateral hypothalamus acts to inhibit panic-like behavioral and autonomic responses; dysregulation of this serotonergic system would be predicted to cause disinhibition of those target areas, leading to panic and/or anxiety-like responses, including tachycardia, hypertension, and a state of hypervigilance or hyperarousal (see Fig. 9 for a hypothetical mechanistic model).

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Amygdala priming with UCN1 also increased *tph2* mRNA expression in the cDRV, and the correlation between *tph2* mRNA expression in the cDRV and anxiety-like behavior in the SI test approached statistical significance ( $P=0.074$ ). The cDRV contains large numbers of neurons projecting to anxiety-related forebrain structures, such as the BL.<sup>[85]</sup> If the hypothesis that increased *tph2* mRNA expression ultimately leads to disinhibition of target areas holds true, this could mean that increased *tph2* expression in the cDRV creates a positive feedback loop to the BL, increasing rather than inhibiting the effects of intra-BL priming. The DRV is also the main source of efferent projections to the sensorimotor



cortex,<sup>[86]</sup> and DR serotonergic neurons recently have been shown to encode specific sensorimotor events.<sup>[87]</sup> Thus, increases in *tph2* in the cDRV may influence encoding of specific sensorimotor events. This hypothesis is consistent with findings that panic disorder patients have altered processing of sensory information.<sup>[88]</sup>

The mechanisms underlying selective increases in *tph2* mRNA expression in the DRVL/VLPAG and cDRV region are not clear, but may involve activation of specific afferents. The BL does not give rise to direct projections to the DR. Most likely, UCN1 priming of the BL causes altered signaling to the central amygdaloid complex (CE), bed nucleus of the stria terminalis (BST), and/or the medial prefrontal cortex (mPFC), which are all known to innervate the DR, with strong input to the caudal DR and to the DRVL/VLPAG region,<sup>[89]</sup> and may thus alter *tph2* expression in the DRVL/VLPAG.

Our observation that expression of *slc6a4* mRNA was not altered by intra-BL priming with UCN1 is consistent with several studies that failed to find associations between allelic variation in *SLC6A4* and panic disorder.<sup>[39–44]</sup> Although SERT binding potential is higher in a number of brain regions in male panic disorder patients,<sup>[90]</sup> unfortunately, no studies have evaluated *TPH2* or *SLC6A4* mRNA expression in postmortem tissues from panic disorder patients. Our results are, however, inconsistent with a previous study investigating the effects of neonatal maternal separation on vulnerability to social stressors later in life. In this study, *slc6a4* mRNA was increased, again, specifically in the DRVL/VLPAG region of rats that had been exposed to a combination of maternal separation as newborns and acute social defeat as adults.<sup>[58]</sup> This may indicate that *slc6a4* expression is more vulnerable to later-life social stressors if a vulnerability was created earlier in life, for example during a critical developmental window, but that *slc6a4* expression is fairly resilient toward repeated stressors that have their first occurrence during adulthood.

## Conclusion

Our study reveals a potential neuroanatomical pathway by which overstimulation of CRF receptors in the BL, for example during chronic stress exposure,<sup>[21]</sup> could lead to dysregulation of serotonergic control of anxiety-related brain circuits. This neuronal pathway may contribute to the development of a chronic anxiety state, including vulnerability to panic. Our finding that a chronic anxiety state is associated with selective dysregulation of the DRVL/VLPAG subset of serotonergic neurons, a panic-related subset of serotonergic neurons that is highly sensitive to hypercapnia<sup>[70]</sup> and sodium lactate,<sup>[50]</sup> is consistent with Klein's suffocation false alarm theory of panic disorder.<sup>[91, 92]</sup> Finally, as suggested by Davies et al.,<sup>[93]</sup> dysregulation of DRVL/VLPAG serotonergic neurons may account for the association between hypertension and panic disorder. Further characterization of the DRVL/VLPAG group of serotonergic neurons may lead to novel therapeutic strategies for the treatment of anxiety disorders, including panic disorder.

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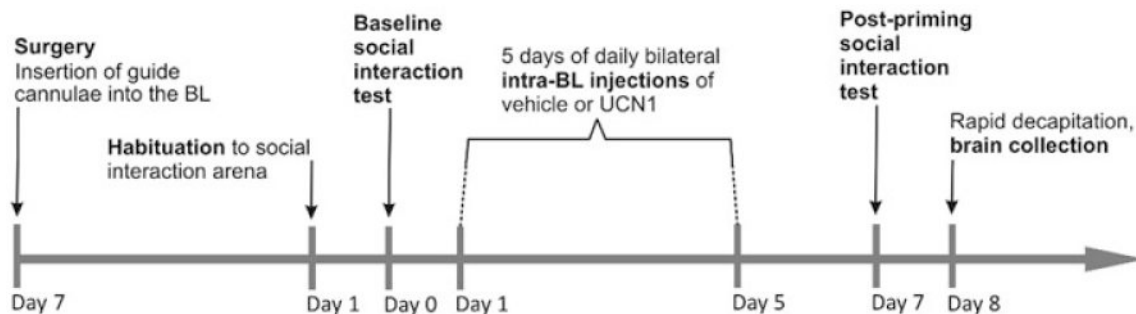
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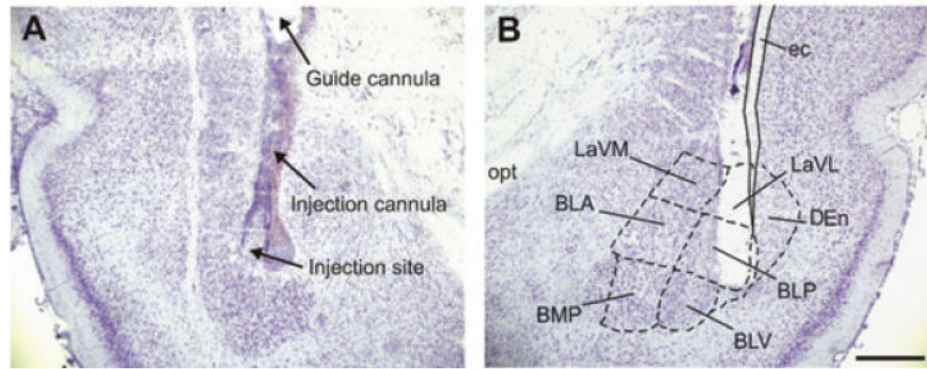
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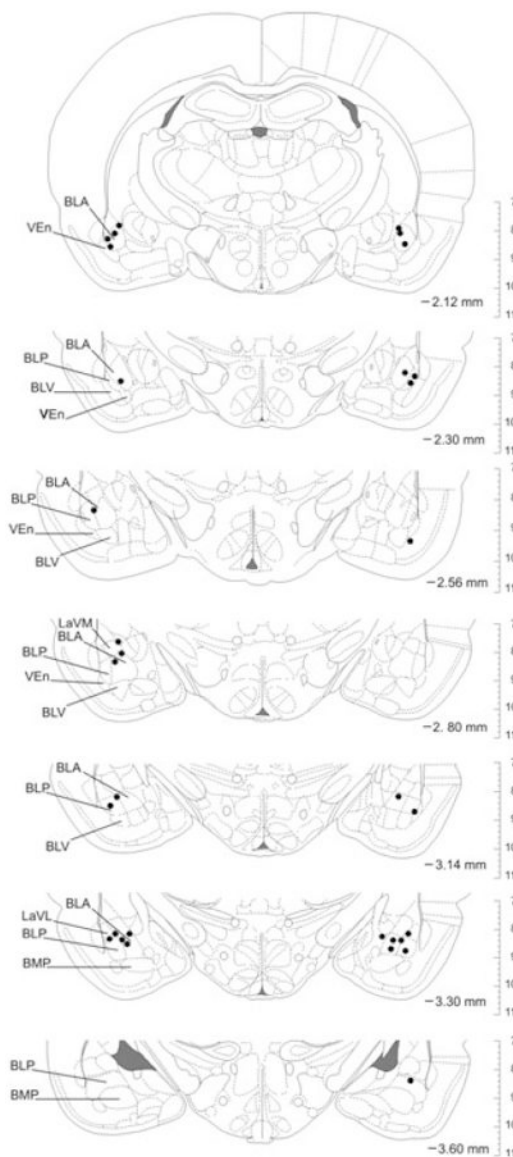
**Figure 1.**

Diagrammatic illustration of the experimental timeline. Adult, male rats underwent stereotaxic surgery to implant bilateral guide cannulae into the basolateral amygdaloid complex (BL) 6 days before being habituated to the social interaction (SI) arena, and 7 days before being tested for baseline anxiety-like behavior in the SI test. On Days 1–5, half of the rats received daily bilateral injections of vehicle (1% bovine serum albumin (BSA) in sterile 0.9% saline,  $n = 8$ ) into the BL, whereas the other half was primed with a daily bilateral dose of urocortin 1 (UCN1, 6 fmoles/100 nl vehicle per side,  $n = 8$ ). On Day 7, 2 days after the last injection, both treatment groups were tested a second time in the SI paradigm. A third group of rats ( $n = 7$ ) did not undergo stereotaxic surgery, behavioral testing, or any other treatment, and served as an undisturbed control group. On Day 8, all rats, including those from the undisturbed control group, were killed by rapid decapitation and their brains were collected for analysis of *tph2* and *slc6a4* expression using *in situ* hybridization histochemistry (ISHH).



**Figure 2.**

Representative photomicrographs of cannula placements within the basolateral amygdaloid complex (BL). Shown is the placement of the injection cannula, and the injection site itself in both the left (A) and right (B) hemisphere of a cresyl violet stained coronal brain section at  $-3.30$  mm bregma from a urocortin 1 (UCN1)-primed rat. BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; BLV, basolateral amygdaloid nucleus, ventral part; BMP, basomedial amygdaloid nucleus, posterior part; DEn, dorsal endopiriform nucleus; ec, external capsule; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part; opt, optic tract. Scale bar: 1 mm.



**Figure 3.**

Verification and mapping of cannula placements. Bilateral stereotaxic placements of guide and injection cannulae within the basolateral amygdaloid complex (BL) were verified via histochemistry and comparison with the rat brain atlas by Paxinos and Watson.<sup>[51]</sup> Depicted are all injection sites (black dots) in rat brain sections from rostral (−2.12 mm bregma) to caudal (−3.60 mm bregma). The orientation of the brain sections matches the orientation of the viewer (left hemisphere on the left, right hemisphere on the right). All injection sites were considered successful because of their location within, or their proximity to, the BL region. The vertical scale on the right side of each brain section indicates the dorsoventral coordinates in millimeters. BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; BLV, basolateral amygdaloid nucleus, ventral part; BMP, basomedial amygdaloid nucleus, posterior part; LaVL, lateral

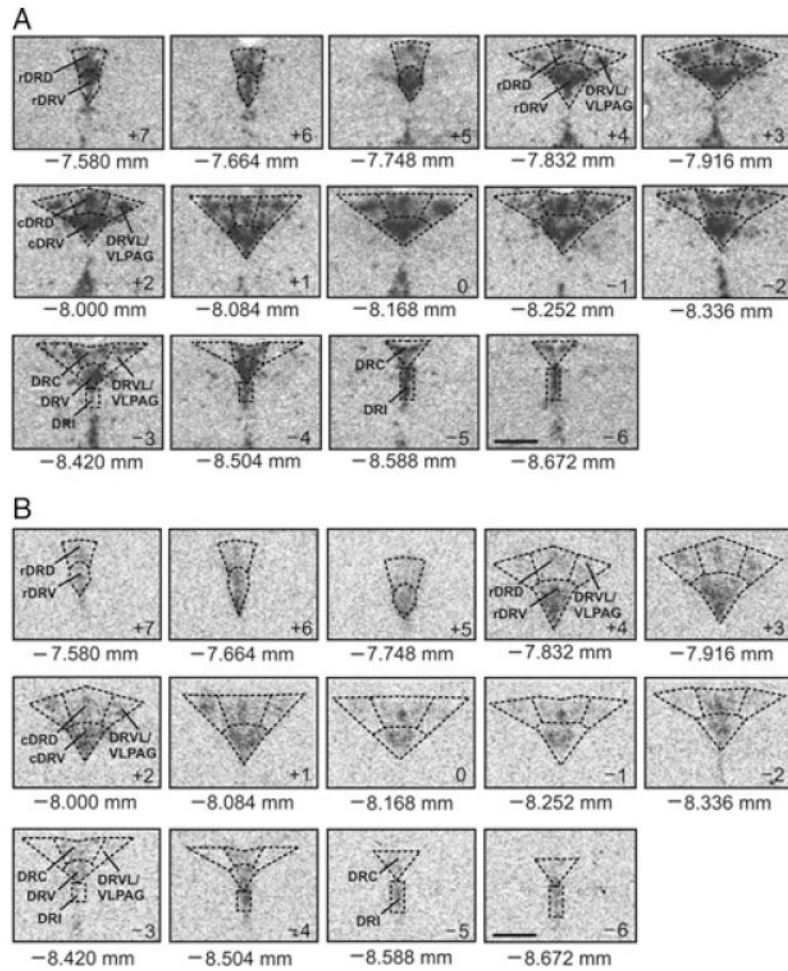
amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part; VEn, ventral endopiriform nucleus.

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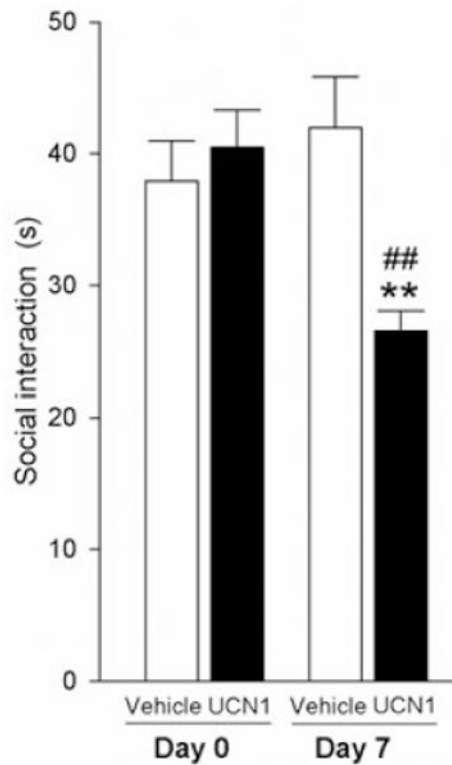
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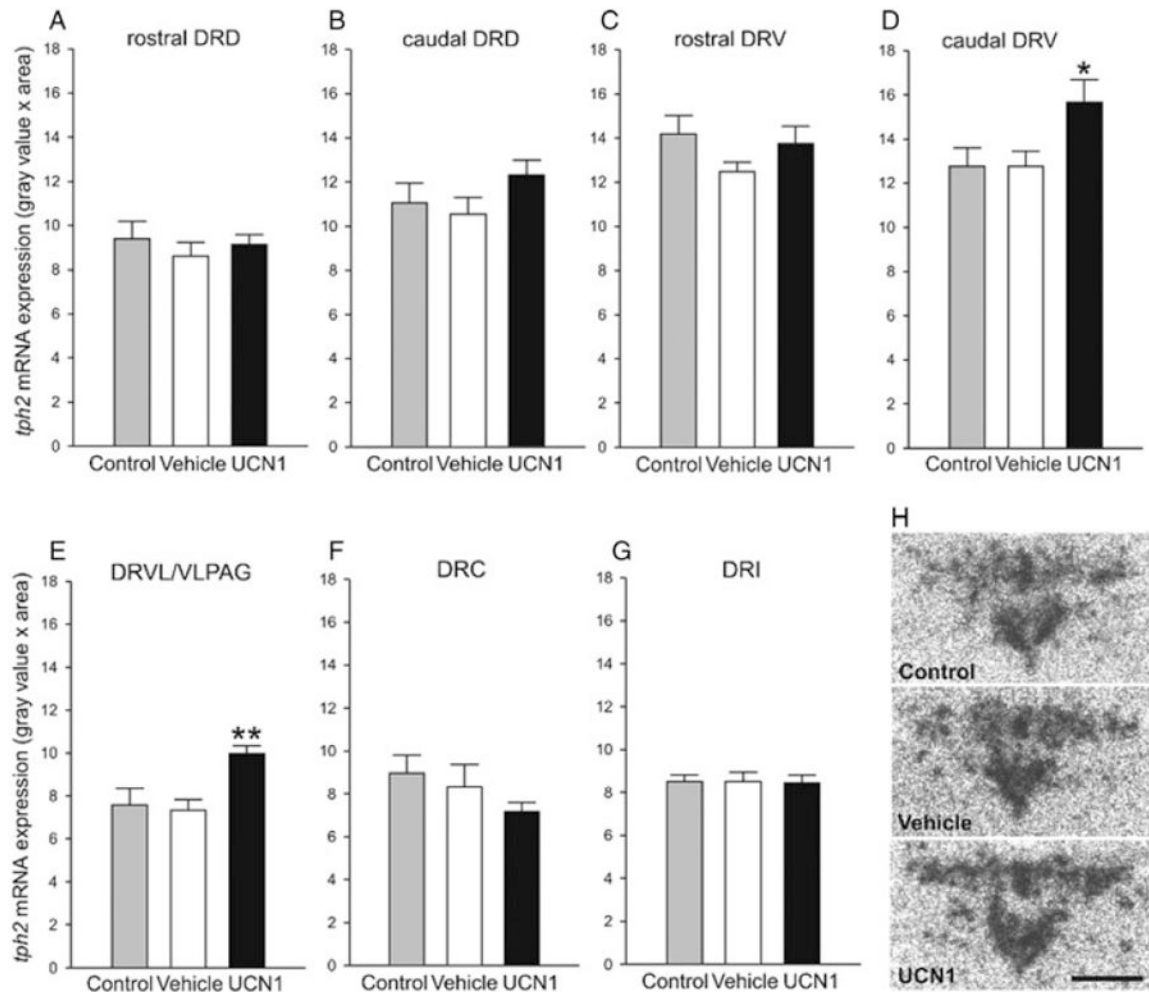
**Figure 4.** Neuroanatomic atlases of rat *tph2* and *slc6a4* mRNA expression. Displayed (from top left to bottom right of each panel) are 14 representative coronal sections ( $12\ \mu\text{m}$ ) that were used to measure *tph2* (A) or *slc6a4* (B) mRNA expression in all subdivisions of the midbrain dorsal raphe nucleus (DR) from rostral ( $-7.580$  mm bregma, designated level +7) to caudal ( $-8.672$  mm bregma, designated level -6). (A) Photomicrographs are autoradiographic images revealing the localization of hybridized  $^{35}\text{S}$ -labeled cRNA probe complementary to *tph2* mRNA. (B) Photomicrographs are autoradiographic images revealing the localization of hybridized  $^{35}\text{S}$ -labeled oligonucleotide probe complementary to *slc6a4* mRNA. Abbreviations are applicable to each following level until indicated differently. cDRD, caudal aspect of the dorsal raphe nucleus, dorsal part; cDRV, caudal aspect of the dorsal raphe nucleus, ventral part; DRC, dorsal raphe nucleus, caudal part; DRI, dorsal raphe nucleus, interfascicular part; DRVL, dorsal raphe nucleus, ventrolateral part; VLPAG, ventrolateral periaqueductal gray; rDRD, rostral aspect of the dorsal raphe nucleus, dorsal part; rDRV, rostral aspect of the dorsal raphe nucleus, ventral part. Scale bar: 1 mm.



**Figure 5.**

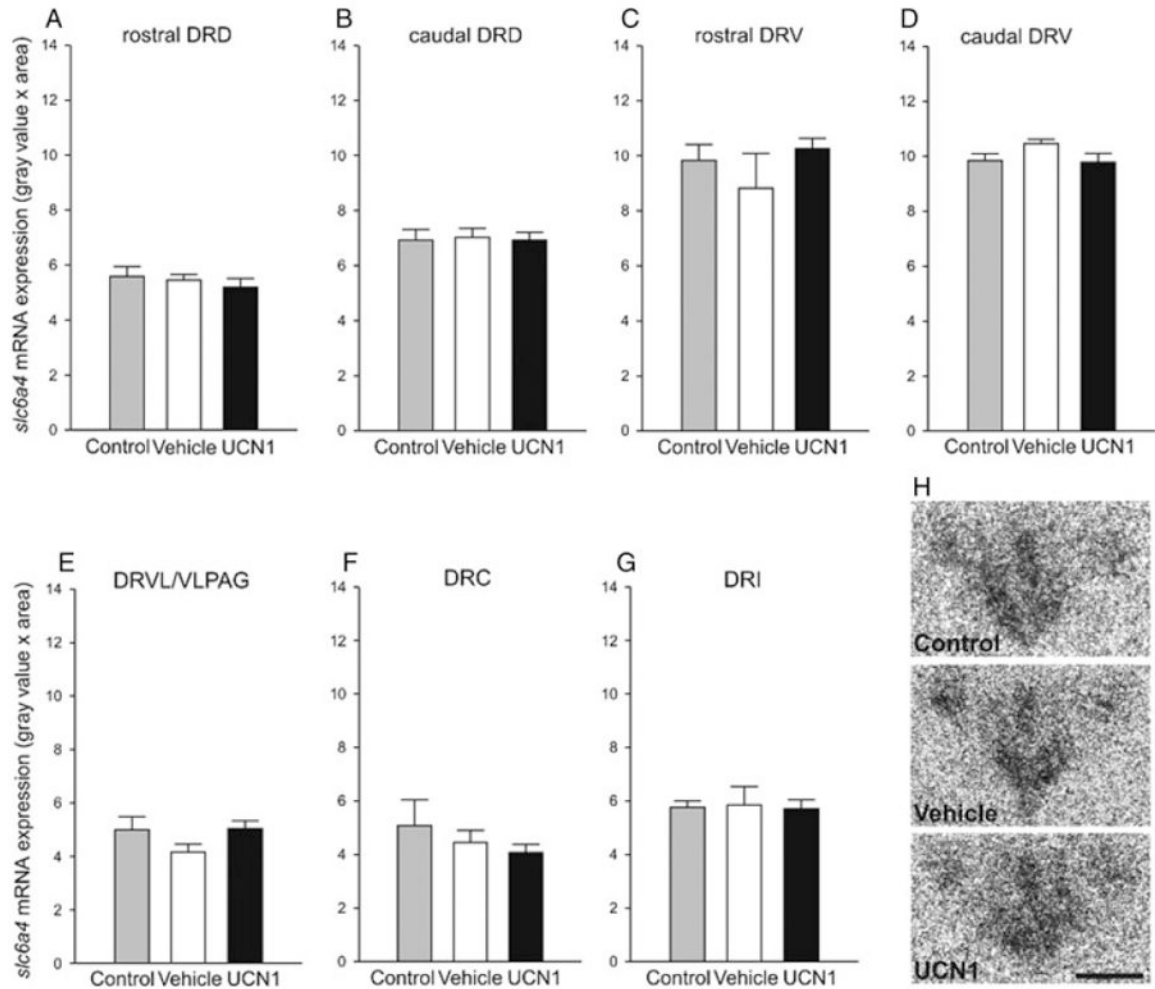
Effects of amygdalar priming on anxiety-related behavior. SI behavior of adult, male rats before (Day 0) and after (Day 7) they received daily bilateral injections of vehicle (1% bovine serum albumin (BSA) in sterile 0.9% saline,  $n = 8$ ) or urocortin 1 (UCN1, 6 fmol/100  $\mu$ l vehicle per side,  $n = 8$ ) into the basolateral amygdaloid complex (BL) for 5 consecutive days. One week prior to the behavioral testing, all rats had been implanted with bilateral guide cannulae targeting the BL. Shown is the mean (+ SEM) time (s, seconds) each treatment group spent interacting with a weight- and age-matched conspecific partner rat on Days 0 and 7 of the experiment.  $**P < .01$  versus postpriming vehicle.  $##P < .01$  versus Day 0.





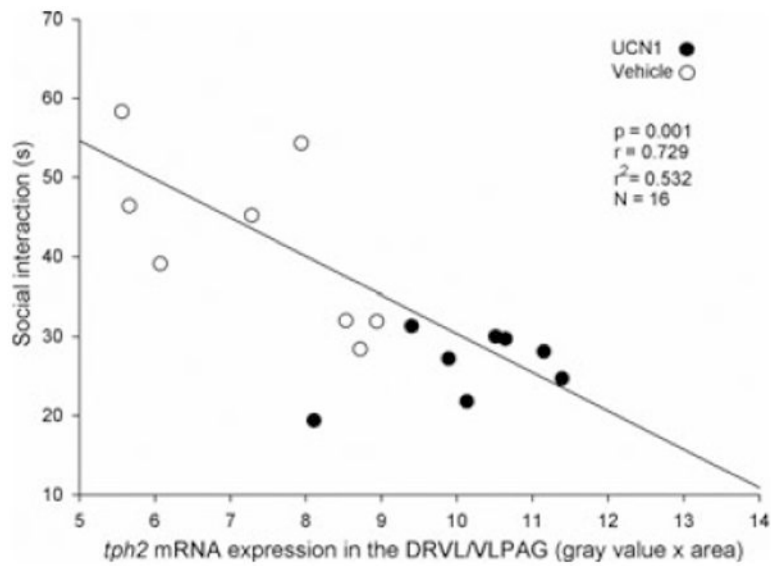
**Figure 6.**

Effect of amygdalar priming on *tph2* mRNA expression. Effect of daily bilateral intra-basolateral amygdaloid complex (BL) priming with either vehicle (1% bovine serum albumin in sterile 0.9% saline,  $n = 8$ ) or urocortin 1 (UCN1, 6 fmoles/100 nl vehicle per side,  $n = 8$ ) for 5 consecutive days on the expression of *tph2* mRNA in the dorsal raphe nucleus (DR). All experimental rats were killed one day after the second SI test, meaning 3 days after the last intra-BL injection, and expression of *tph2* mRNA was compared to that of an undisturbed control group (control,  $n = 7$ ). Shown is the mean (+ SEM) *tph2* mRNA expression in each of the seven subdivisions of the DR (A–G). \* $P < 0.05$  versus vehicle, \*\* $P < 0.01$  versus vehicle; there were no differences between unhandled control rats and vehicle-primed rats. caudal DRD, caudal aspect of the dorsal raphe nucleus, dorsal part; caudal DRV, caudal aspect of the dorsal raphe nucleus, ventral part; DRC, dorsal raphe nucleus, caudal part; DRI, dorsal raphe nucleus, interfascicular part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; rostral DRD, rostral aspect of the dorsal raphe nucleus, dorsal part; rostral DRV, rostral aspect of the dorsal raphe nucleus, ventral part. (H) Representative autoradiograms of *tph2* mRNA expression at  $-8.168$  mm bregma (designated level 0) in undisturbed controls, and in vehicle- or UCN1-treated rats. Scale bar: 1 mm.

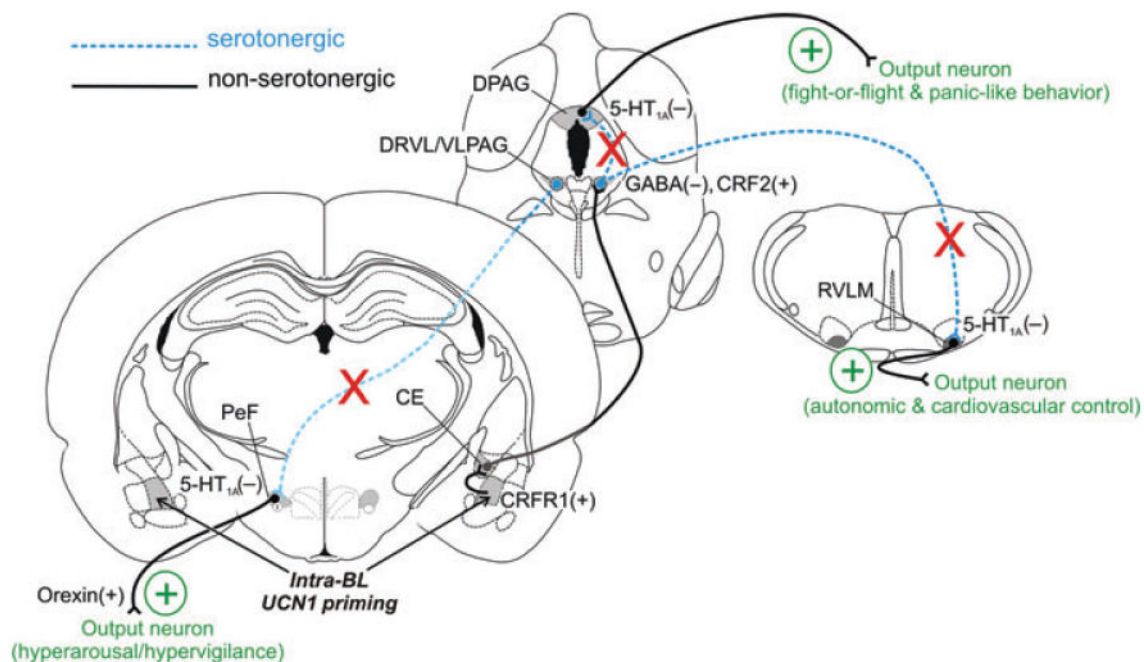


**Figure 7.**

Effect of amygdalar priming on *slc6a4* mRNA expression. Effect of daily bilateral intra-basolateral amygdaloid complex (BL) priming with either vehicle (1% bovine serum albumin in sterile 0.9% saline,  $n = 8$ ) or urocortin 1 (UCN1, 6 fmoles/100 nl vehicle per side,  $n = 8$ ) for 5 consecutive days on the expression of *slc6a4* mRNA in the dorsal raphe nucleus (DR). All experimental rats were killed by rapid decapitation one day after the second SI test, meaning 3 days after the last intra-BL injection, and expression of *slc6a4* mRNA was compared to that of an undisturbed control group (control,  $n = 7$ ). Shown is the mean (+ SEM) *slc6a4* mRNA expression in each of the seven subdivisions of the DR (A–G). caudal DRD, caudal aspect of the dorsal raphe nucleus, dorsal part; caudal DRV, caudal aspect of the dorsal raphe nucleus, ventral part; DRC, dorsal raphe nucleus, caudal part; DRI, dorsal raphe nucleus, interfascicular part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part / ventrolateral periaqueductal gray; rostral DRD, rostral aspect of the dorsal raphe nucleus, dorsal part; rostral DRV, rostral aspect of the dorsal raphe nucleus, ventral part. (H) Representative autoradiograms of *slc6a4* mRNA expression at -8.168 mm bregma (designated level 0) in undisturbed controls, and in vehicle- or UCN1-treated rats. Scale bar: 1 mm.



**Figure 8.** Correlation of SI behavior and *tph2* mRNA expression. Displayed is the correlation of the time (s) that vehicle- or urocortin 1 (UCN1)-primed rats spent socially interacting with a weight- and age-matched conspecific partner rat in the SI test on Day 7 of the experiment and *tph2* mRNA expression in the ventrolateral dorsal raphe nucleus/ventrolateral periaqueductal gray (DRVL/VLPAG), measured on Day 8.



**Figure 9.**

Hypothetical model of mechanisms through which amygdalar priming leads to a chronic anxiety-like state. Urocortin 1 (UCN1) acts on corticotropin-releasing factor type 1 (CRF<sub>1</sub>) receptors in the basolateral amygdaloid complex (BL) to decrease the inhibitory  $\gamma$ -aminobutyric acid (GABA)-ergic tone, leading to a hyperexcitability of BL projection neurons.<sup>[25]</sup> The BL gives rise to projections to both the central nucleus of the amygdala (CE) and the bed nucleus of the stria terminalis, lateral division, dorsal part (BSTLD), brain regions involved in fear- and anxiety-related behavior, respectively,<sup>[94,95]</sup> that in turn project to the lateral wings (dorsal raphe nucleus, ventrolateral part, DRVL/ventrolateral periaqueductal gray, VLPAG) of the DR.<sup>[96]</sup> The finding that amygdala priming enhances acquisition of fear-potentiated startle (dependent on the CE) but not light-enhanced startle (dependent on the BST<sup>[89]</sup>) suggests that amygdala priming preferentially increases the excitability of the CE, leading to activation of the DRVL/VLPAG. Indeed, panic disorder patients show amygdala volume reductions in the corticomедial nuclear group that includes the CE.<sup>[96]</sup> Activation of descending pathways from the CE increases *tph2* mRNA expression selectively in the DRVL/VLPAG region. Increased *tph2* mRNA expression in DRVL/VLPAG serotonergic neurons increases tryptophan hydroxylase 2 (TPH2) protein and 5-HT release within the DRVL/VLPAG, which leads to activation of autoinhibitory 5-HT<sub>1A</sub> receptors, resulting in an overall decreased serotonergic output from the DRVL/VLPAG to distal target sites, such as the dorsal periaqueductal gray (DPAG), the rostral ventrolateral medulla (RVLM), and the perifornical hypothalamus (PeF). In the DPAG, decreased 5-HT action on postsynaptic 5-HT<sub>1A</sub> receptors leads to disinhibition of the DPAG<sup>[97]</sup> and facilitation of panic/escape-like fight-or-flight responses. In the RVLM, decreased 5-HT action on postsynaptic 5-HT<sub>1A</sub> receptors promotes hypertension and sympathoexcitation.<sup>[98]</sup> In the PeF, decreased 5-HT action on postsynaptic 5-HT<sub>1A</sub> receptors leads to hyperactivity of orexin/hypocretin neurons, resulting in hypervigilance, hyperarousal, and panic-like responses.<sup>[83]</sup> Failure to activate DRVL/VLPAG serotonergic

neurons is associated with vulnerability to panicogenic agents such as sodium lactate.<sup>[50]</sup> Decreased serotonergic output from the DRVL/VLPAG leads to a chronic anxiety-like state with increased vulnerability to panic-like responses.

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