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# Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis

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**Abstract**

Neuropeptide Y (NPY) and corticotropin-releasing factor (CRF) have opposing effects on stress and anxiety. Both can modify synaptic activity through their binding to NPY receptors (YRs) and CRF receptors (CRFRs) respectively. The bed nucleus of the stria terminalis (BNST) is a brain region with enriched expression of both NPY and YRs and CRF and CRFRs. A component of the ''extended amygdala'', the BNST is anatomically well-situated to integrate stress and reward-related processing in the CNS, regulating activation of the hypothalamic-pituitary-adrenal (HPA) axis and reward circuits. Using whole-cell recordings in a BNST slice preparation, we found that NPY and CRF inhibit and enhance GABAergic transmission, respectively. Pharmacological experiments suggest that NPY depresses GABAergic transmission through activation of the Y2 receptor (Y2R), while both pharmacological and genetic experiments suggest that CRF and urocortin enhance GABAergic transmission through activation of the CRF receptor 1 (CRFR1). Further, the data suggest that NPY acts to regulate GABA release, while CRF enhances postsynaptic responses to GABA. These results suggest potential anatomical and cellular substrates for the robust behavioral interactions between NPY and CRF.


*Keywords:* Electrophysiology; Whole cell; CRF; Neuropeptide Y; Anxiety; Extended amygdala

## 1. Introduction

Anxiety disorders are serious medical conditions that have been estimated to impact approximately 20% of the population (Greenberg et al., 1999). While anxiety is a normal reaction to stress, repeated or severe stressors can produce pathological behaviors such as those seen in post-traumatic stress disorder (PTSD) and generalized anxiety disorder (Shekhar et al., 2005). An understanding of the neuronal and molecular targets associated with these conditions will likely promote more effective treatments. While classical neurotransmitters, such as gamma-aminobutyric acid (GABA) (Nemeroff, 2003) and glutamate (Javitt, 2004), have been implicated in these disorders, another class of signaling compounds, neuropeptides, are also thought to be involved in these conditions (Valdez and Koob, 2004). Neuropeptides are potent neuromodulators in the CNS whose actions are mediated via G-protein coupled receptors (Ludwig and Leng, 2006). In contrast to classical neurotransmitters, they are released in a frequency dependent fashion and often have a longer half-life of activity after release. These factors among others enable neuropeptides to produce long-lasting effects on cellular functions such as excitatory (Acuna-Goycolea et al., 2005) and inhibitory (Nie et al., 2004) synaptic transmission, neuronal excitability (Acuna-Goycolea et al., 2005) and gene transcription (Zhao et al., 2002). Thus, a long-lasting dysregulation of neuropeptides

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could have significant effects on the activity of neurons and consequentially, behavior.

A large body of literature implicates neuropeptide Y (NPY) as one of several neuropeptides involved in regulation of both anxiety and stress. Early pharmacological studies demonstrated that central infusion of NPY can reduce anxiety-like behaviors (Heilig et al., 1989). Conversely, mice with targeted deletions of NPY (Bannon et al., 2000) exhibit anxiety-like behaviors. Moreover, a recent study conducted in veterans exposed to combat suggested that NPY may play a role as a ''stress protective'' factor for individuals at risk for PTSD (Yehuda et al., 2006).

In contrast to NPY, the neuropeptide corticotrophin releasing factor (CRF) is thought to be primarily anxiogenic. Central administration of CRF causes an increase in anxiety-like behaviors, as well as activation of the stress response (Campbell et al., 2004). CRF knockout mice have significantly lower levels of corticosterone, both basally and following exposure to acute stress (Dunn and Swiergiel, 1999). Further, elevated CRF levels in the cerebrospinal fluid have been reported in persons diagnosed with PTSD (Bremner et al., 1997).

It has been proposed that dysregulation of NPY and CRF play opposing roles in regulation of anxiety disorders (Shekhar et al., 2005; Valdez and Koob, 2004). To date, however, a common site of opposing action has not been identified in situ. The bed nucleus of the stria terminalis (BNST), a brain region associated with anxiety, has enriched expression of both NPY (Walter et al., 1991) and YRs (Parker and Herzog, 1999), and CRF (Ju and Han, 1989) and CRFRs (Van Pett et al., 2000). The BNST receives a dense GABAergic and CRF input from the central nucleus of the amygdala (CeA) (Sakanaka et al., 1986), suggesting that CRF regulation of function in the BNST is critical for shaping BNST output. In keeping with this, pharmacological studies suggest that CRF signaling in the BNST is involved in anxiety (Lee and Davis, 1997; Sahuque et al., 2006) and stress-induced relapse to cocaine self-administration (Erb and Stewart, 1999). Moreover, a stimulus that promotes anxiogenic responses, the withdrawal of rodents from chronic ethanol exposure, produces rises in extracellular levels of CRF in the BNST (Olive et al., 2002), while acute heroin administration increases NPY immunoreactivity in the region (D'Este et al., 2006).

CRF (Nie et al., 2004) and NPY (Cowley et al., 1999; Pronchuk et al., 2002; Sun et al., 2001) have been shown to modulate GABAergic transmission, albeit in different brain regions. Interactions between these neuropeptides and GABAergic transmission have been proposed to underlie aspects of their regulation of animal behavior (Nie et al., 2004). Thus the BNST, in particular GABAergic transmission within the region, provides a potential anatomic and cellular substrate for interaction of CRF and NPY in regulating stress and anxiety. We have explored this possibility by investigating the actions of CRF and NPY on GABAergic transmission in the ventrolateral region of the BNST (vlBNST). This region projects to both the ventral tegmental area (VTA) (Dumont and Williams, 2004; Georges and Aston-Jones, 2002) and the paraventricular nucleus (PVN) of the hypothalamus (Cullinan et al., 1993), thus providing a point of access to both reward and stress pathways. Functionally the vlBNST is critical both for maintaining inhibitory tone on the PVN (Cullinan et al., 1993) and modulating stress induced relapse to drug seeking behavior (Erb et al., 2001). Given these findings, we reasoned that this would be a relevant region to investigate the actions of NPY and CRF, two neuropeptides known to regulate stress/anxiety responses.

## 2. Methods

### 2.1. Brain slice preparation

All procedures were performed according to Institutional Animal Care and Use Committee approved procedures. Male C57Bl/6J mice (6–8 weeks old, Jackson Laboratories) were decapitated under anesthesia (Isoflurane). The brains were quickly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 $NaH_2PO_4$, 10.0 glucose, and 26.0 $NaHCO_3$ saturated with 95% $O_2$/5% $CO_2$. Slices 300 μm in thickness were prepared using a Tissue Slicer (Leica). Rostral slices containing anterior portions of BNST (Bregma 0.26–0.02 mm) (Paxinos and Watson, 1997) were identified using the internal capsule, anterior commissure, fornix, and stria terminalis as landmarks. Slices were then stored in a heated (approximately 28 °C), oxygenated (95% $O_2$/5% $CO_2$) holding chamber containing 'normal' ACSF [ACSF: (in mM) 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 $NaH_2PO_4$, 10.0 glucose, and 26.0 $NaHCO_3$] or transferred to an submerged recording chamber where they were perfused with heated, oxygenated ACSF at a rate of about 2 ml/min. Slices were allowed to equilibrate in normal ACSF for 1 h before experiments began.

### 2.2. Whole-cell voltage clamp recordings

Slices were placed in a submerged chamber (Warner Instruments) and neurons of the vlBNST directly visualized with infrared video microscopy (Olympus). Recording electrodes (3–6 MΩ) were pulled on a Flaming–Brown Micropipette Puller (Sutter Instruments) using thin-walled borosilicate glass capillaries.

### 2.3. Evoked transmission

For analysis of evoked inhibitory postsynaptic currents (eIPSCs), and excitatory postsynaptic currents (EPSCs) electrodes are filled with (in mM) $K^+$-gluconate (135), NaCl (5), HEPES (10), EGTA (0.6), ATP (4), GTP (0.4), and biocytin (0.1%) pH 7.2, 290–295 mOsmol. Twisted nichrome wire stimulating electrodes were placed in the vlBNST, 100–500 μm medial from the recorded neuron. After entering a whole-cell configuration, cells were held at −50 mV and GABA type A receptor ($GABA_A$R)-mediated IPSCs were evoked at 0.2 Hz by local fiber stimulation with bipolar electrodes (5–40 V with a 100–150 μs duration). $GABA_A$-IPSCs were pharmacologically isolated by adding 3 mM kynurenic acid to block AMPA and NMDA receptor-dependent postsynaptic currents and 1 μM CGP 55845 to block $GABA_B$ receptors. EPSCs were obtained in a similar fashion; however, 25 μM picrotoxin was used in place of kynurenic acid and the cells were held at −70 mV. Signals were acquired via a Multiclamp 700B amplifier (Axon Instruments), digitized and analyzed via pClamp 9.2 software (Axon Instruments). Input resistance and series resistance were continuously monitored during experiments. Experiments in which changes in series resistance were greater than 20% were not included in the data analysis. eIPSC experiments were analyzed by measuring the peak amplitude of the synaptic response which was normalized to the baseline period. The baseline period was defined as the 2-min period immediately preceding application of the drug. For all histograms presented, the value presented is a 2-min average 15 min following the application of the neuropeptide (for example, if the peptide was applied from minutes 5–10, then the reported value here would be the average of minutes 20 and 21).

### 2.4. Spontaneous transmission

For analysis of "miniature" IPSCs (mIPSCs), electrodes are filled with (in mM) KCl (70), $K^+$-gluconate (65), NaCl (5), HEPES (10), EGTA (0.6), ATP (4), GTP (0.4), and biocytin (0.1%) pH 7.2, 290–295 mOsmol. To isolate miniature $GABA_A$R-mediated IPSCs (mIPSCs) tetrodotoxin (0.5 μM) was added to the perfusing solution. mIPSCs were recorded in 120-s episodes. In experiments where the role of calcium influx on the modulatory actions of NPY was examined, 100 μM $Cd^{2+}$ was added to the ACSF. The amplitude and frequency of mIPSCs were determined from 120-s recording episodes with the cells held at −70 mV. Signals were acquired via a Multiclamp 700B amplifier (Axon Instruments), digitized at 10 kHz and analyzed using Clampfit 9.2 software (Axon Instruments).

### 2.5. Statistical analysis

Appropriate statistical analyses, including Student's *t*-test, ANOVA and the Kolmogorov–Smirnov test were performed using Microsoft Excel, Graphpad Prism and Microcal Origin. Specifically, when determining if a compound had a significant effect (for example 300 nM NPY), a Student's paired *t*-test was used, comparing the baseline value to the experimental value (outlined above) When comparing the effects of multiple antagonists on an agonist response, ANOVA followed by Dunnett's post test was used to determine the significance of the differences between the groups. Finally, the Kolmorgorov–Smirnov test was used when determining the effect of a drug on the cumulative distribution of a response. All values given for drug effects throughout the paper are presented as mean ± S.E.M. For results given in figures, significance is noted in the figure legend. For results not included in figures, significance is noted in the text.

### 2.6. Pharmacology

All drugs were bath applied. Kynurenic acid (4-hydroxyquinoline-2-carboxylic acid) was purchased from Sigma (St. Louis, MO). GABAzine 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid hydrobromide or SR95531), urocortin, CRF6-33, Antisauvagine-30, NBI 27914 (5-chloro-*N*-(cyclopropylmethyl)-2-methyl-*N*-propyl-*N*′-(2,4,6-trichlorophenyl)-4,6-pyrimidinediamine hydrochloride), neuropeptide Y, L152,804, BIEE 0246 (*N*-[(1*S*)-4-[(aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide), BVD-10 and CRF were purchased from Tocris (Ellisville, MO). NPY13-36 and D-Trp32 NPY were purchased from Phoenix Pharmaceuticals (Belmont, CA). Pro34 NPY was purchased from Anaspec (San Jose, CA). All peptides used in this study were dissolved in $dH_2O$ to a concentration of 0.1 mM and either used immediately or aliquoted and stored at −20 °C until use. Dimethyl sulfoxide (DMSO) (0.001%) was used as the vehicle for NBI 27914 and BIEE 0246. Kynurenic acid solution was prepared fresh daily by stirring directly in the ACSF.

### 2.7. Knockout animals

CRFR2 knockout breeder animals were obtained from Dr. Robert Kesterson and were generated as previously reported (Coste et al., 2000). Animals for this study were generated by the breeding of homozygous knockout males and females.

## 3. Results

We examined the effects of both NPY and CRF on inhibitory synaptic transmission in the vlBNST utilizing whole-cell voltage clamp of neurons from acutely prepared brain slices of adult mice. Local stimulation in the BNST produced an eIPSC that was driven by activation of $GABA_A$Rs, as the selective $GABA_A$R antagonist, SR95531 (GABAzine), completely blocked the response (Fig. 1A). We examined

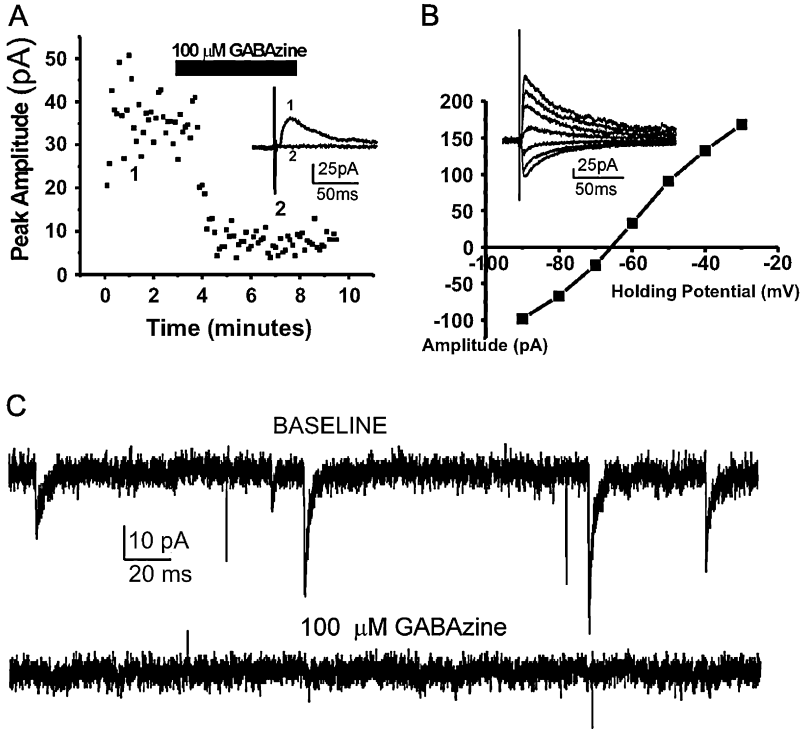


Fig. 1. Characterization of inhibitory transmission in the BNST. (A) Application of the $GABA_A$R selective antagonist, 100 μM GABAzine, blocked evoked IPSCs in the BNST. (B) *I*–*V* plot of evoked IPSC reversed near −70 mV, consistent with a chloride conductance. (C) mIPSCs recorded in a high chloride internal solution were also blocked by 100 μM GABAzine.

the current-voltage relationship for these eIPSCs (Fig. 1B) and found that the reversal potential was approximately −70 mV, consistent with this conductance being mediated primarily by chloride ions. Additionally, using the sodium channel blocker, tetrodotoxin, and a high chloride pipette solution (see Section 2) we observed spontaneous mIPSCs (Fig. 1C; frequency, 0.80 ± 0.13 Hz, $n = 10$ cells from 7 mice; amplitude, 19.0 ± 1.8 pA, $n = 10$ cells from 7 mice). Similar to the eIPSCs, these mIPSCs were completely blocked by GABAzine, suggesting that these events were mediated by $GABA_A$ receptors.

### 3.1. NPY depresses GABAergic transmission through activation of the Y2R

To begin to assess the effects of neuropeptides on GABAergic transmission, we obtained stable baseline recordings of eIPSCs, stimulating and recording every 12 s. After obtaining a stable baseline, we applied NPY for 5 min. A 5-min bath application of 1 μM NPY significantly decreased the peak amplitude of the eIPSC to 65 ± 5% of baseline (Fig. 2A, B). Depression of the eIPSC by this concentration of NPY was observed in 7/7 cells from 5 mice. The NPY-induced depression

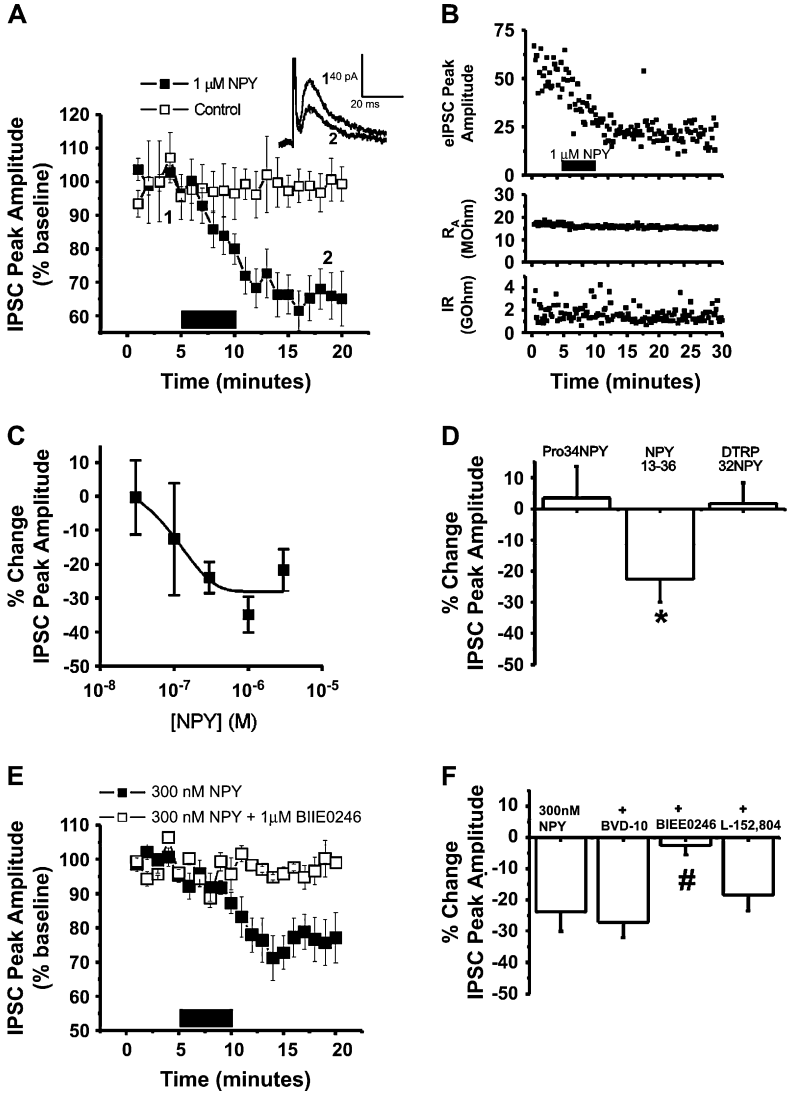


Fig. 2. NPY inhibits $GABA_A$-mediated synaptic transmission in the vlBNST in a concentration dependent fashion via activation of Y2 receptors. (A) A 5-min application of NPY (1 μM) inhibited $GABA_A$-mediated synaptic transmission in the vlBNST ($n = 7$ cells from 5 mice; $^*P < 0.05$, $t$-test). A baseline control showing no change in response is plotted on this graph as well ($n = 3$ cells from 3 mice). (B) Representative experiment showing the effect of 1 μM NPY on peak amplitude of the eIPSC and input resistance and the stability of the access resistance. (C) Concentration–response relationship for NPY inhibition of $GABA_A$-IPSC amplitudes in vlBNST neurons, expressed as percentage of control. The logistic curve, plotted by Origin Software (Microcal Software, Northampton, MA), using $y = A2 + (A1 - A2)/(1 + (x/x_0)^p)$, gives an $EC_{50}$ value of 105 nM NPY for IPSC inhibition. (30 nM, $n = 4$ cells from 3 mice; 100 nM, $n = 4$ cells from 2 mice; 300 nM, $n = 5$ cells from 3 mice; 1 μM, $n = 7$ cells from 5 mice; 3 μM, $n = 4$ cells from 4 mice) (D) The Y2R agonist NPY13-36 (1 μM) significantly decreased the peak amplitude of the IPSC ($P < 0.001$, $n = 6$ cells from 3 mice, $t$-test), whereas the Y1R agonist, [$Pro^{34}$]-NPY (1 μM, $n = 7$ cells from 4 mice), and the Y5R agonist, [D-$Trp^{32}$]-NPY (1 μM, $n = 5$ cells from 4 mice), had no effect. (E) The selective Y2R antagonist (BIEE 0246, 1 μM, $n = 4$ cells from 3 mice) antagonized the IPSC peak amplitude depression of 300 nM NPY, while (F) the Y1R antagonist (BVD-10, 1 μM, $n = 4$ cells from 2 mice) and the Y5R antagonist (L-152804, 1 μM, $n = 5$ cells from 3 mice) had no effect. [ANOVA ($F(3,14) = 4.723$), $P < 0.05$; $^{\#}P < 0.05$ when compared to 300 nM NPY, Dunnett's post test].

of eIPSCs was concentration-dependent, with an $EC_{50}$ of 105 nM (Fig. 2C).

To identify the receptor or receptors underlying the actions of NPY, we assessed the actions of several NPY receptor agonists on eIPSCs. The Y2R agonist NPY13-36 (1 μM) exhibited a strong inhibitory effect on eIPSCs, reducing their peak amplitude by 22 ± 7% (6 cells from 3 mice; Fig. 2D). In contrast, the Y1R agonist [$Pro^{34}$]-NPY (1 μM) and Y5R agonist [D-$Trp^{32}$]-NPY (1 μM) did not change the amplitude of the eIPSC. Additionally, we examined the ability of several different selective YR antagonists to block the actions of NPY. For antagonist experiments, we applied the antagonist for 10–15 min prior to NPY application. Following this pre-application the agonist and antagonist were co-applied for 5 min. None of the antagonists used had any significant effect on the eIPSCs in the absence of agonist (BVD-10, 95 ± 6% of baseline, $n = 5$ cells from 3 mice; BIEE0246, 100 ± 5% of baseline, $n = 4$ cells from 3 mice; L-152,804, 92 ± 6% of baseline, $n = 6$ cells from 4 mice). The non-peptide Y2R antagonist (BIEE 0246, 1 μM) blocked the actions of NPY (Fig. 2E, F), while the peptide Y1R antagonist (BVD-10, 1 μM) and the non-peptide Y5R antagonist (L-152804, 1 μM) had no significant effect (Fig. 2F). Taken together, these results suggest that the NPY-induced depression of eIPSCs was mainly mediated by the activation of the Y2R subtype.

### 3.2. NPY decreases GABAergic transmission through a presynaptic mechanism

To examine the mechanism of NPY inhibition of eIPSCs, we conducted paired-pulse ratio (PPR) experiments. In these experiments a pair of eIPSCs were elicited with a 50-ms interstimulus interval and the ratio of the amplitudes was determined. Alterations in this ratio are suggestive of a presynaptic alteration of function. NPY (1 μM) significantly increased the PPR of eIPSCs, suggesting a decreased release probability of GABA (Fig. 3A, B).

To further explore the mechanism of action of NPY we examined the effect of NPY on mIPSCs. NPY (1 μM) significantly decreased the mean frequency from 0.69 ± 0.21 Hz to 0.40 ± 0.19 Hz (Fig. 3C, D), whereas the mean amplitude was unchanged (from 19.3 ± 3.7 to 19.4 ± 3.5 pA; $n = 5$ cells from 3 mice; Fig. 3C, E).

Presynaptic inhibition of transmitter release can occur through at least two broad mechanisms, modulation of calcium entry or regulation of release machinery. At many CNS synapses, basal mIPSC frequency can be further reduced by the addition of the voltage gated calcium channel blocker, cadmium ($Cd^{2+}$; Nicola and Malenka, 1997). To test whether modulation of $Ca^{2+}$ influx through voltage activated calcium channels contributed to the inhibitory action of NPY on GABA release, we re-examined the actions of NPY on mIPSCs in ACSF containing $Cd^{2+}$ (100 μM). We found that in the presence of $Cd^{2+}$, 1 μM NPY had no effect on either frequency (from 1.05 ± 0.43 Hz to 1.05 ± 0.43 Hz, $n = 5$ cells from 4 animals; Fig. 3F) or amplitude (from 31 ± 12 to 29 ± 9 pA, $n = 5$ cells from 4 animals; Fig. 3G) of the mIPSCs. In total, these data are most consistent with NPY inhibiting GABA release via Y2R-mediated regulation of presynaptic calcium influx.

### 3.3. CRF and urocortin enhance GABAergic transmission through the CRFR1

In contrast to the effects of NPY, a five minute bath application of 1 μM CRF significantly enhanced the peak amplitude of the eIPSC to 116 ± 8% of baseline (Fig. 4A, C). There was some variability in the response, as 2/7 of these cells did not exhibit a response to CRF. Nonetheless, all cells were included in meaned analyses in these and all other experiments. 1 μM urocortin I (Ucn I), also a CRFR agonist, produced a similar enhancement of eIPSC amplitude (Fig. 4B; 116 ± 3% of control). As was seen in CRF, there appeared to be some slight variability in the response, as 2/18 cells did not exhibit a response. The effect of CRF exhibited a concentration dependence, as 100 nM significantly enhanced the peak amplitude of the eIPSC (112 ± 4% of control; $^*P < 0.05$, $n = 6$ cells from 6 mice) but not 10 nM (106 ± 4% of control, $n = 6$ cells from 5 mice). To identify the receptor or receptors underlying the actions of CRF and Ucn I, we tested several selective CRF receptor antagonists. As with the NPY antagonist studies, we applied the antagonist for 10–15 min before a co-application of antagonist and agonist. None of the antagonists used had any significant effects on eIPSCs in the absence of agonist (NBI 27914, 99 ± 5% of baseline, $n = 6$ cells from 4 mice; anti-Sauvagine-30, 96 ± 4% of baseline, $n = 10$ cells from 7 mice). We found that the non-peptide CRFR1 antagonist NBI 27914 (1 μM) blocked the actions of both CRF and Ucn I (Fig. 4C, D). In contrast, the peptide CRFR2 antagonist anti-Sauvagine-30 (300 nM) had no significant effect (Fig. 4C, D). Further, Ucn I was still able to enhance eIPSCs in CRFR2 knockout mice (Fig. 4E). These results provide converging evidence that the CRF/Ucn I induced enhancement of eIPSCs is due to activation of CRFR1.

Given the heterogeneity of neurons in the BNST (Egli and Winder 2003), we sought to determine whether CRF and NPY would target inhibitory transmission in the same population of cells. As shown in a representative experiment (Fig. 4F), Ucn I enhanced the amplitude of the eIPSC, while subsequent application of NPY depressed the eIPSC in the same cell. This experiment was performed in 8 cells. In 7/8 experiments both compounds exhibited a response, in 1/8 cells only NPY elicited a response. The values obtained in these 8 experiments for 1 μM urocortin (115 ± 3% of baseline, $P < 0.05$, $n = 8$ cells from 5 mice) and 1 μM NPY (74 ± 6% of baseline, $P < 0.05$, $n = 8$ cells from 5 mice), were similar to values obtained in single application experiments. A recent study examining the effects of norepinephrine on synaptic transmission in the vlBNST found evidence for a population of projection neurons with distinct physiological characteristics, including small capacitance and an inwardly rectifying potassium conductance (Dumont and Williams, 2004). While we found cells with these characteristics, we did not observe any differences in the abilities of CRF, urocortin or NPY to modulate synaptic inhibition in cells such as these when

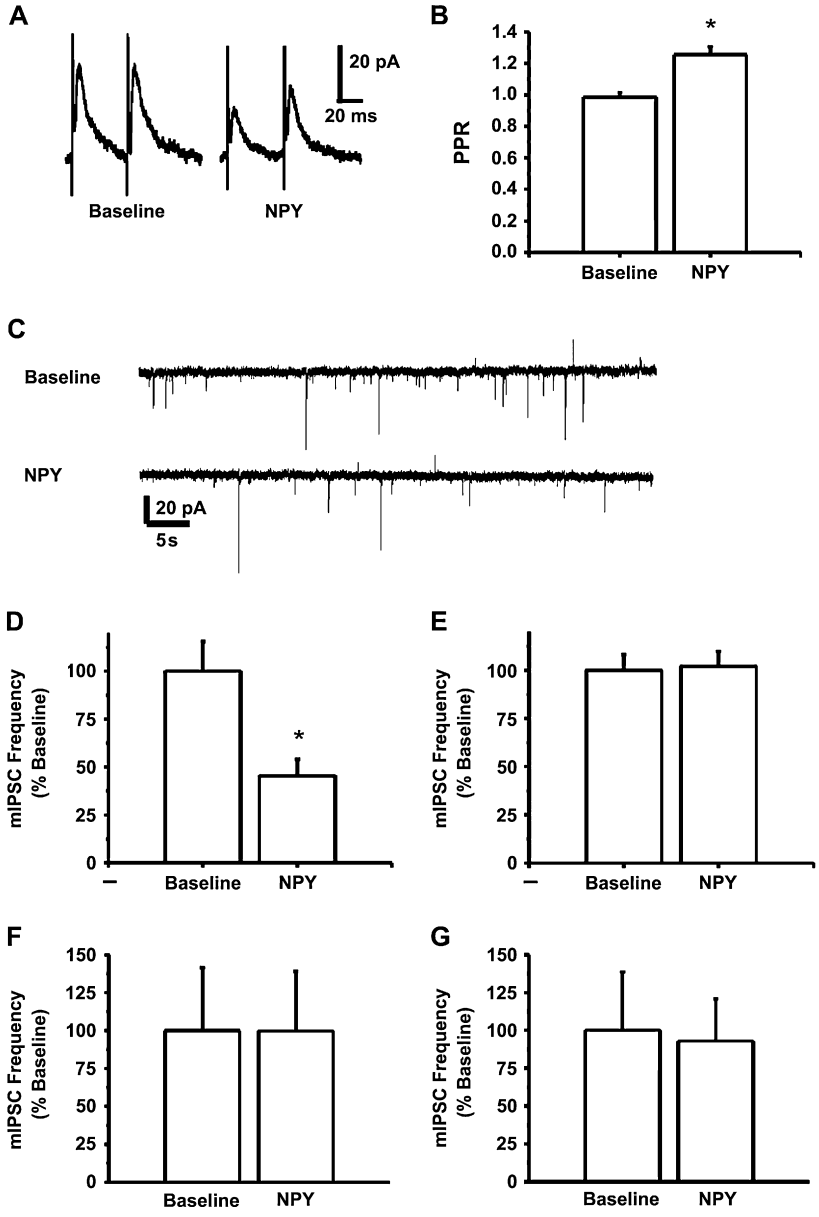


Fig. 3. NPY reduces the frequency of $GABA_A$-mediated miniature inhibitory post-synaptic currents (mIPSCs) in the vlBNST. (A) Representative traces from paired pulse experiments, (B) Bar graph showing the average baseline paired pulse ratio following NPY treatment are increased ($n = 7$ cells from 5 mice; $^*P < 0.05$, *t*-test). (C) Representative baseline (top traces) and mIPSCs 10 min following application of 1 μM NPY (bottom traces). (D) Bar graph showing the average (mean ± S.E.M.) baseline and average frequency following NPY treatment ($n = 5$ cells from 3 mice; $^*P < 0.05$, *t*-test). (E) Bar graph showing the average (mean ± S.E.M.) baseline and average amplitude following NPY treatment. ($n = 5$ cells from 3 mice). (F) Bar graph showing the average (mean ± S.E.M.) baseline and average frequency following 1 μM NPY treatment in the presence of 100 μM $CdCl_2$ ($n = 5$ cells from 4 mice). (G) Bar graph showing the average (mean ± S.E.M.) baseline and average amplitude following 1 μM NPY treatment in the presence of 100 μM $CdCl_2$ ($n = 5$ cells from 4 mice).

compared to cells that did not have those electrophysiological characteristics.

### *3.4. CRF enhances GABAergic transmission through a postsynaptic action*

As with NPY, we examined the effect of CRF on the PPR of eIPSCs. In contrast to NPY, we found that CRF did not alter the PPR (Fig. 5A, B). We next examined the effects of CRF on mIPSCs. In contrast to the effects of NPY, application of CRF (1 μM) did not alter the mean frequency of mIPSCs, $0.92 \pm 0.24$ to $0.88 \pm 0.29$ ($n = 5$ cells from 4 mice) (Fig. 5C, D). However, the mean amplitude was significantly increased from $18.6 \pm 2.0$ to $24.6 \pm 3.3$ pA (Fig. 5E, inset). Further, CRF significantly shifted the normalized cumulative amplitude distribution curve to the right (Fig. 5E), yet did not alter mIPSC kinetics (Fig. 5C, inset; decay time, $20 \pm 5$ ms to $24 \pm 6$ ms, $n = 5$ cells from 4 mice). In total, these data are most consistent with CRF enhancing GABAergic transmission postsynaptically. One possibility suggested by our results is that the enhancement of IPSCs is due to a non-specific enhancement in synaptic transmission via

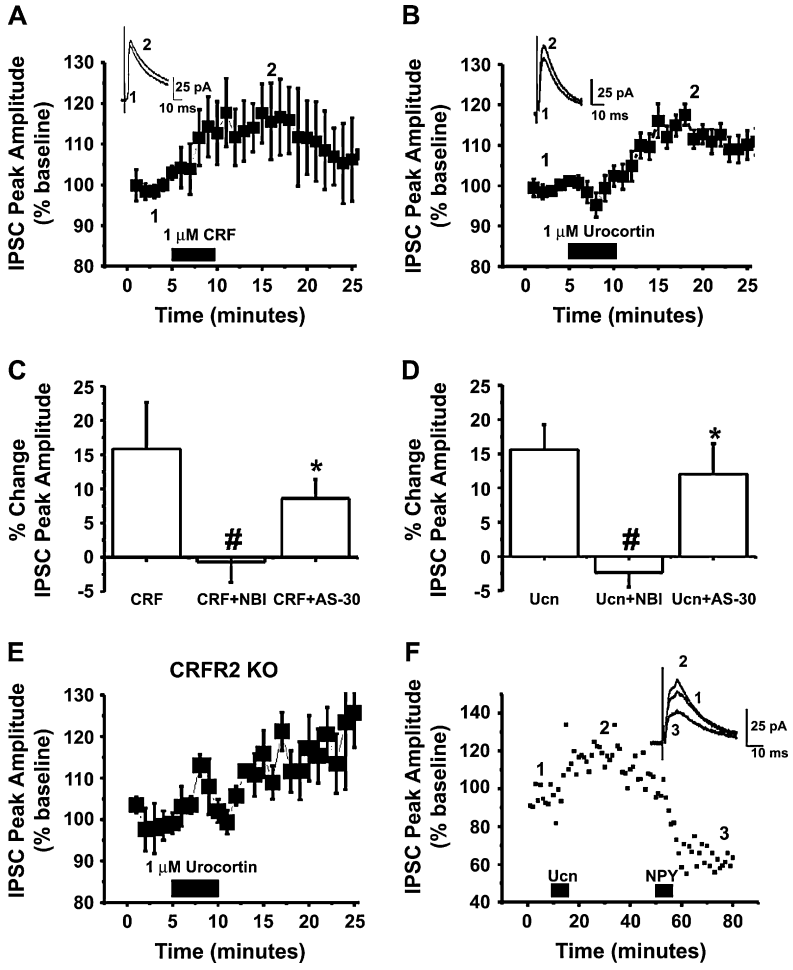


Fig. 4. CRF potentiates $GABA_A$-mediated synaptic transmission in the vlBNST via activation of CRF1 receptors. (A) A 5-min application of either CRF (1 μM) ($n = 7$ cells from 6 mice; $^*P < 0.05$, *t*-test) or (B) urocortin (1 μM) ($n = 18$ cells from 13 mice; $^*P < 0.05$, *t*-test) potentiates $GABA_A$-mediated synaptic transmission in the vlBNST. (C) The CRF2 receptor antagonist, Anti-Sauvagine 30 (AS-30, 300 nM, $n = 6$ cells from 3 mice), was unable to antagonize either CRF or (D) urocortin ($n = 6$ cells from 4 mice) induced potentiation of IPSC peak amplitude. The CRF1 receptor antagonist, NBI27914 (1 μM), was able to antagonize both CRF ($n = 6$ cells from 4 mice) and urocortin ($n = 5$ cells from 2 mice) mediated increases in IPSC peak amplitude [CRF ANOVA ($F(2,14) = 3.960$), $P < 0.05$; urocortin ANOVA ($F(2,26) = 6.464$), $P < 0.05$; $^{\#}P < 0.05$ when compared to 1 μM CRF and urocortin respectively, ANOVA with Dunnett's post test, $^*P < 0.05$, *t*-test when compared to baseline values]. (E) The enhancing effect of urocortin persisted in CRFR2 knockout mice ($n = 4$ cells from 3 mice). (F) Representative experiment showing both urocortin and NPY modulated the ISPC peak amplitude in a single cell.

alterations in excitability. In order to evaluate this, we examined the effects of 1 μM urocortin on AMPA-mediated EPSCs and found no effect (96 ± 7% of baseline, $n = 5$ cells from 4 mice) 15 min following application of 1 μM urocortin.

## 4. Discussion

Abundant evidence suggests an antagonistic relationship of NPY and CRF signaling in the regulation of a variety of neuropsychiatric conditions. Here we provide a potential cellular substrate mediating this interaction by showing that NPY and CRF can bi-directionally modulate synaptic inhibition on a common pool of neurons in the BNST, a region critical for regulation of stress, anxiety and addiction. When presented in context of the connectivity of the BNST, these data thus provide at least one locus at which these peptide systems may act to produce their opposing effects on behavior.

### *4.1. NPY regulation of GABAergic transmission in BNST*

Our results demonstrate that in the vlBNST NPY suppresses GABAergic transmission. Our data are most consistent with this effect being mediated predominantly by the Y2R for the following reasons. First, the effect of NPY is mimicked by another agonist of the Y2R, NPY13-36. It was not mimicked by application of the Y1 agonist [$Pro^{34}$]-NPY (1 μM) or the Y5R agonist [D-$Trp^{32}$]-NPY (1 μM), applied at concentrations shown to produce effects through these receptors in brain slice preparations (Acuna-Goycolea et al., 2005). Second, the effect of NPY was antagonized by the selective Y2R antagonist BIEE 0246, but not by the Y1R antagonist (BVD-10, 1 μM) or the Y5R antagonist (L-152804, 1 μM). These antagonists were applied at concentrations previously shown to block NPY-induced effects mediated by Y1R and Y5R in other brain regions (Acuna-Goycolea et al., 2005; Silva et al., 2003).

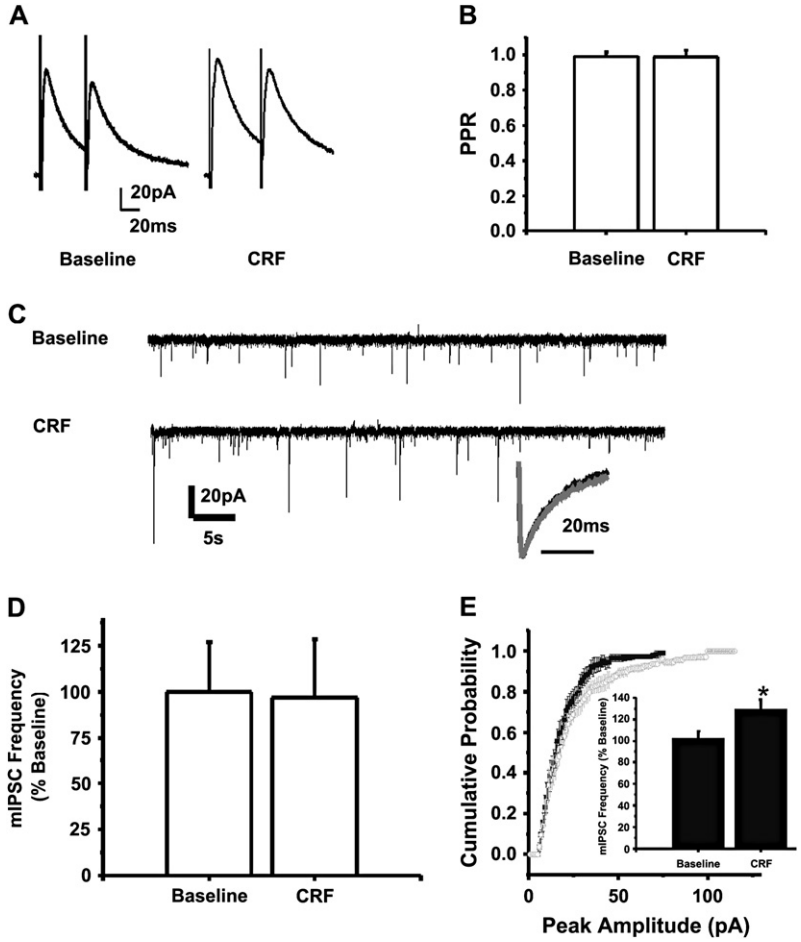


Fig. 5. CRF increases the amplitude of $GABA_A$R-mediated miniature inhibitory post-synaptic currents (mIPSCs) in the vlBNST. (A) Representative traces from paired pulse experiments. (B) Bar graph showing the average baseline paired pulse ratio following CRF treatment ($n = 7$ cells from 6 mice). (C) Representative baseline (top traces) and mIPSCs 15 min following application of 1 μM CRF (bottom traces). Representative normalized average mIPSC traces showing no alteration in kinetics of response (inset). (D) Bar graph showing the average (mean ± S.E.M.) baseline and average frequency following CRF treatment ($n = 5$ cells from 4 mice). (E) Pooled cumulative frequency plot ($n = 5$ cells from 4 mice) for CRF effects on mIPSC amplitude; bin width 1 pA. CRF significantly enhanced mIPSC amplitude ($n = 5$ cells from 4 mice; $P < 0.01$, Kolmorgorov–Smirnov test). Bar graph showing the average (mean ± S.E.M.) baseline and average amplitude following CRF treatment (inset) ($n = 5$ cells from 4 mice; $^*P < 0.05$, *t*-test).

Our data further suggest that the action of the Y2R is to decrease GABA release. First, we show that NPY increases the PPR of eIPSCs, suggesting a decrease in release probability. Consistent with this idea, we further find that NPY decreases the frequency, but not amplitude, of mIPSCs. Further, we examined the effect of NPY in the presence of cadmium, a calcium channel blocker, and found no effect. This suggests that NPY is acting via the Y2R to inhibit GABA release via regulation of presynaptic calcium influx, as has been noted at other synapses (Nicola and Malenka, 1997; Qian et al., 1997). These data are consistent with the reported actions of NPY in both the thalamus (Sun et al., 2001) and PVN (Pronchuk et al., 2002), and with the expression of Y2R in the BNST (Parker and Herzog, 1999). Interestingly, it has been proposed that Y2Rs can function as autoreceptors at NPY terminals, acting to dampen NPY release in response to intense presynaptic activity (Pronchuk et al., 2002). Our data, coupled with those in the thalamus and hypothalamus, suggest that in addition, NPY acts via the Y2 receptor as a heteroceptor on GABAergic terminals.

### 4.2. Potential behavioral significance of NPY action

Based on animal-wide genetic and pharmacological manipulations, NPY signaling in the CNS is thought to play predominantly anxiolytic roles (Heilig et al., 1989). More recent studies, utilizing specific YR agonist and antagonists, along with studies of knockout mice lacking specific YR subtypes, indicate that the specific YR activated is critical for the behavioral outcome. For example, activation of the Y1R (Heilig, 1995), and possibly the Y5R (Sorensen et al., 2004), mediate the anxiolytic responses evoked by NPY, while activation of the Y2R produces anxiogenic effects (Redrobe et al., 2003). It is important to note that these studies reflect the global effect of activating or antagonizing these receptor populations; as local effects of receptors can deviate from this pattern. For example, the anxiolytic effects of NPY in the locus coeruleus appear to be mediated via activation of the Y2R (Kask et al., 1998), in apparent contrast to the reduced anxiety phenotypes observed in the Y2R knockout mouse (Redrobe et al., 2003). These data as well as our own indicate that

region-specific activation of YR subtypes may well evoke distinct behavioral phenotypes. Another possibility is that while the autoreceptor-like functions of the Y2R produce anxiogenic effects, the heteroreceptor-like functions may be predominantly anxiolytic.

It is interesting to consider what effects reduction of GABAergic transmission onto presumed vlBNST projection neurons would have on circuit activity. Globally, reductions in GABAergic transmission are typically associated with anxiogenic responses, while enhancement of GABAergic transmission produces anxiolytic responses. Such interpretations can be complicated, however, by inhibitory control of GABAergic neurons, for example. Indeed, rats having undergone naloxone-precipitated morphine withdrawal, which produces a state of heightened anxiety, display enhanced GABAergic transmission in this same population of neurons (Dumont and Williams, 2004). Evidence indicates that inhibitory projection neurons from the vlBNST contact on neurons within the PVN (Cullinan et al., 1993). Based on this, we would predict that reduced GABAergic input to vlBNST neurons would result in increased GABAergic output to the PVN, resulting in a reduced stress response, a putative anxiolytic effect. However, these interpretations are complicated by the possibility of an excitatory projection from the BNST to the PVN (Spencer et al., 2005).

### *4.3. CRF and urocortin regulation of GABAergic transmission in BNST*

Our results demonstrate that in the vlBNST CRF and Ucn I enhance GABAergic transmission. Our data are most consistent with this effect being mediated predominantly by the CRFR1 for the following reasons. First, the effects of both CRF and Ucn I were antagonized by the CRFR1 selective antagonist, NBI 27914, but not the CRFR2 selective antagonist, AS-30. Second, the effect of Ucn I persisted in mice lacking CRFR2.

Our data further suggest that the action of the CRFR1 is to enhance the responsiveness of $GABA_A$-R postsynaptically. Consistent with this idea, we find that CRF increases the amplitude but not the frequency of mIPSCs, and has no effect on the PPR. Moreover, this appears to be specific to GABAergic transmission, as Ucn I does not significantly effect glutamatergic transmission in the vlBNST. These results are similar to the enhancing effects of CRF on inhibitory transmission in the CeA with one exception (Nie et al., 2004). The actions of CRF on GABAergic transmission in the CeA are also thought to be mediated through CRFR1, however through a presynaptic rather than postsynaptic mechanism. While modulation of GABA release, as observed through alterations in mIPSC frequency, is a common means by which neuromodulators regulate GABAergic transmission, it is important to note that, similar to our data, several studies have provided evidence for neuromodulatory regulation of IPSCs through a postsynaptic mechanism, as observed in part by an enhancement in mIPSC amplitude (Lilly et al., 2003; Nusser et al., 1999).

### *4.4. Potential behavioral significance of CRF and urocortin action*

Both lesion and infusion studies have shown that the BNST is the principle anatomical substrate for CRF enhancement of acoustic startle, a behavioral paradigm thought to closely mimic aspects of anxiety and stress (Walker et al., 2003). Additionally, intact CRF signaling is required for stress-induced relapse to cocaine-seeking behavior (Erb et al., 2001). During ethanol withdrawal, a profoundly anxiogenic state, there is an increase in extracellular CRF levels in the BNST (Olive et al., 2002). Taken together these findings suggest that CRF signaling in the BNST is anxiogenic. A recent study determined, using a pharmacological approach, that the anxiogenic-like effects of CRF in the BNST are mediated via CRFR1 (Sahuque et al., 2006), in agreement with our current results. This possibility agrees with the wealth of pharmacological and genetic evidence that indicates that CRFR1 mediates many of the anxiogenic and ‘‘fight or flight’’ responses evoked by CRF release (Heinrichs et al., 1997). Based on the connectivity we have proposed above, the CRF induced enhancement of GABAergic transmission in the BNST could in turn result in disinhibition of the PVN, leading to activation of the PVN and the subsequent stress response.

### *4.5. Summary*

The results presented in this paper demonstrate that NPY and CRF modulate GABAergic transmission in the vlBNST, providing a potential site of interaction. Given the nature of this interaction, it is conceivable that subtle changes in functional expression of the effects demonstrated in our study could play a role in the pathology of anxiety disorders.

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