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Investigation of a central nucleus of the amygdala/dorsal raphe nucleus serotonergic circuit implicated in fear-potentiated startle

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Abstract

Serotonergic systems are thought to play an important role in control of motor activity and emotional states. We used a fear-potentiated startle paradigm to investigate the effects of a motor-eliciting stimulus in the presence or absence of induction of an acute fear state on serotonergic neurons in the dorsal raphe nucleus (DR) and cells in subdivisions of the central amygdaloid nucleus (CE), a structure that plays an important role in fear responses, using induction of the protein product of the immediate-early gene, *c-fos*. In *Experiment 1* we investigated the effects of fear conditioning training, by training rats to associate a light cue (conditioned stimulus, CS; 1000 lx, 2 sec) with foot shock (0.5 s, 0.5 mA) in a single session. In *Experiment 2* rats were given two training sessions identical to *Experiment 1* on days 1 and 2, then tested in one of four conditions on day 3: 1) placement in the training context without exposure to either the CS or acoustic startle (AS), 2) exposure to 10 trials of the 2 s CS, 3) exposure to 40 110 dB AS trials, or 4) exposure to 40 110 dB AS trials with 10 of the trials preceded by and co-terminating with the CS. All treatments were conducted during a 20 min session. Fear conditioning training, by itself, increased *c-Fos* expression in multiple subdivisions of the CE and throughout the DR. In contrast, fear-potentiated startle selectively increased *c-Fos* expression in the medial subdivision of the CE and in serotonergic neurons in the dorsal part of the dorsal raphe nucleus (DRD). These data are consistent with previous studies demonstrating that fear-related stimuli selectively activate DRD serotonergic neurons. Further studies of this mesolimbocortical serotonergic system could have important implications for understanding mechanisms underlying vulnerability to stress-related psychiatric disorders, including anxiety and affective disorders.

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Keywords

anxiety; fear; fear-potentiated startle; raphe; serotonergic; serotonin

Introduction

Serotonergic systems have been implicated in the regulation of anxiety and fear states (Lowry et al., 2005), while dysregulation of serotonergic systems has been associated with anxiety and affective disorders (Lowry et al., 2008b; Barton et al., 2008; Esler et al., 2007). A number of lines of evidence suggest that stress- and anxiety-related stimuli selectively activate subpopulations of serotonergic neurons within the dorsal raphe nucleus (DR) that in turn modulate the activity of limbic forebrain structures. Studies in rodents suggest that acute stress- and anxiety-related stimuli preferentially alter the activity of a subpopulation of serotonergic neurons located within the dorsal and caudal parts of the DR (Abrams et al., 2005; Evans et al., 2009; Gardner et al., 2005; Hale et al., 2008; Lowry et al., 2005; Lowry et al., 2008a; Lowry et al., 2008b; Staub et al., 2005; Staub et al., 2006; Grahn et al., 1999; Lowry and Hale, 2009), which contribute to facilitation of anxiety states via projections to forebrain limbic structures (Christianson et al., 2010). Recent studies suggest that chronic stress selectively increases *tph2* mRNA expression encoding tryptophan hydroxylase 2, the rate-limiting enzyme in the biosynthesis of serotonin, in the same regions (McEuen and Bale, 2006). Consistent with these studies in rodents, studies from postmortem tissues of depressed suicide patients have reported increased *tph2* mRNA and tryptophan hydroxylase protein expression that is restricted to the dorsal and caudal parts of the DR (Bonkale et al., 2006; Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008); consistent with these findings, *tph2* mRNA expression is also elevated in DRC projection sites (Perroud et al., 2010). Together, these studies in animals, including humans, suggest that understanding the stimulus properties that regulate serotonergic systems in the dorsal and caudal parts of the DR would help understand the regulation and dysregulation of this brain region in normal and pathological anxiety states.

The DR can be divided into several functionally and anatomically distinct regions, each with unique sets of neurochemical properties, anatomical characteristics, afferents, and efferents (Lowry et al., 2008a; Lowry and Hale, 2009). Since subdivisions of the DR have distinct functional and anatomical properties, different stimuli can alter the activity of specific serotonergic circuits through several mechanisms including stimulation of various neuronal afferents (Lowry et al., 2008a). A number of stress- and anxiety-related stimuli activate both the dorsal and caudal parts of the dorsal raphe nucleus. These include anxiogenic drugs, such as 1) N-methyl- β -carboline-3-carboxamide (FG-7142), a partial inverse agonist at the benzodiazepine allosteric site on the γ -amino butyric acid A (GABA_A) receptor, 2) caffeine, an adenosine receptor antagonist, and 3) m-chlorophenyl piperazine (mCPP), a serotonin 5-HT_{2A/2C} receptor agonist (Abrams et al., 2005). A similar pattern of DR activation is observed following treatment with the anxiety-related neuropeptide urocortin 2 (Ucn 2) (Staub et al., 2005; Staub et al., 2006), exposure to social defeat (Gardner et al., 2005), acute noxious stimulation modified by morphine (Bajic and Commons, 2010), or exposure to inescapable, relative to escapable, stress (Grahn et al., 1999).

Acoustic startle is often used as a sensitive behavioral measure of anxiety state in rodents and humans (Davis et al., 1993b; Walker et al., 2003). Anxiogenic drugs increase startle (Grillon et al., 1997; Lee and Davis, 1997; Walker and Davis, 1997), while anxiolytic drugs decrease startle (Davis et al., 1993a; Koch and Schnitzler, 1997; Schweimer et al., 2005). Potentiation of startle by the anxiety- and stress-related neuropeptide, corticotropin-releasing factor (CRF) increases c-Fos expression in serotonergic neurons that project to the medial

prefrontal cortex, an effect that, in the caudal part of the DR, is highly correlated with the magnitude of CRF-enhancement of startle (Meloni et al., 2007). Although a detailed topographical analysis of the effects of CRF-enhancement of startle on c-Fos expression has not been done, a clear topographically organized effect was noted, with a higher percentage of activated neurons in the caudal part of the DR (Meloni et al., 2007). Acoustic startle by itself appears to have no effect on c-Fos expression in the DR (Meloni et al., 2007), suggesting that activation of neurons in the DR is dependent on the anxiogenic neuropeptide CRF alone, or the combination of CRF and acoustic startle.

In order to further define the stimulus conditions sufficient for selective activation of serotonergic neurons in the dorsal and caudal parts of the DR, we compared the effects of fear-potentiated startle with induction of a fear state alone, exposure to acoustic startle alone, or a control condition with neither induction of a fear state nor acoustic startle. To ensure that any effects obtained during these conditions were not simply carryover effects from fear conditioning 24 or 48 h before testing, we conducted a separate experiment comparing the effects of a single session of pairing the CS with foot shock during fear conditioning on c-Fos expression in serotonergic neurons within the DR. Finally, to investigate potential afferent mechanisms controlling serotonergic neuronal activity, we quantified c-Fos expression in subregions of the central amygdaloid nucleus (CE). The CE preferentially innervates the DRD region of the DR (Peyron et al., 1998), and stimulation of the CE is necessary and sufficient for activation of mesolimbocortical serotonergic systems (Forster et al., 2008; Singh et al., 1992) via corticotropin-releasing factor type II (CRF₂) receptors in the DR. It is hoped that a better understanding of the stimulus conditions that selectively engage subpopulations of serotonergic neurons in the dorsal and caudal parts of the DR may increase our understanding of how serotonergic systems become dysregulated in anxiety and affective disorders (Bonkale et al., 2006; Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008; Barton et al., 2008; Esler et al., 2007; Perroud et al., 2010; Boldrini et al., 2005; Underwood et al., 1999b).

Materials and Methods

Animals

Male Sprague-Dawley rats (200-225g; Charles River, Kingston, NY, USA) were maintained under standard environmental conditions on a 12 hour light-dark cycle with lights on at 7 AM in the animal facility of the Department of Psychology and Neuroscience, University of Colorado, Boulder. Rats were housed in standard polycarbonate cages (26.0 × 47.6 × 20.3 cm; Cat. No. RC88D, Alternative Design, Siloam Springs, AR, USA) in groups of four for one week prior to experimentation and housed in groups of two the day before the experiment. Food and tap water were provided *ad libitum* (Teklad Global 18% Protein Rodent Diet, Cat. No. 2018, Harlan, Madison, WI, USA). All studies were consistent with the NIH *Guide for the Care and Use of Laboratory Animals* (N.I.H. Publication No. 85-23) and all procedures were approved by the Institutional Animal Care and Use Committee at the University of Colorado, Boulder. All possible efforts were made to minimize the number of animals used and their suffering.

Apparatus

The apparatus used in this experiment was identical to that described previously (for details see (Masini et al., 2008)) and consisted of a Plexiglas® inner chamber (8 × 15 × 15 cm) inside a darkened box (59.7 × 38.1 × 38.1 cm). The Plexiglas® inner chamber had a wire grid floor for administering foot shocks (0.5 s, 0.5 mA), and was flanked by a panel of LED lights installed on two sides for administration of a visual light cue (2 s, 1000 lx); a speaker on the ceiling of the chamber was used for administration of acoustic stimulation (white

noise, 50 ms, 110 dB). Background white noise (50 Hz - 20 kHz) of 55 dB was provided throughout the experiments. The floor of the chamber was also equipped with an accelerometer in order to measure maximum startle amplitudes in the 200 ms following the onset of each acoustic stimulus.

Experiment 1. Fear conditioning versus home cage controls

Rats (N = 16) were randomly divided into treatment groups consisting of either exposure to fear conditioning (n = 8) or home cage control conditions (n = 8). The fear conditioning group was exposed to one session of conditioning, consisting of 10 pairings of the light cue (conditioned stimulus, CS; 2 s, 1000 lx) always co-terminating with a foot shock (0.5 s, 0.5 mA), a procedure that was identical to the fear conditioning sessions on days 1 and 2 in *Experiment 2* (see below). The rats in *Experiment 1* were not exposed to the fear-potentiated startle test session outlined below in *Experiment 2*. The CS-foot shock pairings in *Experiment 1* occurred at a random interval within two minute blocks over a 20 minute session. Ninety minutes following the onset of the training session, rats were transcardially perfused with fixative and their brains were collected while time-matched home cage control rats were taken directly from their home cages with no training.

Experiment 2. Fear-potentiated startle

Training and testing in the fear-potentiated startle paradigm were conducted as described previously (Campeau et al., 1997) with minor modifications. All rats were trained and tested in the chambers described above and were trained for two sessions on two consecutive days prior to testing on the third day; each training session was identical to that of the fear conditioning group in *Experiment 1*. Rats were tested under one of four conditions as follows: 1) control, placement in the testing chamber without exposure to either the CS or acoustic startle (AS), 2) CS, exposure to 10 trials of the 2 s, 1000 lx CS alone, presented across the same interval as the training session, 3) AS, exposure to 40 110 dB AS trials given at a regular interval occurring every 30 seconds, or 4) CS/AS, exposure to 30 110 dB AS trials with 10 of the trials (intermixed throughout the 30 AS trials to produce regular intervals occurring every 30 seconds) preceded by the CS (Fig. 1). All treatments were conducted during a 20 min period. Mean maximum startle amplitudes were recorded for the AS and CS/AS treatment groups.

Tissue Processing

For both *Experiments 1* and *2* rats were anesthetized using sodium pentobarbital (100 mg/kg) and transcardially perfused 90 min after the start of the fear conditioning or test session, to allow for maximal c-Fos protein expression (Kovacs, 1998), using ice cold 0.05 M phosphate buffered saline until the blood leaving the right atrium was cleared (PBS, 134 g Na₂HPO₄·7H₂O, 90 g NaCl, 2 L distilled H₂O (dH₂O), diluted 1:5 in dH₂O, pH 7.4) followed by ice cold 4% paraformaldehyde (80 g paraformaldehyde, 30 g sucrose, 4.8 mL sodium hydroxide, 808 ml 0.2 M Na₂HPO₄·7H₂O, 192 ml 0.2 M NaH₂PO₄·H₂O, 1 L dH₂O). Following fixation, the brains were removed, post-fixed in 4% paraformaldehyde solution for 16 h, and sunk in 30% sucrose in 0.1 M sodium phosphate buffer (PB, 404 ml 0.2 M Na₂HPO₄·7H₂O, 96 ml 0.2 M NaH₂PO₄·H₂O, 500 ml dH₂O, pH 7.4). Brains were blocked into forebrain and hindbrain pieces with a coronal cut just caudal to the mammillary bodies (approximately -5.80 mm bregma) using a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA) then rapidly frozen using isopentane chilled on dry ice. Once blocked the brains were stored at -80 °C. The brains were then sectioned at 30 µm using a cryostat (Leica CM1900, North Central Instruments, Denver, CO, USA) and stored as 6 alternate sets of sections. The sectioned tissue was stored in cryoprotectant (270 ml ethylene glycol, 160 ml glycerol, 202 ml 0.2 M Na₂HPO₄·7H₂O, 48 ml 0.2 M NaH₂PO₄·H₂O, and 320 ml dH₂O) at -20 °C until immunohistochemistry was performed.

Immunohistochemistry: tryptophan hydroxylase and c-Fos in the midbrain raphe complex

One set of hindbrain sections, including the midbrain raphe complex, was examined with double immunostaining using primary antibodies for the protein product of the immediate-early gene *c-fos* (rabbit anti-c-Fos polyclonal antibody, Cat. No. PC38, Lot No. D00058535, 1:3000; Calbiochem, EMD Chemicals, Gibbstown, NJ, USA) and tryptophan hydroxylase (TPH; sheep anti-TPH antibody, Cat. No. T8575, Lot No. 096K1026, 1:12,000; Sigma-Aldrich, St. Louis, MO, USA). Immunohistochemistry was run simultaneously for both *Experiment 1* and *Experiment 2* using large volume (1 ml) incubations to limit variability. Tissue was washed twice in 0.05 M PBS upon removal from cryoprotectant, then rinsed in 1% H₂O₂ in 0.05 M PBS, followed by washing in 0.05 M PBS and pre-incubation in 0.05 M PBS with 0.3% Triton X-100 (Cat No. T9284, Batch No. 017K00112, Sigma-Aldrich; 0.3% PBST). Sections were incubated overnight at room temperature with rabbit anti-c-Fos antibody (1:3000) in 0.05 M PBS with 0.1% Triton X-100 (0.1% PBST). After 16 h the tissue was washed twice in 0.05 M PBS followed by a 90-min incubation with a biotinylated swine anti-rabbit secondary antibody (Cat. No. E0353, Lot. No. 00047863, 1:200; DAKO, Glostrup, Denmark) in 0.05 M PBS. Tissue was then washed twice in 0.05 M PBS followed by incubation with an avidin-biotin-peroxidase complex (Elite ABC reagent, Cat. No. PK-6100, 1:200; Vector Laboratories, Burlingame, CA, USA) for 90 min. Tissue was then washed twice in 0.05 M PBS, followed by incubation in a peroxidase chromogen substrate (Vector SG; Cat. No. SK4700; Vector Laboratories; diluted as recommended by the vendor) in 0.05 M PBS for 18 min. Immediately upon completion of the chromogen reaction tissue was washed in 0.05 M PBS to stop the reaction, then rinsed with 1% H₂O₂ in 0.05 M PBS, then washed twice in 0.05 M PBS. Tissue was then incubated with sheep anti-TPH antibody (1:12,000) in 0.1% PBST overnight at room temperature. All subsequent steps were repeated as in the immunoperoxidase localization of c-Fos-immunoreactivity, except the secondary antibody and chromogen steps; these used a biotinylated rabbit anti-sheep secondary antibody (Cat. No. PK-6106, 1:200; Vector Laboratories), and a peroxidase chromogen substrate solution consisting of 0.01% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H₂O₂ in 0.05 M PBS (20 min). Immediately after completion of the chromogen reaction tissue was washed twice in 0.05 M PBS to stop the reaction. Immunostaining of c-Fos was a blue/black color localized to the nucleus while immunostaining of TPH was an orange/brown color localized to the cytoplasm. Following immunostaining, sections were rinsed briefly in distilled water with 0.15% gelatin in dH₂O then mounted on microscope slides (VistaVision, Cat No. 16004-390, VWR, West Chester, PA, USA). Mounted tissue was dehydrated using a series of graded alcohols (70%, 95%, and 100% ethanol) then cleared with xylenes. Slides were then mounted with cover slips using Entellan mounting medium (Electron Microscopy Science, Hatfield, PA, USA).

Immunohistochemistry: c-Fos in the central amygdaloid nucleus

Forebrain sections, including the central nucleus of the amygdala (CE), were immunostained using methods identical to those used to immunostain c-Fos in the midbrain raphe complex, except the sections were left in the chromogen reaction for 24 min. Additionally, an adjacent set of Nissl-stained sections was prepared in order to allow analysis of c-Fos expression in subdivisions of the CE. For Nissl staining, sections were mounted on microscope slides and air-dried. Sections were pretreated in a chloroform/ethanol/ether bath (8:1:1, 200 ml chloroform, 25 ml ethanol, 25 ml ether) for 20 min. Tissue was then processed through a hydration series of 100%, 95%, and 70% ethanol in dH₂O for 3 min each and placed in a filtered cresyl violet stain (cresyl violet, 2.5 g; dH₂O, 300 ml; 1 M sodium acetate CH₃COONa, 30 ml; 1 M acetic acid CH₃COOH, 170 ml) for 5 min. Tissue was then rinsed in dH₂O then acidified in 95% ethanol (5 ml glacial acetic acid, 195 ml 95% ethanol) until clear fiber tracts were present. Tissue was then dehydrated in 95% ethanol followed by three

100% ethanol baths before being cleared with xylenes. Microscope slides were then coverslipped as described above.

Cell counting: tryptophan hydroxylase and c-Fos in the midbrain raphe complex

The numbers of c-Fos-immunoreactive (c-Fos-ir) serotonergic neurons (c-Fos-ir/TPH-ir neurons), the numbers of c-Fos-ir, non-serotonergic cells (c-Fos-ir/TPH-immunonegative cells), and the total number of TPH-ir neurons (both c-Fos-ir/TPH-ir and c-Fos-immunonegative/TPH-ir neurons) were counted in subdivisions of the dorsal raphe nucleus at six rostrocaudal levels (−7.28, −7.46, −8.00, −8.18, −8.54, and −8.72 mm bregma). The subdivisions examined at these six rostrocaudal levels were combined into rostral (−7.28 and −7.46 mm bregma), mid-rostrocaudal (−8.00 and −8.18 mm bregma), and caudal (−8.54 and −8.72 mm bregma) levels. Subdivisions are illustrated in Fig. 2, and are as follows: rostral dorsal raphe nucleus, dorsal part (DRD), rostral dorsal raphe nucleus, ventral part (DRV); midrostromcaudal DRD, mid-rostromcaudal DRV, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray (DRVL/VLPAG), dorsal raphe nucleus, caudal part (DRC) and dorsal raphe nucleus, interfascicular part (DRI) (Paxinos and Watson, 1998). Identification of rostrocaudal levels and subdivisions of the midbrain raphe complex was based on comparisons with a stereotaxic rat brain atlas (Paxinos and Watson, 1998), and an atlas of tryptophan hydroxylase staining in the rat brain (Abrams et al., 2004). The only exception was the determination of the border between the DRD and the DRVL/VLPAG, which was moved laterally approximately 100 µm on the left and right sides of the midline to allow complete separation of the cluster of medium-sized serotonergic neurons in the DRD and the morphologically distinct, multipolar, magnocellular serotonergic neurons in the DRVL/VLPAG region. Since the DRVL/VLPAG subdivision is a bilateral structure at both −8.00 and −8.18 mm bregma the cell counts from the left and right sides were combined for analysis. Round or oval-shaped cell nuclei with blue-black immunostaining darker than background were counted as c-Fos-ir cells. Cells with orange-brown cytoplasmic immunostaining darker than background were counted as TPH-ir neurons. Cells with blue-black nuclei and brown/orange cytoplasmic staining were counted as c-Fos-ir/TPH-ir (double-immunostained) neurons. One section at each rostrocaudal level from each rat was used for analysis. The entire cell counting procedure was performed by an investigator (BMS) blind to the treatment groups while using a Nikon E200 microscope with a 10X objective (100X total magnification). Double-immunostained neurons were confirmed using a 40X objective (400X total magnification). Photomicrographs were taken using a Nikon 90i microscope and a Nikon DS-F11 digital camera linked to a computer with NIS Elements 3.00 imaging software (A.G. Heinze Inc., Lake Forest, CA, USA). Photographic plates were prepared using CorelDraw for Windows 12.0 (Viglen Ltd., Wenbley, UK).

Cell Counting: c-Fos in the central amygdaloid nucleus

The numbers of c-Fos-ir cells in the central nucleus of the amygdala (CE) were counted using a camera lucida technique in order to examine the medial (CeM), lateral (CeL), and capsular (CeC) subdivisions of the CE at the rostrocaudal levels of −2.12, −2.56, and −3.14 mm bregma (Fig 3). Identification of rostrocaudal levels and subdivisions of the CE was based on comparisons with a stereotaxic rat brain atlas (Paxinos and Watson, 1998; Paxinos et al., 1999) and CE subdivision outlines were drawn on transparent acetates, using Nissl-stained material, in order to overlay on drawings of c-Fos immunoreactivity in adjacent sections. Adjacent Nissl and immunostained sections were aligned using the camera lucida system using several landmarks such as fiber tracts, distinctive nuclei, and blood vessels, then the number of c-Fos-ir cells within each subdivision was counted.

Statistical analysis

Immunohistochemical data were analyzed using a linear mixed model - a statistical approach ideal for repeated measures data that efficiently accounts for multiple measures derived from the same unit of observation (i.e. rat), while also accommodating missing data and complex covariance structures. In the analysis, we separately modeled standardized c-Fos-ir/TPH-ir, c-Fos-ir/TPH-immunonegative, and total TPH-ir counts measured in different subdivisions of the midbrain raphe complex, or CE, in the same animal, subject to different treatment conditions described above, assuming an unstructured covariance structure. We followed the primary analysis with post hoc analysis using Fisher's Protected Least Significant Difference (LSD) tests when appropriate. Behavioral data were analyzed using independent samples *t*-tests. All statistical procedures were performed using PASW Statistics (18.0.1 for Windows, SPSS Inc., Chicago, IL, USA). Outliers (3.33%) were identified in the raw data set using Grubbs test (Grubbs, 1969) and removed from the data set.

Immunohistochemical data were analyzed separately for *Experiments 1* and *2* using treatment group (*Experiment 1*, home cage, fear conditioning; *Experiment 2* home cage, control, CS, AS, and CS/AS) as a fixed factor and brain region as a within-subjects factor. Cell counts in the midbrain raphe complex and the CE were also analyzed separately. Significance was accepted for the linear mixed model, post hoc Fisher's Protected LSD tests, and independent samples *t*-tests when $p < 0.05$.

Correlations between dependent variables were analyzed using the Pearson Product Moment correlation test.

Results

Experiment 1

c-Fos-ir serotonergic neurons in the DR—Exposure to fear conditioning (10 foot shocks coterminating with a light cue, 20 min session), compared with controls, increased c-Fos expression in serotonergic neurons in subdivisions of the midbrain raphe complex (treatment $\times$ region interaction, $F(6, 13.1) = 68.5$, $p < 0.001$; treatment main effect, $F(1, 13.9) = 155.4$, $p < 0.001$; region main effect, $F(6, 13.1) = 81.4$, $p < 0.001$; Fig 4). Exposure to fear conditioning increased c-Fos-ir/TPH-ir neurons, compared with home cage controls, in all subdivisions of the DR examined (Fig. 4). Photomicrographs in Fig. 5 illustrate c-Fos and TPH immunostaining within the DRD, DRV/VLPAG, and DRV at -8.18 mm bregma in a rat from the fear conditioning group compared with a rat from the home cage control group.

c-Fos-ir non-serotonergic cells in the DR—Exposure to fear conditioning also increased c-Fos expression in non-serotonergic cells in all of the subdivisions of the DR compared with home cage controls (region $\times$ treatment interaction, $F(6, 11.3) = 13.42$, $p < 0.001$; treatment main effect, $F(1, 14.0) = 86.1$, $p < 0.001$; Table 1).

Total numbers of serotonergic neurons in the DR—The total numbers of TPH-ir neurons did not differ between the fear conditioning and home cage control groups (Fig. 4).

c-Fos-ir cells in the CE—Exposure to fear conditioning increased c-Fos expression in subdivisions of the CE in relation to home cage controls (region $\times$ treatment interaction, $F(8, 12.1) = 40.3$, $p = 0.001$; treatment main effect, $F(1, 11.9) = 16.3$, $p < 0.01$; Table 2). Post hoc Fisher's Protected LSD tests detected increases in c-Fos-ir cells in all three rostrocaudal levels of the CeM and CeC in addition to the caudal CeL at -3.14 mm bregma in relation to

home cage controls (Table 2). Photomicrographs in Fig. 6 illustrate c-Fos and Nissl immunostaining within the CE at -2.12 mm bregma in a rat from the fear conditioning group compared with a rat from the home cage control group.

Experiment 2

Behavior—Fear-potential of startle was not evident in three rats, when comparing within-subject trials involving pairings of the CS and AS (10 of the 40 trials) and trials involving exposure to AS alone (30 of the 40 trials). As the purpose of the study was to identify neural correlates of fear-potentiated startle, these rats were not included in analysis of cell counts. The criterion for fear potentiation of startle was a mean % startle potentiation of greater than 125% when comparing AS alone trials versus the CS/AS trials in individual rats within the fear-potentiated startle treatment group. Based on within-subjects analysis of the remaining rats ($n = 5$) in the CS/AS group, startle amplitudes were greater in trials in the presence of the CS compared to trials in the absence of the CS (mean maximum startle on CS/AS trials 4.9 ± 0.6 Newtons (N), AS trials, 2.6 ± 0.5 N, $t = 4.95$, $p < 0.05$).

c-Fos-ir serotonergic neurons—Fear-potentiated startle increased c-Fos expression in serotonergic neurons in subdivisions of the DR (treatment $\times$ region interaction, $F(24, 32.8) = 3.7$, $p < 0.001$; treatment main effect $F(4, 31.8) = 4.5$, $p < 0.01$; Fig. 7). Post hoc Fisher's Protected LSD tests detected increases in c-Fos-ir/TPH-ir neurons in the Control (C) group exposed to the startle chamber, relative to the home cage control group (HC) in the rostral DRD and the DRVL/VLPAG region (Fig. 7). Exposure to CS alone increased the number of c-Fos-ir/TPH-ir neurons in the DRC, compared with the Control group. Exposure to AS alone had no effect on c-Fos expression in serotonergic neurons in any subdivision of the DR, relative to the Control group. However, exposure to CS/AS conditions increased the number of c-Fos-ir/TPH-ir neurons in the midrostrocaudal DRD compared with the CS treatment group. In other words, although acoustic startle had no effect by itself, it increased the number of c-Fos-ir serotonergic neurons in the mid-rostrocaudal DRD in the presence of the CS. Photomicrographs in Fig. 8 illustrate c-Fos and TPH immunostaining within the DRD at -8.18 mm bregma in a rat from the CS/AS group compared with rats from the C, CS, and AS groups.

c-Fos-ir non-serotonergic cells—Fear-potentiated startle had no effect on the numbers of c-Fos-ir/TPH-immunonegative cells in the subdivisions of the midbrain raphe complex examined (data not shown).

Total numbers of serotonergic neurons—The total numbers of TPH-ir neurons did not differ among the home cage control, Control, CS, AS, and CS/AS groups (Fig. 7).

c-Fos-ir cells in the CE—Exposure to fear-potentiated startle increased c-Fos expression in certain subdivisions of the CE in relation to control groups (region $\times$ treatment interaction, $F(32, 31.5) = 2.6$, $p < 0.01$; treatment main effect, $F(4, 31.7) = 2.7$, $p < 0.05$; Table 3). Post hoc Fisher's Protected LSD tests detected increases in c-Fos-ir cells in the C group, in relation to HC group in the CeC at -3.14 mm bregma (Table 3). Exposure to the CS alone had no effect on c-Fos expression in any subdivision of the CE, in relation to the Control group, and exposure to AS alone increased c-Fos expression in the CeL and CeC at -2.12 mm bregma in the AS group, in relation to the Control group. In addition, increases in c-Fos-ir cells were seen in the CS/AS group in all three rostrocaudal levels of the CeM and at -2.12 mm bregma in the CeL, in relation to the CS group (Table 3). In other words, although acoustic startle had no effect by itself on c-Fos expression in the CeM, it increased the number of c-Fos-ir cells in the CeM in the presence of the CS.

Correlations—The numbers of c-Fos-ir/TPH-ir neurons in the DRD subdivision at the rostrocaudal levels of -8.00 and -8.18 mm bregma in *Experiment 2* were positively correlated with percent startle potentiation (calculated as the within-subject CS/AS pairing trial mean startle amplitude / AS only trial mean startle amplitude $\times 100$) in rats within the CS/AS treatment group (DRD -8.00 mm bregma, $r^2 = 0.825$, $p < 0.05$; DRD -8.18 mm bregma, $r^2 = 0.788$, $p < 0.05$, Fig. 9).

When rats from all treatments groups from *Experiment 1* and *Experiment 2* were incorporated, the total numbers of c-Fos-ir/TPH-ir neurons in the mid-rostrocaudal DRD subdivision were positively correlated with the numbers of c-Fos-ir cells in the CeM at -2.12 mm bregma (Fig. 10; $r^2 = 0.495$, $p < 0.001$), but not in the CeM at more caudal levels. The numbers of c-Fos-ir/TPH-ir neurons in the mid-rostrocaudal DRD were also correlated with the numbers of c-Fos-ir cells in the CeL and CeC at -2.12 mm bregma (CeL, $r^2 = 0.319$, $p < 0.05$; CeC, $r^2 = 0.334$, $p < 0.05$), but not in the CeL or CeC at more caudal levels.

Additionally, the numbers of c-Fos-ir/TPH-ir neurons in the mid-rostrocaudal DRD were correlated with the numbers of c-Fos-ir cells, at -2.12 mm bregma, when all three subdivisions examined were combined ($r^2 = 0.334$, $p < 0.05$) and the number of c-Fos-ir cells in the CeM when counts at -2.12 , -2.56 , and -3.14 mm bregma were combined ($r^2 = 0.382$, $p < 0.01$).

Discussion

Fear-potentiated startle selectively activated a subpopulation of serotonergic neurons in the DRD. This fear-potentiated startle-induced increase in c-Fos expression in serotonergic neurons was restricted to the mid-rostrocaudal part of the DRD. The DRD is a region that 1) receives a dense innervation from limbic forebrain structures, including the CE, 2) projects to limbic forebrain structures, and 3) has consistently been shown to be associated with stress- and anxiety-like responses. Based on within-subjects analysis, the c-Fos expression in serotonergic neurons in the DRD was highly correlated with the magnitude of the fear potentiation of startle following AS/CS trials, relative to AS trials. Neither exposure to the AS stimulus by itself, nor exposure to the CS by itself, had the same effect, suggesting that the combination of a fear state and acoustic startle were required for induction of c-Fos expression. In contrast, fear conditioning (*Experiment 1*) indiscriminately increased c-Fos expression throughout the DR, in comparison to home cage control conditions, similar to the effects of tail shock described in previous studies (Takase et al., 2005; Takase et al., 2004). In addition, fear-potentiated startle (*Experiment 2*) activated the medial part of the central nucleus of the amygdala (CeM; the main source of CE afferents to the DR), while fear conditioning (*Experiment 1*) activated the CeM, CeL, and CeC. Together, these findings point toward multiple mechanisms through which stress- or anxiety-related stimuli may influence serotonergic systems by stimulus-specific afferent pathways, one of which could arise from the CeM, to subregions of the midbrain raphe complex.

Acoustic startle, by itself, had no effect on c-Fos expression in serotonergic neurons, consistent with previous studies (Meloni et al., 2007); however, acoustic startle in the presence of the CS (fear-potentiated startle), compared to CS alone, selectively increased c-Fos expression in serotonergic neurons in the mid-rostrocaudal DRD, a region that has consistently been shown to be associated with stress- and anxiety-like responses. As activation of mesolimbocortical tryptophan hydroxylase activity by AS stimuli is mediated by the CE (Singh et al., 1992), this effect may be due to fear-induced increases in excitability of the CE and its output to the DR, which is directed preferentially to the DRD region (Peyron et al., 1998). Consistent with this hypothesis, in our study, exposure to the CS/AS condition increased c-Fos expression within the CeM, the part of the CE that gives

rise to direct projections to the DR (Gray and Magnuson, 1992). Indeed, electrical stimulation of the CE increases serotonin release in forebrain limbic structures in a CRF₂ receptor-dependent manner (Forster et al., 2008), and bilateral lesion of the CE prevents acoustic startle-induced increases in tryptophan hydroxylase activity in forebrain limbic structures (Singh et al., 1992; Boadle-Biber et al., 1993). Consistent with these findings, activation of CRF₂ receptors in the DR using microinjections of CRF or Ucn 2 increases serotonin release in targets of the DRD, including the basolateral amygdaloid nucleus (BL) (Amat et al., 2004) and nucleus accumbens (Lukkes et al., 2008). The DRD is activated by a variety of stress- and anxiety-related stimuli including anxiogenic drugs, such as caffeine and FG-7142 (Abrams et al., 2005), anxiogenic neuropeptides such as the CRF₂ receptor ligand Ucn 2 (Staub et al., 2005; Staub et al., 2006), and stress-related stimuli such as social defeat (Gardner et al., 2005) and inescapable, relative to escapable, stress (Grahn et al., 1999). Multiple lines of evidence suggest that the DRD is an important component of a stress- and anxiety-related neuronal system (Commons et al., 2003; for review, see Lowry et al., 2005; Lowry et al., 2008a; Lowry et al., 2008b; Lowry and Hale, 2009).

Selective activation of serotonergic neurons in the DRD by fear-potentiated startle is likely to result in increased serotonin release in specific limbic forebrain targets regulating emotional behavior. Electrical stimulation of the CE results in increases in serotonin release in the mPFC, and this effect can be blocked by microinjection of the CRF₂ receptor selective antagonist, antisauvagine-30 into the DR (Forster et al., 2008). Studies using intra-DR microinjection of Ucn 2, a selective CRF₂ receptor agonist, reveal an association between increased c-Fos expression in the mid-rostrocaudal DR and extracellular serotonin concentrations in forebrain structures such as the BL (Amat et al., 2004). The DRD sends efferent projections to limbic forebrain sites implicated in the regulation of stress- and anxiety-like states including the BL (Hale et al., 2008; Abrams et al., 2005; Ottersen, 1981), CE (Commons et al., 2003; Li et al., 1990; Rizvi et al., 1991), BNST (Commons et al., 2003; Petit et al., 1995), dorsal hypothalamic area (Commons et al., 2003), mPFC (Van Bockstaele et al., 1993), nucleus accumbens (Van Bockstaele et al., 1993) and ventral hippocampus (Imai et al., 1986). As serotonergic neurons are known to give rise to collateral projections to multiple, functionally related targets (Kohler and Steinbusch, 1982; Van Bockstaele et al., 1993), activation of a subset of serotonergic neurons could impact upon serotonin release in a number of different target sites.

The fact that subsets of serotonergic neurons within the DRD co-express other neuromodulators, such as CRF (Commons et al., 2003), provides further evidence for unique functional properties of serotonergic neurons in this region. Recent studies have described a dense population of vesicular glutamate transporter 3 (VGLUT3)-expressing neurons that is restricted to the DRD region, approximately 25% of which are serotonergic (Hioki et al., 2010). This is of potential interest because other studies have shown that VGLUT3 facilitates serotonergic neurotransmission of a subset of fibers innervating limbic forebrain sites, and plays a critical role in regulation of anxiety states (Amilhon et al., 2010).

The c-Fos expression in the mid-rostrocaudal DRD was positively correlated with the magnitude of the fear potentiation of startle based on within-subjects analysis. In the fear-potentiated startle group, greater numbers of c-Fos expressing serotonergic neurons in the DRD were associated with a greater fear potentiation of startle. Indeed, rats that failed to show a fear potentiation of startle failed to respond with increases in c-Fos expression. This strong correlation could arise because neural systems activated during fear potentiation of startle, for example the CE, selectively activate DRD serotonergic systems. As mentioned above, the DRD receives a dense innervation from the CE, including CRF-containing fibers (Rizvi et al., 1991; Gray and Magnuson, 1992; Gray, 1993). Interestingly, c-Fos expression in the CeM, previously shown to project to the DRD, was correlated with serotonergic

activity in our study. Together, these data are consistent with a hypothetical model in which the combination of the presence of cue-dependent conditioned fear and a motor eliciting stimulus is sufficient for activation of serotonergic neurons in the DRD via a mechanism involving the CE. Acoustic startle stimuli have been shown previously to induce a short-latency, short-duration excitation in DR serotonergic neurons (Rasmussen et al., 1984). It appears that this short duration excitation is not sufficient to induce increased c-Fos expression, although it may be sufficient to increase tryptophan hydroxylase activity (Boadle-Biber et al., 1989), presumably due to action potential-dependent calcium influx (Boadle-Biber and Phan, 1987; Boadle-Biber, 1982; Boadle-Biber et al., 1983; Boadle-Biber, 1978). However, our data suggest that the combination of cue-dependent conditioned fear and an acoustic startle stimulus would result in a greater excitation associated with c-Fos induction in the DRD. It is unclear if a temporal coincidence of the fear-eliciting stimulus and acoustic startle is necessary for activation of DRD serotonergic neurons, as the CS and AS always co-terminated in our study.

The CE is an important output structure of the amygdala that has been implicated in the acquisition, consolidation, and expression of fear (Wilensky et al., 2006) and implicated numerous times in fear conditioning (Campeau and Davis, 1995). The CE can be broken down into three functionally different subdivisions: the medial (CeM), lateral (CeL) and capsular (CeC) parts of the CE (Paxinos and Watson, 1998). Of these subdivisions the CeM projects directly to the DR (Rizvi et al., 1991; Gray and Magnuson, 1992), the CeL contains a high concentration of CRF expressing neurons that project to the BNST (Sakanaka and Lederis, 1986), and the CeC plays an important role in nociception (Neugebauer et al., 2004). Interestingly, our study shows activation of the nociceptive CeC, the CeL and the CeM during fear conditioning, and activation of the CeM and rostral CeL during fear-potentiated startle. Additionally, c-Fos expression in the mid-rostrocaudal DRD was correlated with activation in the CeM when all three rostrocaudal levels of the CeM examined were included, but interestingly, this effect was driven by the most rostral level examined, 2.12 mm bregma. At this rostrocaudal level the CeL and CeC were both correlated with the number of c-Fos-ir/TPH-ir neurons in the mid-rostrocaudal DRD, consistent with inter-subdivision connectivity in the CE and a topographical organization along its rostrocaudal axis in stress-induced c-Fos-like immunoreactivity (Porter and Hayward, 2011). Although the functional consequences of the putative activation of a CeM-DR circuit are not yet clear, electrical stimulation of the CeM, specifically, results in bradycardia, hypotension, behavioral quiescence, and increased respiration (Kapp et al., 1982; Cox et al., 1987), physiologic and behavioral responses that are also observed following stimulation of the DRVL/VLPAG region (Keay and Bandler, 2001). It is likely that several cell types were activated within the CE, including cells that do not project to the DRD. Future studies will attempt to delineate the phenotype of activated cells in these regions and use retrograde tracing to determine if DR-projecting neurons in subdivisions of the CE are activated following fear-potentiated startle.

Although our data support the hypothesis that fear-potentiated startle-induced increases in c-Fos expression in DRD serotonergic neurons is dependent on activation of a CE-DRD pathway, the selective effects of fear-potentiated startle on the DRD could be due to activation of a number of specific afferent pathways that target this region of the DR. A number of forebrain limbic structures give rise to projections that selectively target the DRD region (for review, see Lowry et al., 2005; Lowry et al., 2008a; Lowry et al., 2008b; Lowry and Hale, 2009). For example, the DRD, relative to other subregions of the DR, receives a dense innervation from a number of limbic forebrain structures in addition to the CE, including the bed nucleus of the stria terminalis (BNST) and the medial prefrontal cortex (mPFC) (Peyron et al., 1998; Lee et al., 2003). Consistent with selective afferent projections to the DRD, the DRD receives dense input from CRF-containing fibers (Lowry et al., 2000;

Kirby et al., 2000). An alternative mechanism that could account for a site-specific effect of fear-potentiated startle on serotonergic neurons in the DRD would be selective expression within the DRD of specific receptors for neurotransmitters, neuropeptides, or hormones. Corticotropin-releasing factor type 2 (CRF₂) receptors (Pernar et al., 2004; Staub et al., 2005; Staub et al., 2006; Amat et al., 2004; Lukkes et al., 2008) and α_1 adrenoceptors (Vandermaelen and Aghajanian, 1983), both of which are known to be excitatory to serotonergic neurons in the DR, are expressed throughout its rostrocaudal extent (Day et al., 2004). Likewise, type II glucocorticoid receptors (GR) are widely distributed throughout the midbrain raphe complex (Morimoto et al., 1996). However, the DRD is selectively targeted by fibers that express neurokinin 1 receptors (NK1; Commons et al., 2003), which could, therefore, selectively modulate the activity of DRD serotonergic neurons. A final possibility is that the DR receives widespread excitatory input, but that the DRD has less inhibitory control, mediated by either γ -aminobutyric acid (GABA), 5-HT_{1A} autoreceptors, or other mechanisms. Electrophysiological and functional neuroanatomical studies support the possibility that different subsets of serotonergic neurons are under differential inhibitory control (Commons, 2008; Beck et al., 2004; Crawford et al., 2010).

Control rats were placed into the chamber used for fear conditioning, which likely elicited contextual fear. Rats exposed to the fear conditioning environment responded with increases in c-Fos expression in the rostral DRD and mid-rostrocaudal DRV_L/VLPAG, but no effect was seen in the mid-rostrocaudal DRD where fear-potentiated startle was sufficient for serotonergic activation. The rostral DRD projects to forebrain structures associated with motor control, including the caudate putamen and substantia nigra (Steinbusch et al., 1980; Lowry et al., 2005; Lowry et al., 2008a), and therefore it is possible that changes in muscle tone associated with conditioned fear (e.g. during freezing behavior) are associated with increases in c-Fos expression in the rostral DRD. In contrast, serotonergic neurons in the DRV_L/VLPAG are part of a sympathomotor control system (Kerman et al., 2006) that is involved in inhibition of sympathetically mediated increases in blood pressure (Bago and Dean, 2001; Johnson et al., 2008), via projections to the rostroventrolateral medulla (Bago et al., 2002; Underwood et al., 1999a). This system may provide an inhibitory influence on fear-elicited hypertension, as proposed previously in a model of panic (Johnson et al., 2008).

In contrast to the effects of fear-potentiated startle on specific subdivisions of the DR, fear conditioning indiscriminately increased c-Fos expression throughout the DR, compared with home cage controls. The fear conditioning group only received one training session; it is possible a second session could alter the magnitude or pattern of c-Fos expression. Regardless, the observed widespread serotonergic activity is consistent with previous findings using repeated inescapable tail shock (Takase et al., 2005; Takase et al., 2004). The widespread effects of fear conditioning activating serotonergic neurons throughout the DR may be due to coincident activation of multiple afferent excitatory inputs. Indeed, a number of structures that provide widespread innervation of the DR are activated by foot shock, including brainstem catecholaminergic systems (Braz et al., 2009; Vandermaelen and Aghajanian, 1983; Peyron et al., 1996). Fear conditioning, compared with home cage controls, also increased c-Fos expression in non-serotonergic cells in virtually all regions of the DR.

Together, these findings point toward multiple mechanisms through which stress- or anxiety-related stimuli may influence serotonergic systems, where different stress-related stimuli activate stimulus-specific afferent pathways to subregions of the midbrain raphe complex. Our data suggest pairing of a cue-dependent conditioned fear and a motor eliciting stimulus selectively activates serotonergic neurons in the DRD, potentially in a CeM-dependent fashion, although it is not known whether non-coinciding CS and AS stimuli would elicit a similar effect. These data are consistent with the hypothesis that dysregulation

of a CE-DR circuit may play a role in the pathophysiology of anxiety and affective disorders. Consistent with this hypothesis, depressed suicide patients have elevated calbindin-like immunoreactivity, a marker of a subset of GABAergic neurons, in the CE (Liu et al., 2009). In addition, depressed suicide patients show increases in *tph2* mRNA and tryptophan hydroxylase protein expression that are restricted to the DRD and DRC (Bach-Mizrachi et al., 2006; Bach-Mizrachi et al., 2008; Bonkale et al., 2006) and target regions of these DR subdivisions, such as the mPFC (Perroud et al., 2010). A further understanding of mechanisms underlying regulation of a CE-DR circuit may lead to novel therapeutic approaches to the prevention or treatment of anxiety and affective disorders.

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Abbreviations

AS	acoustic startle
BL	basolateral amygdaloid nucleus
CE	central amygdaloid nucleus
CRF	corticotropin-releasing factor
CS	conditioned stimulus
CS/AS	pairing of conditioned stimulus and acoustic startle
DAB	3,3'-diaminobenzidine tetrahydrochloride
DR	dorsal raphe nucleus
DRC	dorsal raphe nucleus, caudal part
DRD	dorsal raphe nucleus, dorsal part
DRI	dorsal raphe nucleus, interfascicular part
DRV	dorsal raphe nucleus, ventral part
DRV/VLPAG	dorsal raphe nucleus, ventrolateral part/ ventrolateral periaqueductal gray
FPS	fear-potentiated startle
GABA	γ -aminobutyric acid
ir	immunoreactive
PB	sodium phosphate buffer
PBS	phosphate-buffered saline
PBST	0.05 M phosphate-buffered saline containing 0.1% or 0.3% (as indicated) Triton X-100
TPH	tryptophan hydroxylase
Ucn 2	urocortin 2

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Figure. 1.

Schematic illustration of **A) Experiment 1** where rats were trained by pairing a visual CS with a 0.5 sec, 0.5 mA foot shock in a single training session and **B) Experiment 2** where rats were trained in an identical manner to the training session in *Experiment 1* on days 1 and 2 then tested under different treatment paradigms consisting of either control conditions (CS-, AS-); CS, 10 1000 lx, 2 sec visual stimuli; AS, 40 110 dB, 50 msec acoustic stimuli; or CS/AS, 10 CS/AS pairings intermixed among 30 AS-alone stimuli. All training and test sessions were 20 min in duration. Abbreviations: AS, acoustic startle; CS, conditioned stimulus; dB, decibels; lx, lux; mA, milliamperes; s, second.

Figure 2.

Photomicrographs of the rostrocaudal levels and subdivisions of the dorsal raphe nucleus (DR) selected for analysis. Tryptophan hydroxylase (TPH) immunostaining is visible at low magnification as a light brown stain. Subdivisions are outlined by dashed lines based on a standard stereotaxic atlas of the rat brain at the rostrocaudal levels of **A**) -7.28; **B**) -7.46; **C**) -8.00; **D**) -8.18; **E**) -8.54; and **F**) -9.16 mm bregma (Paxinos and Watson, 1998). The subdivisions examined at these six rostrocaudal levels were combined into **A** and **B**) rostral (-7.28 and -7.46 mm bregma); **C** and **D**) mid-rostrocaudal (-8.00 and -8.18 mm bregma); and **E** and **F**) caudal (-8.54 and -8.72 mm bregma) levels for analysis. Abbreviations: 4V, fourth ventricle; Aq, cerebral aqueduct; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus; and MnR, median raphe nucleus. Scale bar, 200 μ m.

Figure 3.

Photomicrographs illustrating the rostrocaudal levels and subdivisions of the central amygdaloid nucleus (CE) that were selected for analysis. Subdivisions are outlined by dashed lines based on Nissl staining and a standard stereotaxic atlas of the rat brain at the rostrocaudal levels of **A**) –2.12; **B**) –2.56; (Paxinos and Watson, 1998), and **C**) –3.14 mm bregma (Paxinos et al., 1999). Solid lines indicate fiber tracts and blood vessels.

Abbreviations: bv, blood vessel; CeC, central amygdaloid nucleus, capsular part; CeL, central amygdaloid nucleus, lateral part; CeM, central amygdaloid nucleus, medial part; cst, commissural stria terminalis; IM intercalated amygdaloid nucleus, main part; lab, longitudinal association bundle; opt, optic tract. Scale bar, 200 μ m.

Figure 4

. Graphs illustrating double immunostained c-Fos-ir/TPH-ir (closed bars) and total TPH-ir cell counts (open bars) in subdivisions of the midbrain raphe complex following exposure to fear conditioning (FC) or home cage control conditions (HC). Graphs are organized into rows representing rostral (−7.28 and −7.46 mm bregma), mid-rostrocaudal (−8.00 and −8.18 mm bregma), and caudal (−8.54, −8.72 mm bregma) levels of the dorsal raphe nucleus. Abbreviations: 4V, fourth ventricle; Aq, cerebral aqueduct; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRV/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; FC, fear conditioning group; HC, home cage control group; mlf, medial longitudinal fasciculus. Data are presented as means + S.E.M., *, $p < 0.05$, fear conditioning group versus home cage control group, Fisher's Protected LSD test. Sample size, $n = 8$.

Figure 5.

Photomicrographs illustrating immunohistochemical staining of c-Fos (blue) and tryptophan hydroxylase (TPH; brown) in subdivisions of the mid-rostromedial DR (−8.18 mm bregma) in *Experiment 1*. Panels **A–D** illustrate immunostaining in a section from a home cage control rat and panels **E–H** illustrate immunostaining in a section from a rat exposed to fear conditioning. Black boxes in **A** and **E** indicate regions shown at higher magnification in panels **B–D** and **F–H**, respectively. Black boxes in **B–D** and **F–H** indicate regions shown at higher magnification in insets in the lower right corner of each panel. Arrows indicate non-serotonergic c-Fos-ir cells, white arrowheads indicate TPH-ir/c-Fos immunonegative neurons, and black arrowheads indicate double immunostained c-Fos-ir/TPH-ir neurons.

Abbreviations: Aq, cerebral aqueduct; bv, blood vessel; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus. Scale bar, **A**, **E**, 200 μm ; **B-D**, **F-H**, 100 μm ; insets, 40 μm .

Figure 6.

Photomicrographs illustrating immunohistochemical staining of c-Fos in subdivisions of the central amygdaloid nucleus (CE) at -2.12 mm bregma following exposure to fear conditioning or home cage control conditions in *Experiment 1*. Panels **A** and **C** demonstrate Nissl staining while panels **B** and **D** demonstrate c-Fos immunoreactivity in adjacent sections. Panels **A** and **B** are sections from a home cage control rat and panels **C** and **D** are sections from a rat exposed to fear conditioning. The location of blood vessels and fiber tracts (solid lines) in alternate Nissl-stained and c-Fos-immunostained sections were used to match adjacent sections in order to define the subdivision borders (dashed lines) of the CE in c-Fos-immunostained sections. Black boxes in **B** and **D** indicate regions shown at higher magnification in panels **B'**, **B''** and **D'**, **D''**, respectively. Arrows indicate c-Fos-immunoreactive (ir) cells. Abbreviations: bv, blood vessel; CeC, central amygdaloid nucleus, capsular part; CeL, central amygdaloid nucleus, lateral part; CeM, central amygdaloid nucleus, medial part; cst, commissural stria terminalis; IM, intercalated amygdaloid nucleus, main part; lab, longitudinal association bundle; opt, optic tract. Scale bar, **A-D**, $200\ \mu\text{m}$; **B'**, **B''**, **D'**, and **D''**, $80\ \mu\text{m}$.

Figure 7.

Graphs illustrating the effects of acoustic startle, conditioned fear, and fear-potentiated startle on c-Fos expression in serotonergic neurons in the dorsal raphe nucleus (DR). Graphs illustrate the effects of exposure to home cage control conditions (HC), control conditions (C; CS-, AS-), the conditioned stimulus (CS) alone, the acoustic startle (AS) stimulus alone, or both the CS and AS (CS/AS) on double immunostained c-Fos-ir/TPH-ir (closed bars) and total TPH-ir cell counts (open bars) in subdivisions of the dorsal raphe nucleus. Graphs are organized into rows representing rostral (-7.28 and -7.46 mm bregma), mid-rostrocaudal (-8.00 and -8.18 mm bregma), and caudal (-8.54, -8.72 mm bregma) levels of the dorsal raphe nucleus. Abbreviations: 4V, fourth ventricle; Aq, cerebral aqueduct; AS, acoustic startle; CS, conditioned stimulus; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus. Data are presented as means + S.E.M., *, $p < 0.05$ C versus HC group; +, $p < 0.05$, CS-, AS- control group versus CS or AS groups; ^a, $p < 0.05$, CS/AS group versus CS group. Fisher's Protected LSD test. Sample size, $n = 8$ for all groups except CS/AS, $n = 5$.

Figure 8.

Photomicrographs illustrating immunohistochemical staining of c-Fos (blue) and tryptophan hydroxylase (TPH; brown) in the dorsal part of the dorsal raphe nucleus (DRD) in the mid-rostrocaudal dorsal raphe nucleus (DR) (−8.18 mm bregma) in *Experiment 2*.

Photomicrographs of sections from representative rats in each treatment are illustrated as follows: **A, E**, control; **B, F**, CS; **C, G** AS, and **D, H**, CS/AS. Black boxes in **A–D** indicate regions shown at higher magnification in panels **E–H**, respectively. Black boxes in **E–H** indicate regions shown at higher magnification in insets in the lower right corner of each panel. Arrows indicate non-serotonergic c-Fos-ir cells, white arrowheads indicate TPH-ir/c-Fosimmunonegative neurons, and black arrowheads indicate double immunostained c-Fos-

ir/TPH-ir neurons. Abbreviations: Aq, cerebral aqueduct; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus. Scale bar, **A-D**, 200 μm ; **E-H** 80 μm ; insets, 40 μm .

Figure 9. Scatter plots illustrating correlations between fear potentiation of startle (based on within-subject analysis) and c-Fos-ir/tryptophan hydroxylase-immunoreactive (TPH-ir) cells in the DRD at **A**) –8.00 and **B**) –8.18 mm bregma in the CS/AS treatment group. Data are presented as the mean percent fear potentiation of startle (CS/AS pairing/AS alone $\times$ 100) versus the number of c-Fos-ir/TPH-ir double immunostained neurons. Open circles represent data from the three rats in the CS/AS treatment group that were excluded from analysis of cell counts based on the absence of fear potentiation of startle; closed circles represent data from the remaining 5 rats. Dashed line at 125% potentiation represents cutoff point for fear potentiation of startle.

Figure 10.

Scatter plot illustrating a positive correlation between the numbers of c-Fos-ir/TPH-ir neurons in the mid-rostrocaudal DRD and the number of c-Fos-ir cells in the CeM at -2.12 mm bregma ($r^2 = 0.495$, $p < 0.001$) based on between-subject analysis when rats from all treatment groups from *Experiment 1* and *Experiment 2* were included.

Table 1

Effects of fear conditioning on c-Fos expression in non-serotonergic cells in the midbrain raphe complex (*Experiment 1*).

Region	Rostrocaudal level (mm bregma)	Home cage	Fear conditioning
DRD	Rostral (−7.28 and −7.46)	30.9 ± 2.94	67.6 ± 7.32 *
DRV		15.0 ± 3.10	33.9 ± 3.82 *
DRD	Mid-rostrocaudal (−8.00 and −8.18)	14.6 ± 2.33	54.6 ± 6.82 *
DRVL		32.3 ± 5.45	105.6 ± 6.51 *
DRV		2.75 ± 0.49	18.5 ± 2.69 *
DRC	Caudal (−8.54 and −8.72)	8.88 ± 1.87	22.8 ± 1.87 *
DRI		9.75 ± 0.88	17.1 ± 1.86 *

* $p < 0.05$, Home cage versus Fear conditioning group; Fisher's Protected Least Significant Difference test.

Table 2

Effects of fear conditioning on c-Fos expression in subdivisions of the central nucleus of the amygdala (*Experiment 1*).

Region	Rostrocaudal level (mm bregma)	Home cage	Fear conditioning
CeM		23.0 ± 3.34	57.8 ± 9.06*
CeL	−2.12	17.1 ± 4.37	18.0 ± 2.57
CeC		15.1 ± 1.70	30.1 ± 4.02*
CeM		22.3 ± 3.27	30.2 ± 1.40*
CeL	−2.56	34.8 ± 8.18	20.4 ± 5.24
CeC		16.8 ± 2.39	35.4 ± 4.95*
CeM		35.5 ± 5.66	68.0 ± 14.0*
CeL	−3.14	15.3 ± 3.92	57.9 ± 8.02*
CeC		27.3 ± 4.55	56.9 ± 6.80*

* $p < 0.05$, Home cage versus Fear conditioning group; Fisher's Protected Least Significant Difference test.

Table 3Effects of fear-potentiated startle on c-Fos expression in subdivisions of the central nucleus of the amygdala (*Experiment 2*).

Region	Rostrocaudal level (mm bregma)	Home cage	Control	CS	AS	CS/AS
CeM		23.0 ± 3.34	21.0 ± 2.80	17.8 ± 2.56	36.4 ± 6.77	35.0 ± 8.17 ^a
CeL	-2.12	17.1 ± 4.37	7.29 ± 1.63	7.37 ± 1.80	20.6 ± 4.78 ⁺	21.1 ± 5.86 ^a
CeC		15.1 ± 1.70	15.4 ± 3.26	16.0 ± 3.06	25.8 ± 4.40 ⁺	17.4 ± 3.70
CeM		22.3 ± 3.27	19.8 ± 3.92	14.5 ± 3.36	28.4 ± 3.63	33.6 ± 7.09 ^a
CeL	-2.56	34.8 ± 8.18	22.1 ± 6.23	10.9 ± 2.45	26.5 ± 2.87	21.2 ± 6.30
CeC		16.8 ± 2.39	27.9 ± 6.51	17.4 ± 3.80	26.8 ± 1.81	23.4 ± 6.74
CeM		35.5 ± 5.66	54.1 ± 6.49	29.8 ± 6.58	54.0 ± 4.16	36.6 ± 9.75 ^a
CeL	-3.14	15.3 ± 3.92	30.4 ± 6.67	24.1 ± 6.00	21.3 ± 2.69	22.0 ± 3.30
CeC		27.3 ± 4.55	45.4 ± 5.81 [*]	38.0 ± 6.85	53.6 ± 5.33	57.8 ± 8.03

* $p < 0.05$, Home cage versus Control group⁺ $p < 0.05$, Control group versus AS group^a $p < 0.05$, CS group versus CS/AS group; Fisher's Protected Least Significant Difference test.