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Differential distribution of serotonin receptor subtypes in $BNST_{ALG}$ neurons: Modulation by unpredictable shock stress

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Abstract

The bed nucleus of the stria terminalis (BNST) plays a critical role in regulating the behavioral response to stress. Stressors that activate the BNST also activate serotonergic (5-HT) systems. Hence, maladaptive changes of 5-HT receptor expression may contribute to stress-induced anxiety disorders. The BNST contains three neuronal types, Type I – III neurons. However, little is known about 5-HT receptor subtypes mRNA expression in these neurons, or whether it can be modulated by stress.

Whole-cell patch clamp recording from Type I – III neurons was used in conjunction with single cell RT-PCR to characterize 5-HT receptor mRNA expression, and examine the effects of stress on this expression. We report that Type I neurons expressed mRNA transcripts predominantly for 5-HT_{1A} and 5-HT_7 receptors. Type II neurons expressed transcripts for every 5-HT receptor except the 5-HT_{2C} receptor. Type II neurons were divided into three sub-populations: Type IIA in which transcripts for 5-HT_3 and 5-HT_7 receptors predominate, Type IIB that mainly express 5-HT_{1B} and 5-HT_4 receptor transcripts, and Type IIC in which transcripts for 5-HT_{1A} and 5-HT_{2A} receptors predominate. Type III neurons were also subdivided into two sub-populations; one that predominantly expressed transcripts for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2A} receptors, and another that mainly expressed transcripts for 5-HT_{2C} receptor.

Unpredictable shock stress (USS) caused a long-lasting increase in anxiety-like behavior, and a concomitant decrease in 5-HT_{1A} transcript expression in Type I – III neurons, as well as an up-regulation of a transcriptional repressor of 5-HT_{1A} gene expression, deformed epidermal autoregulatory factor 1(Deaf-1). Significantly USS decreased 5-HT_{1A} protein level, and increased the level of Deaf-1. USS also increased 5-HT_{1B} transcript expression in Type III neurons, as well as 5-HT_7 expression in Type I and II neurons. These data suggest that cell type-specific disruption of 5-HT receptor expression in $BNST_{ALG}$ neurons may contribute to stress-induced anxiety disorders.

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Keywords

Bed nucleus of the stria terminalis; chronic stress; Deaf-1; serotonin receptors; single cell RT-PCR; 5-HT_{1A}

Introduction

The stress response is an adaptive process essential for an animal to face challenging and threatening situations. Chronic stress, however, may produce maladaptive consequences such as anxiety disorders and depression (Lupien et al., 2009). Extra-hypothalamic responses to stress are mediated by several interconnected nuclei, including the bed nucleus of the stria terminalis (BNST), which play a key role in regulating the autonomic, neuroendocrine, and behavioral responses to stress (Walker et al., 2003, Choi et al., 2007, Crestani et al., 2010, Kuwaki, 2011). Importantly, the BNST is considered as the major integrative center of excitatory and inhibitory inputs that regulate the HPA axis (Forray and Gysling, 2004), and has been reported to mediate stress-responding and anxiety-like behavior resulting from persistent stress (Walker et al., 2009). Consistent with this observation, long-term plastic changes occur in the BNST in response to chronic stress, including alterations in the volume of the BNST that is accompanied by changes in the dendritic length and number of branch points in individual neurons (Vyas et al., 2003).

However, the BNST is a complex structure that is divided into anterior and posterior subdivisions by the fibers of the stria terminalis, into dorsal and ventral subdivisions by the fibers of the anterior commissure (De Olmos, 1985, Ju et al., 1989) and that contains approximately 16 different nuclei (Bota et al., 2012). Notably, the anterior BNST, and in particular the dorsolateral cell group ($BNST_{ALG}$), contains a high density of neurons that express the stress neuropeptide, corticotropin releasing factor (Swanson et al., 1983) and has been reported to play a prominent role in modulating the behavioral response to chronic stressors (Dunn, 1987, Casada and Dafny, 1991, Shepard et al., 2006, Hammack et al., 2009, Davis et al., 2010, Christianson et al., 2011, Conrad et al., 2011). Recently, we identified three electrophysiolgically distinct cell types in the $BNST_{ALG}$ (Type I – III neurons, see Hammack et al., 2007) and we were interested to see how stress may affect the different cell types.

Stressors that activate the BNST also activate central serotonergic (5-HT) systems (Dilts and Boadle-Biber, 1995, Grahn et al., 1999b, Funada and Hara, 2001, Lowry, 2002, Summers et al., 2003, Takase et al., 2004). Interestingly, uncontrollable stress and anxiogenic drugs have been shown to activate a subset of serotonin (5-HT) containing neurons in the dorsal raphé nucleus (DRN) that preferentially target limbic forebrain regions such as the BNST (Grahn et al., 1999a, Lowry et al., 2000, Singewald et al., 2000). Previously, we have shown that activation of inhibitory 5-HT_{IA} receptors in the $BNST_{ALG}$ has an anxiolytic action (Levita et al., 2004), and we have posited that activation of these receptors may function as an inhibitory-feedback mechanism to terminate the stress response (Hammack et al., 2009). Conversely, activation of excitatory 5-HT receptors, such as the 5-$HT_{2A,2C,and7}$ receptors in $BNST_{ALG}$ neurons may have an opposing anxiogenic action (Guo et al., 2009). Hence, the net behavioral response to 5-HT release in the $BNST_{ALG}$ would be determined by the relative level of activation of these key receptor subtypes. It is noteworthy, therefore, that transgenic mice lacking the 5-HT_{1A} receptor display decreased exploratory activity and increased fear of aversive environments compared to their wild type counterparts (Gordon et al., 2005), suggesting that reduced 5-HT_{1A} receptor expression might result in heightened anxiety. Significantly, chronic stress, or chronic administration of stress hormones, has been shown to decrease 5-HT_{1A} receptor expression and/or increase 5-HT_{2A} receptor expression

in other brain regions (Katagiri et al., 2001, Ossowska et al., 2001). A similar stress-induced alteration in the expression of either of these two receptors in the $BNST_{ALG}$ would be predicted to have a significant impact on stress-induced affective behavior.

Recent studies suggest that the 5-HT_{1A} receptor gene is tightly regulated by an upstream dual repressor element (DRE) that inhibits gene expression in neuronal and non-neuronal systems (Ou et al., 2000). Subsequently, several novel DNA binding proteins have been shown to potently regulate 5-HT_{1A} gene expression by binding to the DRE site, including hairy and enhancer of split 5 (Hes-5), five prime repressor element under dual binding protein (Freud-1) and deformed epidermal autoregulatory factor-1 (Deaf-1, also called NUDR for review see (Albert et al., 2011). Significantly, Deaf-1 is reported to selectively co-localize with 5-HT_{1A} receptors in neurons of the DRN and limbic forebrain (Lemonde et al., 2003), and alterations in its expression have been associated with depression, suicide, and panic disorder (Szewczyk et al., 2009). However, nothing is known about the expression of the selective repressor elements in the $BNST_{ALG}$, or whether their expression or that of any of the 5-HT receptor subtypes is altered following chronic stress.

Recently, we reported that mRNA transcripts for the 5-$HT_{1A, 2A, 2C}$ and$_7$ receptor subtypes are differentially expressed in Type I–III $BNST_{ALG}$ neurons. Hence, the 5-HT_{1A} receptor was seen to be expressed by all $BNST_{ALG}$ neurons, whereas 5-HT_{2A} and 5-HT_{2C} receptors were expressed exclusively by Type II, and Type III neurons, respectively. Similarly, 5-HT_7 receptors were expressed mainly in Type I and II neurons but not in Type III neurons (Guo et al., 2009). Here, we extend these initial observations to examine 1) the mRNA expression pattern for all of the known 5-HT receptor subtypes in Type I–III $BNST_{ALG}$ neurons, and 2) the presence or absence of 5-HT_{1A} receptor transcriptional repressors in these same neurons. We then further extend these studies to examine the effects of a repeated unpredictable shock stress (USS) paradigm on the expression of 5-HT receptor subtypes and/or 5-HT_{1A} repressor elements in physiologically defined cell types in the $BNST_{ALG}$.

Experimental Procedures

Animals

All experiments were conducted on male Sprague-Dawley rats (Charles River, NC) aged between 35 and 45 days of age. All rats were housed four per cage and had unrestricted access to food and water. Care was taken to minimize the number of animals used, and all procedures were done in accordance with policy guidelines set by the National Institutes of Health and were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Single Cell RT-PCR

Whole cell patch clamp recordings were obtained from visually identified $BNST_{ALG}$ neurons in 350 μm brain slices, and individual cell types were determine as previously described (Hammack et al., 2007, Guo et al., 2009, Guo and Rainnie, 2010). Briefly, three electrophysiologically distinct cell types have been defined (Hammack et al., 2007); Type I neurons are characterized by a regular firing pattern in response to membrane depolarization, and a depolarizing sag in the voltage response to hyperpolarizing current injection that is mediated by the hyperpolarization-activated cation current, I_h. Type II neurons are characterized by a burst-firing pattern that is mediated by activation of the low-threshold calcium current, I_T, and also express a prominent I_h. Type III neurons are characterized by a regular firing pattern, have no prominent I_h, and a pronounced fast hyperpolarization-activated voltage rectification indicative of the inwardly rectifying potassium current, $I_{K(IR)}$.

At the end of each recording session, the cell cytoplasm was aspirated into the patch recording pipette containing ~5 μl of RNase-free patch solution under visual control, by applying gentle negative pressure. The contents of the patch pipette were then expelled into a microcentrifuge tube containing 5 μl of the reverse transcription cocktail (Applied Biosystems, Foster City, CA). The RT product was amplified in triplicate and screened for 18S rRNA. Only those cell samples that were positive for 18S rRNA were subjected to amplification with primers. The procedure used to determine mRNA transcript expression in single cells has been described (Hazra et al, 2011). The sequence for the oligonucleotide primers of the 5-HT receptor subtypes used in this study have been reported previously (Guo et al, 2009). The primers used for Deaf-1 is forward 5'- GGT TTG TGC AGT GGT AGA TG-`3 and reverse 5'- GAG CGT GCC ACT GAT GTT C-`3 (Accession # NM_031801, 521bp); Freud-1 is forward 5'- CGC CAG CTG CAC TTC TAT AC -`3 and reverse 5'- CTC ACT CTC CAC CAG GTT CC -`3 (Accession # NM_001013869, 400bp); and Hes-5 is forward 5'- CGC ATC AAC AGC AGC ATT GAG -`3 and reverse 5'- TGG AAG TGG TAA AGC AGC TTC-`3 (Accession # NM_024383, 350bp). PCR products were visualized by staining with ethidium bromide and separated by electrophoresis in a 1% agarose gel.

Controls for the RT-PCR

PCR conditions were optimized using total RNA isolated from rat $BNST_{ALG}$ so that a PCR product could be detected from (250pg–1ng) of total RNA without contamination caused by non-specific amplification. For each PCR amplification, sterile water was used instead of cDNA as a control for contaminating artifacts. A second control with no RT present was also used in each amplification step. Both the controls gave negative results throughout the study. All primers were intron-spanning to exclude amplification of genomic DNA.

Acoustic startle test

In the acoustic startle response (ASR), rats were placed in an acoustic startle chamber for 5 minutes prior to testing to acclimatize the animals to the chambers. Four rats were tested simultaneously in identical 8 × 15 × 15 cm Plexiglas and wire mesh cages, each suspended between compression springs within a steel frame located within a custom-designed sound-attenuating chamber. Details of the recording apparatus have been reported previously (Walker and Davis, 1997, 2002, Sink et al., 2011). Subsequently, the ASR was measured following each of 30 acoustic stimuli. Startle responses were evoked by 50-ms 100, 105 and 110 dB white-noise bursts generated in a pseudorandom order by a Macintosh G3 computer sound file, amplified by a Radio Shack amplifier (100 W, Model MPA-200; Tandy, Fort Worth, TX), and delivered through speakers located 5 cm in front of each cage. Startle amplitude was defined as the maximum peak-to-peak voltage of the Instrument's output during the first 200 ms after each noise burst. The presentation and sequencing of all stimuli was under the control of a Macintosh G3 computer using custom designed software (The Experimenter; Glassbeads Inc.,Newton, CT). On day 10 a similar ASR paradigm was followed in both non stress (NS) and 4 day unpredictable shock stress rats (USS).

Repeated stress procedure and behavioral analysis

The USS paradigm used in these experiments was adapted from previous studies in rat and human (Walker et al., 2003, Moberg and Curtin, 2009). Here, rats were placed inside a modular operant conditioning chamber, 59.7 × 34.3 × 26.35 cm, with aluminum and polycarbonate walls (Lafayette Instruments, Lafayette, IN). The floor of the chamber is made of 0.4 cm diameter stainless steel bars spaced at 1.1 cm that conducts the electric shock. Before administering the USS, rats, 35 days of age, were first matched for their basal anxiety level using a standard acoustic startle paradigm (see above). In total, 32 rats were matched according to their initial startle response, and then divided into two groups, 16 NS rats and 16 USS rats. Eight rats (NS=4; USS=4) were used for total RNA isolation, eight rats

for protein isolation, eight rats for single cell RT-PCR, and eight rats for contextual freezing. NS animals received exactly the same handling procedures as the USS group and were placed in the shock chamber for the same duration without being shocked. On day 1, the USS rats were placed in the shock chamber and allowed to habituate to their environment for 5 minutes. Rats then received two eight minute periods of eight randomly applied footshocks (0.5s, 0.5 mA) separated by an eight minute period of no shock. The USS paradigm was repeated on each of the following three days for a total of four consecutive days shock stress. Each rat was shocked at approximately the same time every day (9 AM) to control for diurnal hormone variations. Rats in both groups were then returned to their home cages for six days. On the tenth day, the rats were first measured for their post-stress startle response, and then placed in the chamber in which they received the shock and their contextual freezing behavior assessed over a 5 minute period, recorded on video, and analyzed off-line using the Freezescan software program (Cleversys Inc, VA). Rats were then immediately sacrificed; the brain removed, and processed for RNA and protein isolation using standard procedures (Dabrowska and Rainnie, 2010, Hazra et al., 2011).

Quantitative PCR measures of transcript expression

RNA Isolation—Total RNA was isolated from $BNST_{ALG}$ tissue by homogenizing each sample in Trizol (Invitrogen, Carlsbad, CA). The isolated RNA was then reverse transcribed using a cocktail containing 5 μl of 10×RT buffer, 10mM dNTP mix, 10× random hexanucleotide and Multiscribe RT 5U/ul and RNAase free water. The mixture was incubated in a thermal cycler at 25°C for 10 min and then at 37°C for 120 min, the resulting cDNA samples were stored at −20°C. All reagents were obtained from Applied Biosystems (Foster City, CA).

Quantitative PCR—Real-time PCR reactions were performed using an Applied Biosystems 7500 Fast-Real Time PCR system (Applied Biosystems, Foster City, CA). Here, 2μl of cDNA obtained from the isolated RNA were combined with Taqman probes specific for 18S rRNA (Accession # X03205), 5-HT_{1A} (NM_012585), 5-HT_{1B} (NM_022225), 5-HT_{1D} (NM_012852), 5-HT_{1F} (NM_021857), 5-HT_{2A} (NM_017254), 5-HT_{2C} (NM_012765),5-HT_3 (NM_024394), 5-HT_4 (NM_012853), 5-HT_5 (NM_013148), 5-HT_6(NM_024365),5-HT_7 (NM_022938), Deaf-1 (NM_031801),Freud-1 (NM_001013869) and Hes5 (NM_024383) and 1×Taqman universal PCR Master Mix (Applied Biosystems). The reaction for each cell sample was performed in triplicate, and using a 40 cycle thermal cycling program: cycle 1– 20 min at 95°C; cycles 2 through 50– 95°C for 3 sec, followed by 60°C for 30 min. The relative levels of mRNA expression were normalized in all the samples with expression levels of 18S rRNA. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to calculate the fold change in expression of genes. For statistical analysis, we used mean ΔΔCt ±SEM between NS and USS groups (Livak and Schmittgen, 2001).

Western blotting

Western blots were used to determine the relative expression of 5-HT_{1A} receptor and Deaf-1 protein in isolated $BNST_{ALG}$ samples using procedures mentioned previously (Dabrowska and Rainnie, 2010). In brief, 25 μg of protein per sample was loaded onto polyacrylamide-SDS mini-gels (Bio-Rad, Hercules, CA, USA), separated electrophoretically, blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and blocked for 1 h in blocking buffer containing 2% nonfat dry milk, 0.1% Tween 20, 0.05 M NaCl, and 1 M HEPES (pH 7.4). To examine the relative expression of 5-HT_{1A} and Deaf-1 the membranes were incubated with the following primary antibodies: rabbit polyclonal anti-5-HT_{1A} (1:1000, AB 15350, Chemicon-Millipore, Billerica, MA), and rabbit monoclonal against Deaf-1 (1:1000, ab75792, Abcam, Cambridge, MA). On the following day, the immunoblots were incubated with an HRP-labeled specific secondary antibody (peroxidase conjugated anti-rabbit IgG

antibody, Vector Labs, 1:2000) for 1 h at room temperature. The level of 5-HT_{1A} and Deaf-1 proteins in the $BNST_{ALG}$ homogenates was determined using SuperSignal West Chemiluminescence (Pierce Biotechnology) and visualized with an Alpha Innotech Fluorochem imaging system (Alpha Innotech, San Leandro, CA).

Data analysis

Statistically significant differences were determined by Student's t test. The results are presented as mean ± SEM.

Results

Previously, we reported that mRNA transcripts for almost every 5-HT receptor subtype were expressed in whole tissue homogenates of the $BNST_{ALG}$ (Guo et al, 2009). Here, we extended our initial PCR observations to look at the expression profile for all of the 5-HT receptor subtypes in physiologically defined Type I – III $BNST_{ALG}$ neurons.

Transcriptome analysis of serotonin receptor subtypes in Type I – III $BNST_{ALG}$ neurons

For this study, we recorded the membrane properties and extracted the cytosolic mRNA from 75 visually identified neurons in the $BNST_{ALG}$. Of these 75 neurons, 19/75 were Type I neurons, 34/75 were Type II neurons, and 22/75 were Type III neurons (see Methods for characterization criteria). It should be noted that the sample size of Type I and III neurons were inflated in order to allow comparisons between cell types, and do not represent their normal distribution in the general population (Hammack et al., 2007).

The relative transcriptome distribution for each of the 5-HT receptor subtypes in the three $BNST_{ALG}$ cell types is illustrated in Table 1. Here, Type I neurons (n=19) were seen to express mRNA transcripts predominantly for 5-HT_{1A} (63%) and 5-HT_7 (53%) receptors, at the expense of almost every other receptor subtype apart from 5-HT_{2C} (5%) and 5-HT_3 (11%) receptors. Phenotypically, there appear to be two sub populations of Type I neurons: one that expresses both 5-$HT1_A$ and 5-HT_7 receptor transcripts (n = 10), and another that only expresses 5-HT_{1A} receptor transcripts (n = 9).

Type II neurons (n = 34) expressed transcripts for almost all of the 5-HT receptor subtypes, with the exception of 5-HT_{2C} receptors. There are, however, six 5-HT receptor subtypes that are predominantly expressed by Type II neurons; namely 5-HT_{1A} (32%), 5-HT_{1B} (21%), 5-HT_{2A} (32%), 5-HT_3 (44%), 5-HT_4 (21%) and 5-HT_7 (44%). In a previous paper we have argued that Type II neurons could be subdivided into 3 distinct subpopulations based on their ion channel mRNA expression pattern (Hazra et al, 2011). A similar tripartite division of Type II neurons could be seen in the current study, based on their 5-HT receptor subtype transcript expression pattern. One population (Type IIA) in which transcripts for 5-HT_3 and 5-HT_7 (n=15) receptors predominate, a second population (Type IIB) that mainly express 5-HT_{1B} and 5-HT_4 receptor transcripts (n=7), and a third population (Type IIC) in which transcripts for 5-HT_{1A} and 5-HT_{2A} receptors predominate (n=11). By comparison, expression of transcripts for 5-HT_{1D} (12%), 5-HT_{1F} (9%), 5-HT_5 (9%), and 5-HT_6 receptors (9%) appear to be uniformly distributed throughout the three sub-populations.

Finally, Type III neurons (n = 22) also expressed transcripts for multiple 5-HT receptor subtypes, with the exception of 5-HT_{1D}, 5-HT_4, 5-HT_6 and 5-HT_7 receptors. The most frequently expressed transcripts were those for the 5-HT_{1A} (41%), 5-HT_{1B} (41%), 5-HT_{2A} (32%), 5-HT_{2C} (59%), and 5-HT_5 receptor subtypes (18%). Like Type I neurons, there appear to be two sub-populations of Type III neurons; a subpopulation that predominantly expressed transcripts for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2A} receptors (n=9), and another that predominantly expressed transcripts for 5-HT_{2C} receptor (n=13).

Together these data suggest that Type I–III neurons may represent heterologous cell populations that could be differentially affected by prolonged stress manipulations. Stress has been reported to alter the expression of several 5-HT receptor subtypes including $5HT_{1A}$ and 5-HT_7 receptors (Chaouloff et al., 1999, Chaouloff, 2000, Xu et al., 2011). Hence, we next examined the effects of repeated USS on anxiety-like behavior and looked to see if there were any correlated changes in the expression pattern of transcripts for the different 5-HT receptor subtypes in Type I – III neurons.

Effects of repeated USS on baseline startle and freezing behavior

Rats were first tested for their baseline startle response prior to the repeated USS and again six days after the last shock presentation (Walker and Davis, 1997, 2002). No significant difference was observed between the NS and USS groups prior to training. However, as shown in Figure 1, rats receiving repeated USS ($n = 16$, $P<0.05$) showed a significantly enhanced startle response compared to NS rats ($n = 16$). Moreover, when NS and USS rats were re-exposed to the shock chamber, as expected, the USS group showed significantly ($P<0.001$) more contextual freezing on day 10 than the NS group. Together these data strongly suggest that repeated USS induces a long-lasting increase in anxiety-like behavior and contextual fear.

Effects of repeated USS on the mRNA expression of 5-HT receptor subtypes in whole tissue $BNST_{ALG}$ and in Type I–III neurons

We next examined the effects of repeated USS on mRNA expression for all of the 5-HT receptor subtypes and the transcriptional regulators (Deaf-1, Freud-1 and Hes-5) in whole tissue homogenates of the $BNST_{ALG}$ using quantitative RT-PCR. As illustrated in Figure 2, exposure to repeated USS failed to cause any significant change in mRNA expression for the 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_5, and 5-HT_6 receptor subtypes compared with the mRNA expression pattern in NS control rats. However, a 2.8 fold decrease in 5-HT_{1A} receptor mRNA expression was observed, together with a concomitant 2 and 3.5 fold increase of 5-HT_{1B} and 5-HT_7 receptor mRNA expression, respectively.

Given that we had previously shown selective expression for these receptor transcripts in distinct subpopulations of $BNST_{ALG}$ neurons, these data further suggested that repeated USS may preferentially disrupt mRNA expression in discrete subpopulations of $BNST_{ALG}$ neurons. However, whole tissue mRNA expression is derived not only from $BNST_{ALG}$ neurons but also from Schwann cells, glia, and vascular tissue in the surrounding neuropil. Consequently, we next examined the effects of repeated USS on 5-HT receptor subtype mRNA expression in Type I–III neurons using scRT-PCR. The results of these studies are summarized in Table 2. Here, 5-HT receptor mRNA transcripts in Type I – III neurons showed a similar subtype specific change in expression pattern to the changes seen in mRNA expression in tissue homogenates. Hence, no significant change was observed in the number of neurons showing mRNA transcript expression for the 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_3, 5-HT_4, 5-HT_5, and 5-HT_6 receptor subtypes in Type I–III neurons. However, repeated USS caused a marked reduction in the number of neurons showing 5-HT_{1A} receptor mRNA transcript expression, down from 63% to 10% of Type I neurons, from 32% to 16% of Type II neurons, and from 41% to 28% of Type III neurons. In contrast, repeated USS caused a significant increase in the number of Type III neurons showing 5-HT_{1B} receptor mRNA expression (78%), without affecting the number of Type II neurons expressing the same receptor transcripts. Finally, the number of Type II neurons showing 5-HT_7 mRNA transcript expression increased from 44% to 60% following USS, and the number of Type I neurons rose from 53% to 70%. Together these data suggest that repeated USS caused a widespread reduction in the expression of transcripts for the 5-HT_{1A} receptor,

and a cell type-specific modulation of expression for the 5-$HT_{1B\ and\ 7}$ receptor subtypes in $BNST_{ALG}$ neurons.

Effects of repeated USS on the mRNA expression for 5-HT_{1A} transcriptional repressor elements in $BNST_{ALG}$ tissue and Type I–III neurons

We next determined whether concomitant changes could be observed in the expression of transcripts for the transcriptional repressors of 5-HT_{1A} receptor gene expression. Following repeated USS, Deaf-1 mRNA expression was seen to be significantly, 3 fold, up-regulated in whole tissue homogenates ($P<0.05$). In contrast, repeated USS caused no significant change in Freud-1 or Hes-5 mRNA transcript expression (Figure 2). Because expression of Freud-1 and Hes-5 mRNA did not show any changes in whole tissue homogenates, we restricted our scRT-PCR screening of mRNA transcripts in single neurons to Deaf-1. Low level Deaf-1 mRNA expression was observed in all three cells types in NS control animals (Type I 21%, Type II 29%, and Type III 32%). Following repeated USS, Deaf-1 mRNA expression increased in all three cell types to 40%, 40%, and 50% respectively. The global decrease of 5-HT_{1A} receptor mRNA expression and the concomitant increase in Deaf-1 mRNA expression in Type I – III neurons suggested a potential cause-effect relationship, which could induce a functional change in the response to local 5-HT release following repeated USS. However, these changes may be negated by post-translational modification of protein expression. Hence, we next determined if repeated USS could also induce changes in protein expression for these two key elements of 5-HT signaling in the $BNST_{ALG}$.

Effects of repeated USS on 5-HT_{1A} receptor and Deaf-1 protein expression in the whole tissue homogenates of the $BNST_{ALG}$

Here, the expression levels of 5-$HT_{1A,}$ and Deaf-1 peptides were determined using Western blot analysis of whole tissue $BNST_{ALG}$ extracts from NS and repeated USS rats. To normalize the data and control for variability in sample loading, protein expression was determined relative to the expression of a house-keeping peptide, GAPDH. As illustrated in Figure 3, single bands were observed for all three proteins at the predicted molecular weights. In agreement with results from our RT-PCR study, quantitative analysis revealed a significant decrease ($P<0.001$) in $5HT_{1A}$ receptor protein levels in $BNST_{ALG}$ homogenates from repeated USS rats compared to the NS control group. Conversely, protein levels for Deaf-1 were significantly increased ($P<0.001$) in $BNST_{ALG}$ homogenates from the repeated USS group compared to the NS group.

Discussion

The results of our study have confirmed and extended our original observations of neuronal diversity in the $BNST_{ALG}$ and revealed that subgroups of Type I – III neurons differentially express specific combinations of 5-HT receptor subtypes. Hence, two subpopulations of Type I neurons were identified, one of which expressed transcripts for $5HT_{1A}$ receptors only, and one which expressed transcripts for both 5-HT_{1A} and 5-HT_7 receptors. Three Type II subpopulations were identified; for comparative purposes we have named these subpopulations Type IIA – IIC. Type IIA neurons predominantly expressed transcripts for 5-HT_3 and 5-HT_7 receptors. Type IIB neurons predominantly expressed transcripts for 5-HT_{1B} and 5-HT_4 receptors, and Type IIC neurons predominantly expressed transcripts for 5-HT_{1A} and 5-HT_{2A} receptors. Lastly two subpopulations of Type III neurons were identified, one of which expressed transcripts for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2A} receptors, and one which only expressed transcripts for 5-HT_{2C} receptors. Intriguingly, Deaf-1 transcript expression in all $BNST_{ALG}$ neurons from NS control animals was low (~20–30%), and restricted primarily to those subpopulations that did not normally express transcripts for the 5-HT_{1A} receptor, with the exception of the Type I subpopulation.

Consistent with our previous observation (Hammack et al., 2009), repeated USS caused a long-lasting increase in anxiety-like behavior as measured, 6 days after the last shock delivery, by an elevated baseline startle response and enhanced contextual freezing. Using quantitative RT-PCR we then showed that the increase in anxiety-like behavior and contextual fear was associated with 1) a decrease in transcript expression of the inhibitory 5-HT_{1A} receptor, 2) an increase in transcript expression of excitatory, 5-HT_{2C}, and 5-HT_7 receptors, and 3) an increase in 5-HT_{1B} receptor expression in $BNST_{ALG}$ homogenates. Repeated USS also caused a significant increase in the mRNA and protein expression for the 5-HT_{1A} receptor repressor element, Deaf-1, in the $BNST_{ALG}$. Moreover, our scRT-PCR study further revealed that whereas the reduction in 5-HT_{1A} receptor transcript expression and increase in Deaf-1 expression was observed across the board in Type I – III neurons, stress-induced alterations in 5-HT_{1B}, 5-HT_{2C}, and 5-HT_7 receptor transcript expression were cell type-specific. Hence, 5-HT_{1B} receptor transcript expression only increased in one of the two Type III neuron subpopulations, and 5-HT_{2C} expression only decreased in the other. Intriguingly, 5-HT_7 transcripts appeared to increase in the same subpopulation of Type I neurons in which 5-HT_{1A} receptor expression decreased, and also increased in Type IIA neurons, which do not normally express 5-HT_{1A} receptor transcripts.

Together these data strongly suggest that repeated stress exposure would markedly affect the response of Type I – III neurons to local release of 5-HT in the $BNST_{ALG}$. Globally, inhibitory control by postsynaptic $5HT_{1A}$ receptor activation would be reduced in Type I – III neurons, whereas excitatory drive would increase in subpopulations of Type I and IIA neurons through 5-HT_7 receptor activation, and in Type III neurons through 5-HT_{2C} receptor activation. Given that electrical stimulation of the $BNST_{ALG}$ increases anxiety-like behavior (Shaikh et al., 1986, Casada and Dafny, 1991, Dunn and Williams, 1995) a net increase in 5-HT-induced excitation in the $BNST_{ALG}$ may contribute to the long-lasting anxiety-like behavior induced by repeated USS.

Functional correlates of 5-HT receptor subtype expression in the $BNST_{ALG}$

Our study is the first to systematically investigate baseline mRNA expression for all of the known 5-HT receptor subtypes (5-HT_{1A}-5-HT_7) in whole $BNST_{ALG}$ tissue homogenates as well as in individual $BNST_{ALG}$ neurons (Type I–III). As previously reported, 5-HT can both inhibit and excite neurons of the $BNST_{ALG}$ (Levita et al., 2004; Guo et al., 2009). Inhibition was the most prevalent response via activation of 5-HT_{1A} receptors, whereas excitation was mediated by activation of 5-HT_{2A}, 5-HT_{2C}, and/or 5-HT_7 receptors. However, most neurons within the $BNST_{ALG}$ responded to 5-HT with a mixed response whereby an initial inhibition was followed by excitation. In these neurons, the 5-HT_{1A} receptor-mediated inhibitory response was followed by excitation mediated by either 5-HT_{2A} or 5-HT_7 receptor activation. The results of our present study are consistent with these initial observations in that our single cell RT-PCR data revealed that ~ 50% of all $BNST_{ALG}$ neurons (32/65) expressed mRNA transcripts for the 5-HT_{1A} receptor. We have shown that selective *in vivo* activation of 5-HT_{1A} receptors in the $BNST_{ALG}$ elicits an anxiolytic-like behavioral response in rats (Levita et al., 2004). Activation of 5-HT_{1A} receptors in the BNST has been reported to mediate the facilitation of the baroreflex response by induced cannabinoids in response to blood pressure increases (Alves et al., 2010, Gomes et al., 2011). Together these data suggest that activation of a distinct population of $BNST_{ALG}$ neurons comprised of the majority of Type I neurons, Type IIC neurons, and a subpopulation of Type III neurons may play a critical role in the acute response to adverse environmental stimuli. Consistent with this premise, Type I neurons co-expressed mRNA transcripts for the 5-HT_7 receptor, whereas Type IIC neurons and Type III neurons co-expressed transcripts for the 5-HT_{2A} receptor. Recent studies have suggested that activation of 5-HT_{2A} and 5-HT_7 receptors may facilitate anxiety-like behavior (Delgado et al., 2005, Hedlund, 2009), and that ligands with

mixed 5-HT_{1A} receptor agonist and 5-HT_{2A} receptor antagonist properties may make more effective anxiolytics (Delgado et al., 2005).

Significantly, Type IIA, Type IIB, and the remainder of the Type III neurons never expressed mRNA transcripts for the $5HT_{1A}$ receptor. Notably, the Type IIA neurons expressed transcripts for the 5-HT_3 and 5-HT_7 receptor subtypes, whereas the subpopulation of Type III neurons lacking 5-HT_{1A} receptor transcripts expressed transcripts for the 5-HT_3 and 5-HT_{2C} receptor subtypes suggesting that this population of neurons could respond to local 5-HT release with a rapid excitation mediated by 5-HT_3 receptor activation (Farber et al., 2004) as well as a slower excitation mediated by 5-$HT_{2C/7}$ receptor activation (Guo et al., 2009). Like activation of 5-HT_7 receptors, activation of 5-HT_{2C} and 5-HT_3 receptors has been reported to have anxiogenic-like actions (Delgado et al., 2005, Harada et al., 2006, Dekeyne et al., 2008) suggesting that these neurons may play a role in the rapid anxiogenic response to acute stressors. However, a caveat to this hypothesis is that the $BNST_{ALG}$ is primarily a GABAergic system and it is possible that a subset of these neurons act as local circuit inhibitory interneurons, and function to inhibit the activity of $BNST_{ALG}$ output neurons.

Intriguingly, the subpopulation of Type III neurons that expressed mRNA transcripts for 5-HT_{1A} and 5-HT_{2A} receptors also expressed 5-HT_{1B} receptor transcripts. 5-HT_{1B} receptors not only act as autoreceptors to modulate serotonergic transmission, but also act as heteroreceptors to modulate release of other neurotransmitters (Morikawa et al., 2000). Several studies have reported high levels of 5-HT_{1B} receptor binding sites in the BNST (Bonaventure et al., 1997, Cloez-Tayarani et al., 1997, Cloez-Tayarani et al., 1998), and we have shown that activation of presynaptic 5-HT_{1B} receptors reduced glutamate transmission in the $BNST_{ALG}$ (Guo et al., 2010). However, it is most likely that any protein resulting from transcription of the 5-HT_{1B} receptor mRNA would be shipped to the axon terminals of these neurons to regulate release of their endogenous neurotransmitters. It is interesting to note, therefore, that 5-HT_{1B} receptor knockout mice show an exaggerated autonomic response to stress (Bouwknecht et al., 2000, Groenink et al., 2003) just like 5-HT_{1A} receptor knockout mice (Sibille and Hen, 2001). Hence, activation of 5-HT_{1A} and 5-HT_{1B} receptors in these neurons may act synergistically to limit transmitter release to downstream targets.

5-HT receptor subtype mRNA expression is altered after USS

Previously, we have shown that acute CRF receptor activation or a mild stress (one week isolation housing) could facilitate the 5-HT_{1A}-mediated inhibitory response in $BNST_{ALG}$ neurons (Hammack et al, 2009), whereas repeated restraint stress facilitated anxiety-like behavior and altered whole tissue expression of selected 5HT receptor subtypes. Moreover, Chattarji and colleagues have shown that chronic stress can result in dendritic remodeling in BNST neurons (Vyas et al., 2003). Hence, stress intensity and/or duration appear to be critical factors in regulating affective behavior by promoting neuronal plasticity and/or remodeling within the BNST. The results of the current study extend these observations and show that repeated USS caused a prolonged increase in anxiety-like behavior that was associated with a 2.8 fold reduction in 5-HT_{1A} mRNA expression in $BNST_{ALG}$ tissue homogenates and a concomitant decrease in 5-HT_{1A} mRNA expression in Type I – III $BNST_{ALG}$ neurons. These data are consistent with previous studies showing that chronic stress or corticosterone administration resulted in reduced $5HT_{1A}$ receptor expression in other brain regions (Ferretti et al., 1995, McKittrick et al., 1995, Crayton et al., 1996, Fernandes et al., 1997, Takao et al., 1997, Lopez et al., 1998, Maines et al., 1999). Moreover, 5-HT_{1A} knockout mice show increased anxiety-like behavior compared to their wild-type litter mates (Parks et al., 1998), and show increased freezing and tachycardia in response to footshock (Gross et al., 2000, Alves et al., 2010). As noted above, activation of 5-HT_{1A} receptors in the BNST is thought to facilitate the baroreflex response to increased

blood pressure (Alves et al., 2010). Hence, reduced 5-HT_{1A} receptor expression in response to repeated USS may directly contribute to the enhanced anxiety-like behavior observed in these animals. A similar down-regulation of 5-HT_{1A} receptor expression has been observed in the prefrontal cortex in response to chronic social defeat (Kieran et al., 2010). Significantly, dysfunction of the 5-HT_{1A} receptor has been associated with anxiety and major depressive disorder in humans (Savitz et al., 2009), and stress is known to be a major precipitating factor in the etiology of both of these disorders (Heim and Nemeroff, 2002).

It is important to note that unpredictable shock has also been shown to cause a prolonged and elevated release of 5-HT in the limbic forebrain (for review see (Maier and Watkins, 2005), and that this response was dependent on CRF release within the dorsal raphe nucleus (DRN). We have shown that CRF neurons in the $BNST_{ALG}$ of transgenic mice that selectively express green fluorescent protein (GFP) in this cell population (Martin et al., 2010) have identical physiological properties to those of Type III neurons of the rat $BNST_{ALG}$ (Rainnie DG, 2010). Here, we show that USS caused a significant reduction in the expression of 5-HT_{1A} receptor transcripts in Type III neurons of the rat, suggesting that USS may result in a dis-inhibition of $BNST_{ALG}$ CRF neurons. The BNST has been shown to send afferent projections to the DRN (Peyron et al., 1998) and hence dis-inhibition of BNST CRF neurons may contribute to the hyper-activation of the DRN induced by unpredictable stress. Significantly, USS also reduced 5-HT_{1A} mRNA transcript expression in Type I and Type IIC neurons and the resultant dis-inhibition of these neurons may also contribute to the heightened anxiety-like behavior observed following USS. Studies are in progress to determine the neuropeptide phenotype of these subpopulations of $BNST_{ALG}$ neuron. Consistent with the results presented here, a recent human study has shown that tryptophan depletion increases anxiety, but not fear, and that this response may result from reduced serotonergic inhibition of CRF neurons in the BNST (Robinson et al., 2012).

Dis-inhibition is not the only mechanism by which USS may increase the output of BNST neurons. As noted above, 5-HT release is increased in the forebrain following USS, and we have shown that the expression of transcripts for several 5-HT receptor subtypes that could potentially mediate excitation of BNST neurons is unaffected by USS and in some cases expression is up-regulated. Thus, activation of excitatory 5-HT receptors together with prolonged 5-HT release may also contribute to the heightened anxiety-like behavior. For example, in the subpopulation of Type III neurons that normally express 5-HT_{1A} and 5-HT_{2C} receptor transcripts, USS reduced the number of neurons expressing 5-HT_{1A} transcripts but had no effect on the number of neurons expressing 5-HT_{2C} receptor transcripts. Our Western blot data suggest that alterations in mRNA expression are mirrored in receptor protein expression; hence, the inhibitory-excitatory balance in these Type III neurons would shift heavily in favor of excitation. Similarly, USS also caused a significant reduction in the number of Type I neurons expressing transcripts for the 5-HT_{1A} receptor (63% NS–vs-10% USS) and a concomitant increase in the number of these neurons expressing transcripts for the 5-HT_7 receptor (53% NS–vs-70% USS). We have shown that activation of 5-HT_7 receptors causes a depolarizing shift in the membrane potential of $BNST_{ALG}$ neurons (Guo et al., 2009); hence, USS would also cause a shift in the inhibitory-excitatory balance in these neurons to favor 5-HT-induced excitation. Significantly, 5-HT_7 knockout mice show anti-depressant-like activity in the Porsolt forced swimming test (Guscott et al., 2005), and in the tail suspension test (Hedlund et al., 2005), as well as impaired contextual fear conditioning (Roberts et al., 2004), suggesting a potential role for 5-HT_7 receptors in both depression or anxiety-like behavior. Consistent with this premise, chronic treatment with antidepressants has been shown to down-regulate 5-HT_7 receptor binding (Sleight et al., 1995, Mullins et al., 1999).

Another receptor that is up-regulated following USS is the 5-HT_{1B} receptor. In non-stressed animals transcripts for 5-HT_{1B} receptor was detected in 21 % of Type IIB neurons, and in 41% of Type III neurons. Following USS the number of Type IIB neurons expressing mRNA for the receptor remains constant, but the number of Type III neurons expressing transcripts for the 5-HT_{1B} receptor increased from 41% to 78%. Consistent with this observation, Ferguson and co-workers reported that chronic mild stress increased the expression of 5-HT_{1B} receptors on the terminals of nucleus accumbens neurons that project to the ventral tegmental area (Ferguson et al., 2009).

In this study, we did not see any significant changes in the expression of 5-$_{HT2A}$, 5-HT_{2C}, and 5-HT_3 receptor transcripts following USS. However, resistance to modulation by stress does not necessarily imply that these receptors do not contribute to the enhanced anxiety-like behavior following USS. As noted above, removal of inhibitory control by 5-HT_{1A} receptor down-regulation may unmask an anxiogenic profile for these receptors. Indeed, Weisstaub and co-workers reported that 5-HT_{2A} receptor knockout mice show a decrease in anxiety-like behavior in several behavioral tasks (Weisstaub et al., 2006). Similarly, gene knockout of 5-HT_{2C} receptors leads to a decrease in anxiety-like behavior (Heisler et al., 2007), an effect that is mimicked by administration of 5-HT_{2C} receptor antagonists (Dekyne et al., 2008; Harada et al., 2006). Conversely, selective over expression of 5-HT_{2C} receptors in the forebrain resulted in increased anxiety and hypoactivity (Kimura et al., 2009), suggesting that activation of these receptors contributes to the expression of anxiety-like behavior even under conditions of basal 5-HT release. It remains to be determined if activation of 5-HT_{2C} receptors in the $BNST_{ALG}$ contribute to the altered behavioral state. Finally, systemic administration of 5-HT_3 receptor antagonists, such as ondansetron and tropisetron, have also been shown to have anxiolytic–like effects in rodent behavioral assays (Griebel, 1995, Millan et al., 2003, Costall and Naylor, 2004). Hence, by reducing 5-HT_{1A} receptor expression USS may favor the expression of anxiety-like behavior as a result of local activation of any combination or permutation of these excitatory 5-HT receptors.

A potential mechanism for stress-induced down-regulation of 5-HT_{1A} receptor expression

Transcriptional regulation of 5-HT_{1A} receptor expression is an important determinant of the basal response of $BNST_{ALG}$ neurons to local 5-HT release. Here, expression of the 5-HT_{1A} gene is regulated by a TATA-driven promoter and also by upstream repressors that inhibit gene expression (Parks and Shenk, 1996, Ou et al., 2000) including Deaf-1, Freud-1, and Hes-5 (Albert and Lemonde, 2004). Here, we show that under basal conditions mRNA transcripts for all three repressors were expressed in $BNST_{ALG}$ tissue homogenates suggesting that 5-HT_{1A} receptor expression may be tightly regulated in $BNST_{ALG}$ neurons. However, USS failed to alter the expression levels of Freud-1 and Hes5, but caused a significant up-regulation of Deaf-1 mRNA transcripts. These data were consistent with the significant reduction in 5-HT_{1A} receptor expression following USS, and suggest that Deaf-1 may be the principal regulator of 5-HT_{1A} receptor expression in $BNST_{ALG}$ neurons. Differential regulation of 5-HT_{1A} gene repressor expression has also been observed in the PFC (Iyo et al., 2009). Here chronic restraint stress caused a significant reduction in Freud-1 mRNA and protein expression but had no effect on Deaf-1 expression. These data suggest that transcriptional regulation of Deaf-1 and Freud-1 expression, and by extension 5-HT_{1A} receptor expression, may be brain region and context-specific. Intriguingly, chronic social defeat was observed to reduce 5-HT_{1A} mRNA expression but did not reduce either Deaf-1 or Freud-1 expression levels (Kieran et al., 2010). It should be noted that Deaf-1, Freud-1, and Hes-5 are not the only transcriptional regulators of 5-HT_{1A} gene expression. The 5-HT_{1A} receptor promoter also contains a glucocorticoid response element (GRE) that can bind heterodimers of type 1 (mineralocorticoid, MR) and type 2 (glucocorticoid, GR) receptors and repress 5-HT_{1A} receptor expression (Ou et al., 2001). The $BNST_{ALG}$ contains

moderate to high levels of both MR and GR (Ahima and Harlan, 1990, Pietranera et al., 2001) and it is possible that stress-induced glucocorticoid release may also contribute to the down-regulation of 5-HT_{1A} receptor expression following USS.

At the single cell level, Deaf-1 expression was detected in distinct subpopulations of $BNST_{ALG}$ neurons. Significantly, Deaf-1 expression was observed only in those subpopulations of Type I and III neurons that did not show basal mRNA expression for the 5-HT_{1A} receptor. Similarly, Deaf-1 was highly expressed in Type IIA neurons, which did not express 5-HT_{1A} receptor transcripts. These data raise the intriguingly possibility that this population of neurons may be capable of expressing 5-HT_{1A} receptor transcripts, but that even under basal conditions gene expression is strongly repressed by Deaf-1. Conversely, Deaf-1 transcripts were never observed in Type I – III $BNST_{ALG}$ neurons that expressed 5-HT_{1A} receptor mRNA transcripts, suggesting that these cells either do not co-express Deaf-1, or that under basal conditions Deaf-1 was itself repressed. Consistent with the latter premise, following USS the number of Deaf-1 expressing neurons increased in Type I – III neurons. Significantly, Deaf-1 expression was now observed in some Type I and Type III neurons that also expressed transcripts for the 5-HT_{1A} receptor. These data are consistent with studies in the prefrontal cortex which report co-expression of Deaf-1 and 5-HT_{1A} receptor protein in cortical neurons (Szewczyk et al., 2009). Our results suggest that enhanced expression of Deaf-1 may bind to its repressor sequence on the promoter region of the 5-HT_{1A} receptor gene and inhibit transcription. Hence, induction of Deaf-1 in select subpopulations of $BNST_{ALG}$ neurons may contribute to the prolonged elevation of anxiety-like behavior following repeated USS. Selective targeting of factors that regulate Deaf-1 induction and/or translation may offer novel avenues of approach for the development of new pharmacotherapeutics for anxiety disorders and depression.

Questions still to be answered

Notwithstanding decades of research into the putative role of the serotonergic system in the behavioral response to stress stimuli, no clear picture has emerged thus far. Understanding the role of selective expression of the different 5-HT receptor subtypes in subpopulations of $BNST_{ALG}$ neurons seems a more promising approach to unraveling the role these receptors in the etiology of anxiety. However, a key issue that has yet to be addressed is how activation of the different cell types modulates the output activity of the $BNST_{ALG}$ as a whole.

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT_{1A}	serotonin 1A receptor
5-HT_{1B}	serotonin 1B receptor
5-HT_{1D}	serotonin 1D receptor
5-HT_{1F}	serotonin 1F receptor
5-HT_{2A}	serotonin 2A receptor
5-HT_{2C}	serotonin 2C receptor

5-HT$_3$	serotonin 3 receptor
5-HT$_4$	serotonin 4 receptor
5-HT$_5$	serotonin 5 receptor
5-HT$_6$	serotonin 6 receptor
5-HT$_7$	serotonin 7 receptor
ASR	acoustic startle response
BNST	the bed nucleus of the stria terminalis
BNST$_{ALG}$	anterolateral cell group of the bed nucleus of the stria terminalis
CRF	corticotrophin releasing factor
Deaf-1	deformed epidermal autoregulatory factor 1
DRN	dorsal raphe nucleus
Freud-1	five prime repressor element under dual binding protein 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GR	glucocorticoid receptors
GRE	glucocorticoid response element
Hes5	hairy and enhancer of split 5
MR	mineralocorticoid receptors
NS	non stress
PCR	polymerase chain reaction
PFC	prefrontal cortex
scRT-PCR	single cell reverse transcriptase polymerase chain reaction
USS	unpredictable shock stress
VTA	ventral tegmental area

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Highlights

Cell type specific distribution of 5-HT receptors in $BNST_{ALG}$ neurons.

5-HT receptors and transcriptional regulators are modulated by stress.

Stress decreased 5-HT_{1A} mRNA and protein in the $BNST_{ALG.}$

Stress up-regulates the transcriptional repressor of the 5-HT_{1A} gene, Deaf-1.

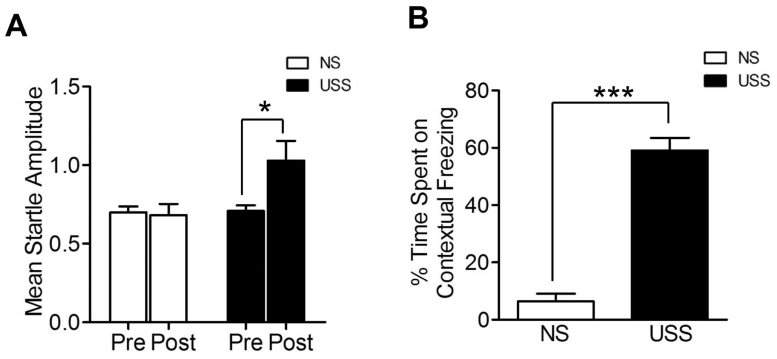

Figure 1. Effect of unpredictable shock stress (USS) in startle and contextual freezing behavior

Four daily sessions of USS causes a significant long-lasting enhancement of baseline startle and contextual freezing in Sprague-Dawley rats. Six days after the last presentation of the USS **(A)** Mean baseline startle amplitude were enhanced significantly ($P<0.05$) and **(B)** percentage of time spent on contextual freezing behavior was increased significantly ($P<0.001$) as well.

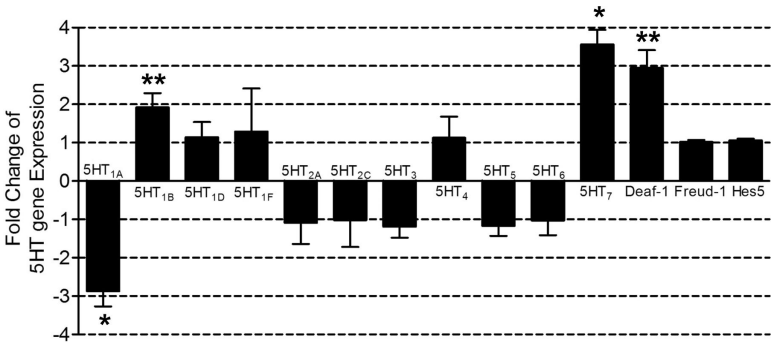

Figure 2. Effect of unpredictable shock stress (USS) on 5-HT receptors and regulators gene expression in the $BNST_{ALG}$ tissue

Quantitative RT-PCR was performed in whole $BNST_{ALG}$ tissue to determine the mRNA expression of 5-HT receptors (5-HT_{1A-7}) and transcriptional regulators (Deaf-1, Freud-1, Hes-5) in USS rats. Each bar represents the relative fold change of a specific gene as determined by $2^{-\Delta\Delta Ct}$ method of quantification in non-stress (NS) and USS rats (n=4). The data shows 5-HT_{1A} receptor mRNA expression was down-regulated by 2.8 fold and 5-HT_{1B}, 5-HT_7 and Deaf-1 mRNA expression was up-regulated by 2, 3.5 and 3 fold respectively. Mean $\Delta\Delta$Ct ±SEM values show significant difference in 5-HT_{1A} ($P<0.05$), 5-HT_{1B} ($P<0.01$), 5-HT_7 ($P<0.05$) and Deaf-1($P<0.01$) mRNA expression in USS rats.

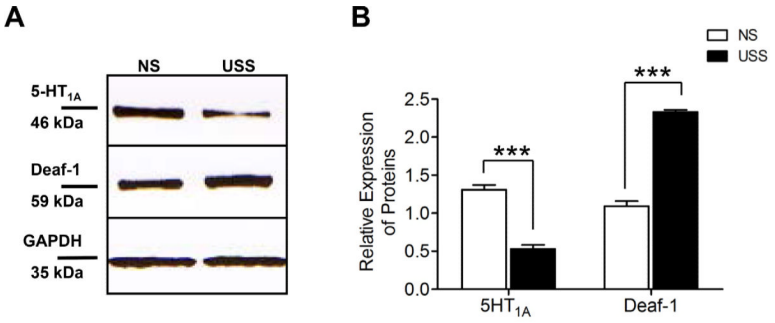

Figure 3. Expression of 5-HT$_{1A}$ and Deaf-1 protein in unpredictable shock stress (USS) rats

(A) Representative western blot analysis of BNST$_{ALG}$ tissue revealed reduced expression of 5-HT$_{1A}$ (46 kDa) protein following USS. Consequently Deaf-1 protein expression, showing distinct band at 59 kDa, was enhanced considerably after USS. Lower bands represent GAPDH loading controls. **(B)** A bar chart showing the quantitative data of relative protein expression in all western blots experiments (n=4) performed. A significant decrease in 5-HT$_{1A}$ ($P<0.001$) and a significant increase in Deaf-1 ($P<0.001$) protein expression was noted in the BNST$_{ALG}$ of USS rats relative to their non-stress counterparts.

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Table 1

Single-cell RT-PCR analysis of the relative distribution of 5-HT receptor subtypes mRNA in $BNST_{ALG}$ neurons.

No of cell expressed	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1F}	5-HT_{2A}	5-HT_{2C}	5-HT_3	5-HT_4	5-HT_5	5-HT_6	5-HT_7	Deaf-1
Type I (19)	63%	0%	0%	0%	0%	5%	11%	0%	0%	0%	53%	21%
10/19	10	0	0	0	0	0	0	0	0	0	10	0
9/19	2	0	0	0	0	1	2	0	0	0	0	4
Type II (34)	32%	21%	12%	9%	32%	0%	44%	21%	9%	9%	44%	29%
A (15/34)	0	0	2	1	0	0	15	0	0	2	15	10
B(7/34)	0	7	1	1	0	0	0	7	0	1	0	0
C(11/34)	11	0	1	1	11	0	0	0	3	0	0	0
Type III (22)	41%	41%	0%	18%	32%	59%	27%	0%	18%	0%	0%	32%
9/22	9	9	0	4	7	0	0	0	4	0	0	0
13/22	0	0	0	0	0	13	6	0	0	0	0	7

The table shows the percentage of 5-HT receptor subtypes expression in different $BNST_{ALG}$ neurons (Type I–III). The number of neurons expressed 5-HT receptors subtypes and Deaf-1 mRNA in Type I–III subpopulation of $BNST_{ALG}$ neurons, has been shown.

Table 2

Unpredictable shock stress changes the mRNA expression profile for 5-HT receptor subtypes in Type l-lll neurons.

No of cells expressed	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2C}$	5-HT$_{3}$	5-HT$_{4}$	5-HT$_{5}$	5-HT$_{6}$	5-HT$_{7}$	Deaf-1
Type I (10)	10%	0%	0%	0%	0%	0%	20%	0%	0%	0%	70%	40%
10/10	1	0	0	0	0	0	2	0	0	0	7	4
Type II (25)	16%	24%	16%	16%	16%	0%	40%	16%	16%	12%	60%	40%
A15/25	0	0	4	0	0	0	10	0	0	3	15	10
B 6/25	0	6	0	4	0	0	0	4	0	0	0	0
C 4/25	4	0	0	0	4	0	0	0	4	0	0	0
Type III (18)	28%	78%	0%	17%	28%	22%	17%	0%	17%	0%	0%	50%
12/18	5	12	0	2	5	0	0	0	3	0	0	6
6/18	0	2	0	1	0	4	3	0	0	0	0	3

The table shows the percentage of 5-HT receptor subtypes expression in USS BNST$_{ALG}$ neurons (Type I–III). The number of neurons expressed 5-HT receptors subtypes and Deaf-1 mRNA in Type I–III subpopulation of BNST$_{ALG}$ neurons, has been shown.