

Stress-Hyperresponsive WKY Rats Demonstrate Depressed Dorsal Raphe Neuronal Excitability and Dysregulated CRF-Mediated Responses

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Major depression is a debilitating psychiatric disease that may be precipitated by a dysregulation of stress neurocircuitry caused by chronic or severe stress exposure. Moreover, hyperresponsivity to stressors correlates with depressed mood and may contribute to the etiology of major depression. The serotonergic dorsal raphe nucleus (DRN) is an important site in the neurocircuitry underlying behavioral responses to stressors, and is tightly regulated, in part, by a combination of intrinsic cell properties, autoinhibition, and GABAergic synaptic transmission. The stress-related neurotransmitter corticotropin-releasing factor (CRF) modulates DRN neuronal excitability and subsequent 5-HT release in the forebrain. Wistar Kyoto (WKY) rats exhibit exaggerated behavioral responses to stressors, that is, stress hyperresponsivity, and are considered an animal model of depression. To better understand the neurobiological basis of the stress hyperresponsivity, we used a combination of mRNA analysis and whole-cell electrophysiological techniques to measure differences in intrinsic activity and receptor response, in 5-HT- and non-5-HT-containing neurons of the DRN in WKY rats compared with Sprague-Dawley controls. In the WKY rat, there was a decrease in the neuronal excitability of 5-HT neurons coupled with decreased TPH2 production. Additionally, we found that CRF did not increase GABAergic activity in 5-HT neurons as is normally seen in 5-HT neurons of Sprague-Dawley controls. The CRF modulation of 5-HT DRN neurotransmission at the single-cell level is selectively disrupted in the WKY animal model of depression and may be one of the cellular correlates underlying depression.

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INTRODUCTION

Major depressive disorder (MDD) is a debilitating psychiatric disease that is often co-morbid with anxiety and addiction. A leading theory of MDD is the monoamine depletion hypothesis that posits a causal link between lower monoamine tone and depressed mood (Ruhe *et al*, 2007). However, numerous preclinical and clinical studies have shown that either acute severe or chronic stress can precipitate the onset or relapse to psychiatric disorders, including depression, anxiety, and addiction (Drake *et al*, 2003; Schmidt *et al*, 2008; Walker *et al*, 2008). Therefore, a more accurate characterization of MDD may be a basal

decrease in monoamine tone combined with dysregulated stress neurocircuitry.

The serotonergic dorsal and median raphe nuclei (DRN, MRN) are important sites in the neurocircuitry underlying behavioral responses to stressors (Adell *et al*, 1997; Dilts and Boadle-Biber, 1995; Leonard 2005; Maier and Watkins, 2005), and have been implicated in several stress-related pathologies including depression and anxiety (Deakin, 1991; Graeff *et al*, 1996; Kinney *et al*, 1997; Leonard, 2005; Maier and Watkins, 2005; Robinson *et al*, 1989; Stockmeier, 1997; Tao and Auerbach, 2002). Humans and rodents exhibiting depression-like symptoms demonstrate evidence of 5-HT dysfunction (Deakin, 1991; Stockmeier, 1997) that may be initiated by changes in the excitability of 5-HT neurons. The excitability of raphe neurons is mediated and modulated by intrinsic properties, norepinephrine input, fast inhibitory (GABA) and excitatory (glutamate) synaptic inputs, 5-HT1A receptor (5-HT1A)-mediated autoinhibition, and neuropeptidergic regulation (eg, orexin, opioid, and corticotropin-releasing factor (CRF)).

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Wistar Kyoto (WKY) rats exhibit exaggerated behavioral responses to stressors or stress hyperresponsivity; in tests of depression and anxiety, these animals show enhanced immobility in forced swim, reduced exploration in open field tests, hyponephagia, and rapidly acquired learned helplessness when compared with Sprague-Dawley (SD) control rats (Lahmame *et al*, 1997; Pare, 1989b, 1994b; Pare and Redei, 1993a, b; Pare and Tejani-Butt, 1996; Will *et al*, 2003). In comparison with Wistar rats, WKY rats have prolonged elevation of corticosterone levels following acute stress (De La Garza and Mahoney, 2004). Dopamine, norepinephrine, and serotonin release, uptake, and modulation are altered in WKY rats. Specifically, when compared with SD rats, WKY rats show upregulation of both dopamine and norepinephrine transporter mRNA and protein expression, leading to a decrease in extrasynaptic catecholamine tone, a reduction in 5-HT levels in several brain regions (Scholl *et al*, 2010), and decreases in the efficacy of 5-HT antidepressants to alleviate symptoms of depression (Lopez-Rubalcava and Lucki, 2000; Tejani-Butt *et al*, 2003; Zafar *et al*, 1997) and anxiety (Pollier *et al*, 2000). Thus, WKY rats represent a useful animal model of stress-related depression and anxiety; yet, changes in cellular characteristics, 5-HT_{1A} receptor-mediated responses, excitatory and inhibitory synaptic inputs, and peptidergic effects in the DRN have not been investigated in these animals.

Based on evidence that serotonin levels are reduced in the forebrain of depressed individuals (Coppen and Doogan, 1988; Deakin, 1991; Grahame-Smith, 1989), we hypothesized that WKY rats would demonstrate changes in both intrinsic properties and modulation of synaptic currents, consistent with a depression in raphe 5-HT neuron excitability. These alterations may be because of up- or down-regulation of various G-protein-coupled receptor or ion channel expression levels in the cell or alterations in the function of these proteins at the cell surface. Therefore, we used a combination of mRNA analysis and *in vitro* electrophysiology to more accurately assess the mechanism(s) underlying various phenomenological cellular differences between SD and WKY rats.

A major hypothesis of our laboratory is that dysregulation of stress neurocircuitry is caused by desensitization of the receptor because of a chronic or prolonged elevation of the corresponding endogenous stress-related neurochemical. This phenomenon alters the response of the receptor system to acute stressors. Therefore, we predicted that CRF-mediated changes in 5-HT neuronal function would be abolished in 5-HT neurons of WKY rats because of an overactive stress system. This study extends the work done by our lab using electrophysiological techniques in a DRN and MRN *in vitro* slice preparation from SD rats to characterize intrinsic properties of 5-HT and non-5-HT neurons, 5-HT_{1A} receptor-mediated responses (Beck *et al*, 2004), and CRF receptor-mediated responses (Kirby *et al*, 2008), by elucidating how these responses may be altered in the WKY animal model of depression.

MATERIALS AND METHODS

Subjects

Male SD and WKY rats (75–150 g) from Taconic Farms (Taconic, Germantown, NY) were housed two to three per cage on a 12-h light schedule (lights on at 0700 h) in a temperature-controlled (20 °C) colony room. Rats were given

access to standard rat chow and water *ad libitum*. All animals were used in accordance with the US Public Health Services Policy on Humane Care and Use of Laboratory Animals. All protocols were reviewed and approved by the IACUC committee. The majority of behavioral studies specifically examining the depression-like behaviors in the WKY rat have used SD rats as the control(s) (see Introduction). Therefore, SD rats were used as the control group for both the mRNA analysis and electrophysiology. As is standard for *in vitro* electrophysiology studies, slices from adolescent rats were used because of the high technical difficulty in maintaining healthy recordings from adult rats.

Laser Capture Microdissection (LCM) and mRNA Analysis

The rats were killed via decapitation, their brains removed, and frozen on dry ice. The brains were sectioned at 10 μ m intervals with a Leica CM3000 microtome/cryostat (Leica, Allendale, NJ) at -20°C , and thaw-mounted onto microscope slides (Fisher Scientific, Waltham, MA). Subsequently, sections were stored in a -80°C freezer until they were processed for immunostaining. The sections were fixed with dithiobis(succinimidyl propionate) (DSP, 1 mg/ml) for 5 min, rinsed for 30 s in DEPC water, and incubated for 15 min in 1:250 dilution of antibody directed against TPH (Sigma, St Louis, MO). After a quick DEPC water rinse, they were placed in the DaKoCytomation antibody/enzyme complex for 20 min, rinsed in DEPC water, and added to DaKoCytomation coloring solution for 5 min (the above solutions contained 200 U/ml RNasin). The slides were quickly dried at room temperature before the microdissections were performed. TPH-positive cells (~ 1000 cells) were dissected from 10 raphe sections from four animals per strain, using PALM MicroLaser system. The total RNA was isolated from the dissected cells using TRIzol reagent. The cDNA was synthesized using Superscript II Reverse Transcriptase with oligo dT-T7 primer. The double-stranded cDNA carrying a T7 RNA polymerase promoter was transcribed using the Ampliscribe transcription kit (Epicentre, Madison, WI). The aRNA was then again synthesized into a single-stranded cDNA template for a second round of amplification. The second round of aRNA was cleaned up using the RNeasy kit (Qiagen, Valencia, CA). Then, the P³²-dCTP-labeled cDNA probe was synthesized using Superscript II. The probe was incubated at 95°C for 3 min, purified and transferred to a prehybridized filter array, and incubated for 18 h in a hybridization chamber at 42°C . After the hybridization, filter arrays were washed four times with agitation in the following solutions: $2 \times \text{SSC}/1\%$ SDS for 20 min, $0.5 \times \text{SSC}/1\%$ SDS for 20 min, $0.2 \times \text{SSC}/1\%$ SDS, and $0.1 \times \text{SSC}/0.5\%$ SDS for 20 min. Over 1000 5-HT-containing neurons throughout the rostral-caudal extent of the DRN were necessary to produce enough mRNA for analysis. The DRN is a heterogeneous brain region of which approximately one-third of the neurons in the DRN are non-5-HT containing. These non-5-HT neurons have various neurochemical identities and subfield locations. Therefore, it was not possible to use only one neurochemical marker for that population. Hence, only differences in 5-HT-containing neurons of SD and WKY rats were measured.

Slice Preparation

Procedures were similar to those previously reported (Beck *et al*, 2004; Lemos *et al*, 2006). Animals were rapidly decapitated, head placed in ice-cold sucrose buffer, and the brain dissected and blocked. The blocked tissue was mounted on the stage of a Leica microslicer and surrounded in oxygenated sucrose buffer. Sections that were 200 μm thick were cut through the raphe nuclei in sucrose and then placed in oxygenated 95% O_2 /5% CO_2 ACSF with 50 μM L-tryptophan (Sigma) and incubated in a 35–37 $^\circ\text{C}$ bath for 1 h. Addition of L-tryptophan is necessary to ensure 5-HT production *ex vivo* and helps prevent against false negatives during the *post hoc* immunohistochemical classification. The slices were then removed from the bath and kept in oxygenated ACSF at room temperature. ACSF was composed of (in mM): NaCl 124, KCl 2.5, NaH_2PO_4 2, CaCl_2 2.5, Dextrose 10, and NaHCO_3 26. The sucrose buffer was ACSF in which NaCl was substituted with 248 mM sucrose.

Electrophysiology

Slices were placed in a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with oxygenated ACSF at ~ 1.5 ml/min, and maintained at 30–32 $^\circ\text{C}$ by an inline solution heater (TC-324; Warner Instruments). Healthy recordings cannot be maintained for long periods of time if the ACSF is heated > 35 $^\circ\text{C}$. For recording GABAergic synaptic activity, electrodes were filled with an intracellular solution (in mM): 70 Kgluconate, 70 KCl, 2 NaCl, 10 Hepes, 4 EGTA, 4 Mg ATP, 0.3 Na_2GTP , and 0.1% biocytin at 7.3 pH to yield a resistance of 4–8 M Ω . To record cell characteristics, the electrolyte contained (in mM): 140 Kgluconate, 2 NaCl, 10 Hepes, 4 EGTA, 4 Mg ATP, 0.3 Na_2GTP , and 0.1% biocytin at a 7.3 pH to yield a resistance of 6–10 M Ω .

Whole-cell recordings, specifically to obtain cellular characteristics in current clamp and inhibitory postsynaptic currents (IPSCs) in voltage clamp, were performed on cells in the ventromedial aspect of the DRN. The cell was voltage clamped at -70 mV using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA) or HEKA EPC-10 amplifier (HEKA Elektronik, Pfalz, Germany). Signals were digitized by a Digidata 1320 A/D converter (Molecular Devices) and stored using pClamp 9 software (Molecular Devices). For the HEKA amplifier, signals were stored online using Pulse software (HEKA). Junction potentials between the electrode and the ACSF were ~ 10 –15 mV, as calculated using a program in Clampex, and were not subtracted out.

For voltage clamp experiments, once a whole-cell recording was established, 20 μM DNQX and 1 μM TTX were added to the ACSF to eliminate glutamatergic synaptic activity and action potential-dependent GABA release. Previously, we determined that there were no differences between spontaneous IPSCs and miniature IPSCs (mIPSCs), and hence TTX was added simultaneously with DNQX (Lemos *et al*, 2006). Traces of mIPSCs were recorded and stored online. A total of 10 nM ovine CRF (oCRF; selective CRF receptor 1 (CRF R1) agonist) or Urocortin II (Ucn II, selective CRF R2 agonist) were added to the perfusion buffer and recording continued for 9–12 min. This concentration was chosen based on the reported

affinities for specific receptors (Lovenberg *et al*, 1995). At the end of some of the protocols, bicuculline (20 μM) was added to the bath to confirm that mIPSCs were mediated by GABA_A receptors.

Immunohistochemistry

Standard immunohistochemistry procedures were used to assess the neurochemical identity of the recorded cell, as described in Beck *et al* (2004). Slices were incubated for at least 1 h in paraformaldehyde (4%), and then placed in PBS, 0.5% Triton, and BSA for 30 min. Slices were then incubated for 12–24 h in mouse anti-TPH primary antibody (1:500) (Sigma). Slices were subsequently incubated in donkey anti-mouse Alexa 488 secondary antibody (1:200; Molecular Probes, Eugene, OR). Biocytin was visualized using streptavidin-conjugated Alexa 633 (1:200; Molecular Probes). Slices were imaged using a confocal microscope. Recorded cells were identified via the biocytin labeling. If a cell co-labeled with the TPH immunoreactivity, the cell was identified as 5-HT containing. Cells were confirmed as non-5-HT containing only if TPH immunoreactivity was seen in the same plane as the biocytin-filled cell. For GABA_A $\beta 3$ immunohistochemistry, 20 μm cryosections were made through the rostrocaudal axis of the raphe from perfusion-fixed SD and WKY rat brains. Tissue was thaw mounted onto Superfrost slides and immunohistochemistry was carried out on the tissue, co-staining for TPH (1:500) and GABA_A $\beta 3$ protein (1:500) (courtesy of Stephen J Moss, PhD, Tufts University). Tissue from SD and WKY rats were stained in parallel and images captured using the same exposure time and gain controls.

Data Analysis and Statistics

Current clamp experiments were analyzed using Clampfit 9.2 software. mIPSCs were analyzed using MiniAnalysis 6.0.3 (Synaptosoft, Dacatur, GA). Initially, noise analysis was conducted for each cell and amplitude detection thresholds set to exceed noise values. In most cases the threshold was set at ~ 5 pA. Events were automatically selected, analyzed for double peaks, and then visually inspected and confirmed. Event amplitude histograms were generated and compared with the noise histogram to ensure that they did not overlap. Mean and median frequency (Hz), amplitude (pA), rise time (10–90%, ms), and holding current (pA) were measured for each cell and plotted in a summary table by the MiniAnalysis program. An averaged trace from ~ 200 events was fitted with a double exponential function, and subsequently fast and slow components of the decay phase were computed.

Following a stable baseline, drug was added and events recorded for 15–20 min. The last minute of each 3-min interval was analyzed. For experiments in which a single pharmacological agent was added, the baseline measurements were compared with the maximum steady-state response (in either direction) elicited by the drug using paired *t*-tests. For experiments in which there were multiple drugs added to a neuron, repeated-measures one-way ANOVAs with the *post hoc* Student–Newman–Keuls *t*-tests were performed. One-way ANOVAs with *post hoc t*-tests were performed for between-group comparisons using SigmaStat 3.0 software (SPSS, Chicago, IL) or Excel

(Microsoft, Bellevue, WA). Repeated-measures two-way ANOVAs were used to compare the effect of strain and drug on mIPSCs. Statistical tests are reported for raw data measurements. A result was considered significant with a probability of $p < 0.05$. Results are reported as means \pm SEM. Unless otherwise indicated, Ns are reported as number of cells. For each set of electrophysiology experiments, data were collected from a minimum of three animals, but more typically was collected from more than five animals.

Specifically for CRF and Ucn II experiments on GABA mIPSC activity conducted in 5-HT and non-5-HT cells of the DRN, data collected from WKY rats are presented next to previously published data from SD rats (see Kirby *et al*, 2008). The data from the previously published work were collected in parallel to the data collected from WKY rats on adjacent rigs on the same day or interleaved on different days throughout the same year. The data from both SD and WKY rats for the mRNA analysis, cellular characteristics, 5-carboxyamidotryptamine (5-CT), zolpidem, loreclezole, and ethanol experiments as well as the CRF experiments conducted in the MRN are all new to this manuscript.

Drugs

All chemicals for making the ACSF, electrolyte solution, L-tryptophan, antalarmin, 5-CT, and ethanol were obtained from Sigma-Aldrich or Fisher Scientific. DNQX, zolpidem, and loreclezole were purchased from Tocris (St Louis, MO) and TTX was obtained from Calbiochem (San Diego, CA). Antalarmin and DNQX were dissolved in DMSO (final DMSO concentration in bath 0.015%). oCRF, Ucn II, and antisauvagine 30 were generously supplied by Dr Jean Rivier of the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute (La Jolla, CA).

RESULTS

Figure 1 contains high-power photomicrographs of a 5-HT- and a non-5-HT-containing cell in the DRN. The left panel contains biocytin-labeled cells, the middle panel is the TPH-stained cells, and the right panel contains the merged images.

LCM and RNA Analysis

LCM techniques were implemented to isolate TPH-positive cells from the heterogeneous cell population in the DRN. Following microdissection, RNA was extracted from 5-HT-containing cells in the DRN of SD and WKY rats. Table 1 represents a subpopulation of measurements taken from the two strains. Values are listed as the fraction of RNA against β -actin, percentage change from SD to WKY as well as p -values for unpaired t -tests. We focused our analysis on mRNA that encode for proteins known to have a role in stress responses, anxiety, depression, or addiction, and also those critical for regulating the excitability of DRN neurons. In 5-HT neurons in the DRN of WKY rats, there was a significant reduction (66% decrease) in mRNA encoding the predominant isoform of TPH expressed in the brain, TPH2, compared with SD rats. Differences in 5-HT receptor mRNA included that encoding for 5-HT1A, 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2B, 5-HT6, and 5-HT7A. Notably, 5-HT neurons in the DRN of WKY rats demonstrated a significant reduction in 5-HT1A and 5-HT6 receptor mRNA (21 and 8%, respectively), and there was an 87% increase in 5-HT7A mRNA. The CRF R1 receptor mRNA expression was not significantly different based on strain; however, there was a 54% increase in CRF R2 mRNA expression in WKY rats. There was relatively little expression of mineralocorticoid receptor (MR) mRNA in the DRN. Moreover, although glucocorticoid receptors (GRs) were expressed

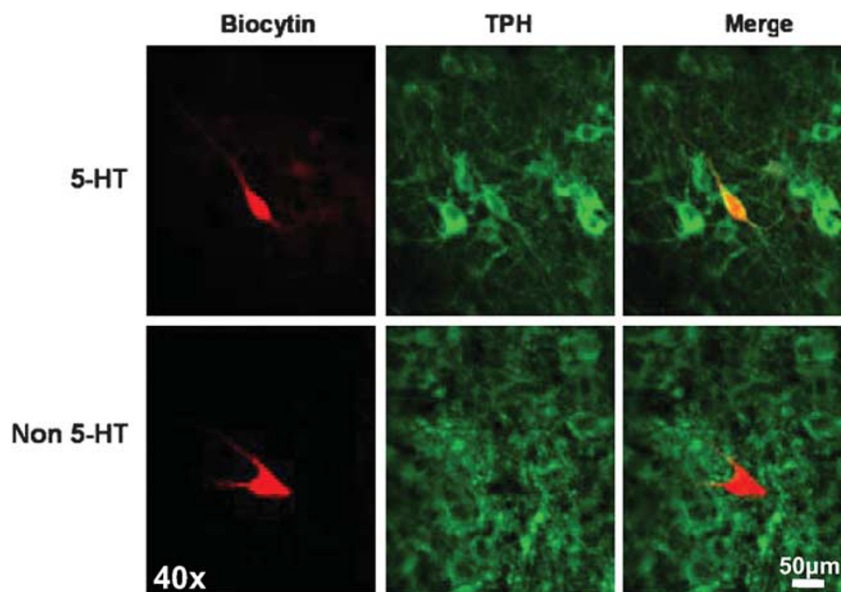


Figure 1 Immunohistochemical identification of 5-HT- and non-5-HT-containing neurons. Immunofluorescent photomicrographs of recorded neurons filled with biocytin (red) and co-stained for tryptophan hydroxylase (TPH) (green). The merged image is depicted in the left panels. Recorded neurons colocalized with TPH were classified as 5-HT containing (top panel). Neurons without TPH staining that were surrounded by TPH-positive cells in the same plane of focus were classified as non-5-HT containing (bottom panel).

Table 1 Normalized aRNA Levels That Encode for Anxiety- and Depression-Related Proteins in 5-HT DRN Neurons of SD vs WKY Rats

Name	5-HT SD (N = 4)		5-HT WKY (N = 4)		% Change WKY/SD *100	t-test P-value
	Average	SEM	Average	SEM		
5-HT 1A*	0.775	0.029	0.612	0.055	79	0.04
5-HT 1B	0.007	0.003	0	0	N/A	NS
5-HT 1D*	0.013	0.005	0	0	N/A	0.037
5-HT 2A	0.96	0.023	0.894	0.021	93.1	NS
5-HT 6*	1.063	0.016	0.982	0.023	92	0.029
5-HT 7A**	0.015	0.001	0.028	0.004	187	0.002
TPH 1	0.002	0.001	0.006	0.001	279.5	0.058
TPH 2***	0.157	0.007	0.051	0.017	32	0.001
Adcy 2**	0.126	0.005	0.071	0.012	56	0.006
CRF R1	0.03	0.001	0.04	0.005	133	NS
CRF R2*	0.024	0.002	0.037	0.003	154	0.011
GR	0.027	0.003	0.035	0.004	129	NS
MR	0.01	0.002	0.008	0.002	81	NS
GABA-A α 1*	0.947	0.026	0.837	0.026	88	0.023
GABA-A α 2	0.779	0.052	0.636	0.077	82	NS
GABA-A α 4	0.783	0.051	0.668	0.052	85	NS
GABA-A α 5	0.714	0.058	0.809	0.075	113	NS
GABA-A β 1	0.6	0.037	0.577	0.138	96	NS
GABA-A β 2	0.625	0.056	0.653	0.079	104	NS
GABA-A β 3***	0.14	0.009	0.017	0.007	12	0.001
GABA-A γ 2	0.761	0.064	0.692	0.063	91	NS

Abbreviation: N, number of animals.

Amplified RNA levels have been normalized to β -actin levels. Data are expressed as the mean \pm SEM.

Significant difference (in bold) between SD and WKY 5-HT-containing cells ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) by unpaired t-test.

in the DRN, there was not a significant difference in expression between strains. Finally, there was a significant reduction (12%) of GABA_A α 1 subunits and remarkably an 88% reduction in GABA_A β 3 subunits in 5-HT neurons of WKY rats.

Electrophysiological studies were focused on receptor-mediated responses with known functional effects that have been well characterized in either the DRN or other regions, namely, 5-HT_{1A}, CRF R1 and R2, and GABA_A subunit effects. Electrophysiological data were recorded from both 5-HT- and non-5-HT-containing neurons and results are reported for both cell types in the text and tables; however, only data from 5-HT neurons are shown graphically.

Cellular Characteristics of 5-HT and Non-5-HT Cells in the DRN of SD and WKY Rats

Using current clamp configuration, cellular characteristics were recorded from 5-HT- and non-5-HT-containing cells in the DRN of SD and WKY rats. Table 2 contains the summary data (means \pm SEM). The cellular characteristics of the 5-HT and non-5-HT neurons in SD rats were similar to those previously reported (Beck *et al*, 2004). However, 5-HT cells of WKY rats had a more hyperpolarized resting membrane potential (RMP) when compared with SD 5-HT cells ($p < 0.05$), suggesting a decreased intrinsic excitability of WKY 5-HT neurons. Interestingly, there were no differences in either membrane input resistance or tau between SD and

WKY 5-HT neurons. The response to 5-HT_{1A} receptor activation was also assessed. A saturating concentration of the 5-HT_{1,7} receptor agonist 5-CT (100 nM) was bath applied to assess the hyperpolarization (mV) associated with 5-HT_{1A} receptor activation (Beck *et al*, 2004). Based on the mRNA data, we hypothesized that 5-HT neurons of WKY rats would show an attenuated response to 5-HT_{1A} receptor activation. The response to 5-CT administration was not different between SD and WKY rats for both 5-HT- and non-5-HT-containing neurons in the DRN. However, using a criterion of 5 mV hyperpolarization minimum, there was a trend for a difference in the percentage of neurons exhibiting a 5-HT_{1A} receptor-mediated response, that is, 85% for neurons recorded from SD rats, but only 75% for neurons from WKY rats. It is possible that a nonsaturating concentration of 5-CT would reveal a more substantial difference between strains. Additionally, there was a difference between the non-5-HT neurons, with 50% of the neurons from SD rats exhibiting a response, but only 20% of the neurons from the WKY rats. The χ^2 analysis revealed that the data were not significant ($p = 0.07$).

Functional Effects of GABA_A Subunit Changes in WKY 5-HT Neurons

The rise and decay kinetics of the mIPSC are entirely dependent on the GABA_A receptor subunit composition. The RNA data indicated only a 12% reduction in α 1 subunit

Table 2 Cellular Characteristics and 5-CT Response of 5-HT and Non-5-HT Neurons in the DRN of SD and WKY Rats

Cell type	RMP (mV)	Res (mΩ)	Tau (ms)	AHP Amp (mV)	AHP $\tau_{1/2}$ (ms)	AHP Dur (ms)	AP threshold (mV)	AP Amp (mV)	AP Dur (ms)	Response (mV) ^a	% of cells with 5-CT response ^b
SD 5-HT (N = 18)	-65 ± 2.1	614 ± 51	52 ± 4.4	18 ± 1.4	162 ± 15	444 ± 26	-23 ± 1.7	72 ± 2.2	2.0 ± 0.2	8 ± 1.2 (N = 13)	85%
SD non-5-HT (N = 13)	-62 ± 2.1	544 ± 64	51 ± 7.2	16 ± 1.3	127 ± 20	440 ± 38	-24 ± 1.8	69 ± 3.5	1.6 ± 0.2	4 ± 1.4 (N = 11)	50%
WKY 5-HT (N = 19)	-72 ± 2.2*	620 ± 49	54 ± 4.0	17 ± 0.9	160 ± 15	426 ± 31	-23 ± 2.3	76 ± 1.0	2.0 ± 0.1	8 ± 1.6 (N = 16)	75%
WKY non-5-HT (N = 9)	-67 ± 1.7	492 ± 74	47 ± 7.0	17 ± 1.6	127 ± 13	398 ± 57	-21 ± 2.0	72 ± 4.0	2.0 ± 0.2	1 ± 0.85 (N = 5)	20%

Data are expressed as mean ± SEM and were attained from current clamp protocols run on cells recorded from the DRN of SD and WKY rats.

^aNot all cells included in the cellular characteristics data were maintained long enough to apply 5-CT, and hence there is a second set of N values reported in the 5-CT response column.

^bA criterion of an hyperpolarization ≥ 5 mV was used to assess the percentage of cells with a 5-CT response.

*Significant difference between SD and WKY 5-HT-containing cells ($*p < 0.05$) by unpaired *t*-test. Unpaired *t*-tests comparing non-5-HT cells from SD and WKY rats resulted in no significant differences.

expression, yet demonstrated an 88% reduction in $\beta 3$ subunit expression in WKY rats. Based on previous studies from $\beta 3^{-/-}$ mice (Ramadan *et al*, 2003), we hypothesized that mIPSCs recorded from 5-HT neurons in the DRN of WKY rats would have significantly faster decay kinetics than SD rats. The baseline data from all of the experiments were combined for the analysis of baseline mIPSC parameters. There were no significant differences in baseline mIPSC characteristics between experiments from the same cell type and strain. The mIPSC parameters from SD and WKY 5-HT and non-5-HT neurons are presented in Table 3. A one-way ANOVA with *post hoc t*-test revealed that non-5-HT neurons in SD rats exhibited significantly larger mIPSC amplitude compared with all other groups. However, this was the only significant difference in basal mIPSC properties. Surprisingly, there were no significant differences specifically in rise or decay kinetics in the mIPSC measured between SD and WKY 5-HT neurons. There were also no significant differences in mIPSC frequency or amplitude between 5-HT neurons based on rat strain, suggesting a similar probability of release of GABA from presynaptic terminals as well as similar basal receptor occupancy/receptor density.

The $\beta 3$ subunit is important in the activation of the receptor, and therefore the difference between SD and WKY rats may manifest when the receptor is activated by selective agonists. To confirm the mRNA data, we used standard immunohistochemistry protocols to assess $\beta 3$ protein expression on 5-HT neurons. Consistent with the quantitative mRNA data, qualitative inspection of changes in GABA_A $\beta 3$ immunohistochemistry in SD and WKY rats revealed an overall reduction in fluorescent intensity and $\beta 3$ -positive cells in WKY animals (Supplementary Figure S1). Despite the fact that we did not see a change in basal mIPSC kinetics between SD and WKY 5-HT neurons, it is possible that this difference in GABA_A receptor subunit expression would manifest as a change in pharmacological efficacy.

To test the hypothesis that there was a functional effect of GABA_A $\beta 3$ reduction in WKY rats, loreclezole, an anticonvulsant and GABA_A modulator that selectively binds to $\beta 2/3$ subunits, was bath applied. We reasoned that as there was no change in $\beta 2$ mRNA expression, the potential differences in loreclezole (10 μ M)-mediated mIPSC prolongation would be because of alterations in $\beta 3$ expression. Loreclezole experiments were conducted only in 5-HT neurons. Figure 2a depicts representative traces before and after application of loreclezole in 5-HT neurons of SD and WKY rats. Loreclezole did not significantly increase mIPSC frequency in SD or WKY rats, nor did it significantly augment mIPSC amplitude ($N = 7$ for both SD and WKY; Supplementary Table S1 and Figure 2b). Loreclezole significantly prolonged mIPSC decay only in SD 5-HT neurons, but not in WKY 5-HT neurons (Supplementary Table S1 and Figure 2c). Based on these data, we concluded that the large reduction in GABA_A $\beta 3$ mRNA in WKY 5-HT neurons results in a functional decrease in GABA_A receptor activation.

The alteration in $\beta 3$ function may have implications for the functional effects of the $\alpha 1$ subunit, although only a small change in $\alpha 1$ subunit expression itself was found. In hippocampal cultures from $\beta 3^{-/-}$ mice, there was a significant enhancement in the efficacy of zolpidem that allosterically binds selectively to $\alpha 1$ containing GABA_A

Table 3 mIPSC Characteristics for 5-HT and Non-5-HT Neurons in the DRN of SD and WKY Rats

Cell type	Frequency (Hz)	Amplitude (pA)	Rise time (msec)	Fast decay time (msec)	Slow decay time (msec)
SD 5-HT (N = 77)	6.9 ± 0.4	17.1 ± 0.7	1.3 ± 0.04	3.9 ± 0.3	35.9 ± 5.9
SD non-5-HT (N = 35)	5.7 ± 0.5	23.3 ± 1.9 ^a	1.3 ± 0.05	3.7 ± 0.2	18.7 ± 2.8
WKY 5-HT (N = 62)	6.1 ± 0.5	18.3 ± 0.9	1.3 ± 0.05	3.9 ± 0.2	54.9 ± 17.7
WKY non-5-HT (N = 26)	4.9 ± 0.5	16.6 ± 1.0	1.5 ± 0.06	3.6 ± 0.2	26.1 ± 5.4

^aSignificantly different from all other groups by Kruskal–Wallis ANOVA, Dunn's follow-up $p < 0.05$.

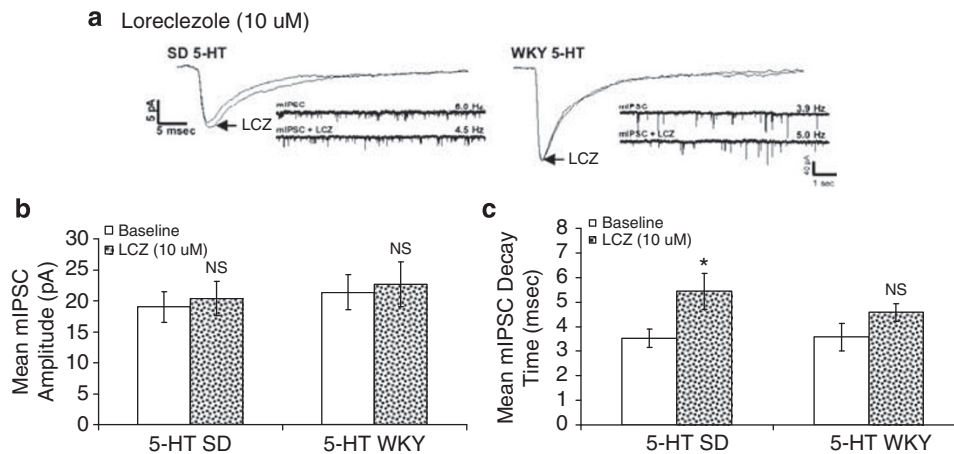


Figure 2 Response to loreclezole is absent in 5-HT neurons of WKY rats. (a) A representative averaged mIPSC and trace (inset) from a 5-HT neuron in a SD (left) or WKY (right) rat before and after 10 μ M loreclezole application, an anti-convulsant that selectively binds to the $\beta 2/3$ subunits. (b) Loreclezole had no effect on mIPSC amplitude in either SD or WKY rats ($p > 0.05$), (c) and only significantly prolonged mIPSC fast decay in SD animals ($p < 0.05$), but not WKY animals ($p > 0.05$).

channels, because of loss of $\alpha 2/3$ subunit expression at the cell surface. Similar to $\beta 3^{-/-}$ mice, we found a zolpidem-elicited increase in mIPSC amplitude in 5-HT neurons of WKY rats compared with SD rats (Supplementary Table S1).

CRF Receptor Activation Effects on mIPSCs in Cells Recorded from the DRN of WKY Rats

In a recent paper from our laboratory we demonstrated that CRF R1 activation enhances GABAergic synaptic transmission both pre- and post-synaptically in 5-HT-containing neurons in the DRN of SD rats (Kirby *et al*, 2008). Additionally, in both 5-HT and non-5-HT neurons, CRF produced a small, yet physiologically relevant, inward current, through activation of different receptors: CRF R2 in 5-HT neurons and CRF R1 in non-5-HT neurons. It has been demonstrated that WKY rats are behaviorally similar to rats that have been previously exposed to stressors and, therefore, we predicted that there would be an attenuation of the *in vitro* CRF effects on GABAergic synaptic transmission and cell excitability established by Kirby *et al* (2008). However, in light of the mRNA data, we further predicted that some, if not all, of the CRF R2 effects may be retained as there was a significant upregulation of CRF R2 mRNA in 5-HT neurons of WKY rats.

The same protocol and concentrations of oCRF (selective R1 agonist) and Ucn II (selective R2 agonist) were used as described in Kirby *et al* (2008). Data were collected from WKY rats in parallel using the same drugs and stock

solutions. Therefore, the data from the SD rats are the controls for the data obtained from the WKY rats (see Materials and Methods).

Figure 3a displays representative traces (left) and the averaged mIPSC (right) recorded from cells before and after the administration of oCRF from SD 5-HT (upper panel) and WKY 5-HT (lower panel) neurons. Figure 3b and c contains summary graphs of the mIPSC frequency (Figure 3b) and amplitude (Figure 3c) following oCRF bath application for 5-HT neurons recorded in the DRN of SD and WKY rats ($N = 26$ and 19 , respectively). In direct contrast to our findings in SD rats, oCRF did not increase mIPSC frequency or amplitude in neurons recorded from WKY rats (Supplementary Table S2 and Figure 3b and c). Interestingly, in non-5-HT WKY neurons, oCRF did significantly increase both mIPSC frequency and amplitude ($p < 0.05$, $N = 7$); these effects were not apparent in non-5-HT neurons in SD rats (Supplementary Table S2).

In a separate set of experiments, the selective CRF R2 agonist, Ucn II, was bath applied to the slice. Figure 4a contains representative traces (left) and averaged mIPSCs before and after Ucn II administration in recordings made from 5-HT neurons in SD (upper panel) and WKY (lower panel) rats. In SD rats, Ucn II had no effect on frequency but significantly increased the amplitude of mIPSCs ($p < 0.01$, $N = 17$; Figure 4c and Supplementary Table S2). Similarly to what was reported in SD rats, Ucn II had no effect on mIPSC frequency in WKY 5-HT neurons ($N = 13$; Figure 4b). Interestingly, similarly to SD 5-HT neurons, Ucn

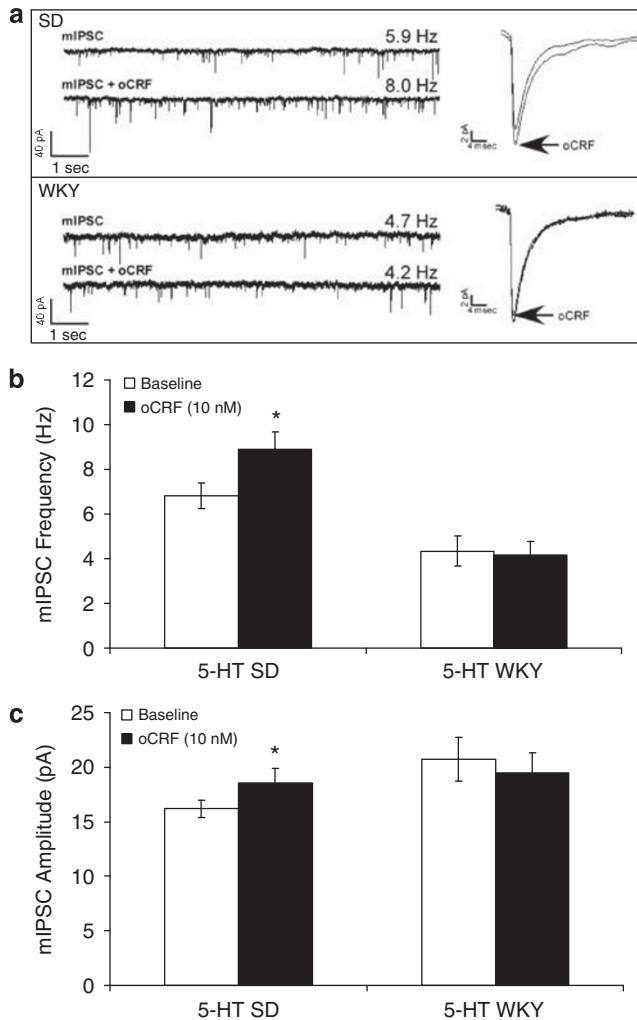


Figure 3 CRF responses are attenuated in 5-HT DRN neurons of WKY rats. (a) Representative traces (left) and averaged mIPSC (right) from recordings made from 5-HT neurons of SD (top) and WKY (bottom) rats before and after 10 nM oCRF bath application. (b) Summary bar graph depicting the baseline mean mIPSC frequency and the change following oCRF administration to neurons recorded from SD and WKY rats. oCRF significantly increased mIPSC frequency ($p < 0.05$). This effect was absent in WKY rats ($p > 0.05$). (c) Summary bar graph depicting the baseline mean mIPSC amplitude and change following oCRF in SD and WKY rats. oCRF significantly increased mIPSC amplitude ($p < 0.05$). This effect was absent in WKY rats ($p > 0.05$).

II significantly augmented mIPSC amplitude in WKY 5-HT neurons (Figure 4c). Kirby *et al* (2008) reported no effect of Ucn II on mIPSC frequency or amplitude in non-5-HT-containing neurons of SD rats. However, Ucn II did significantly augment mIPSC amplitude in WKY non-5-HT neurons ($p < 0.05$, $N = 8$; Supplementary Table S2).

Therefore, in the WKY stress-hyperresponsive rats, the CRF R1-mediated increase in frequency was attenuated in 5-HT neurons, but enhanced in non-5-HT neurons. Ucn II significantly increased mIPSC amplitude in WKY 5-HT-containing cells. In WKY non-5-HT neurons, both oCRF and Ucn II augmented mIPSC amplitude. Thus, in the DRN 5-HT neurons of stress-hyperresponsive rats, the CRF R2-mediated response on GABAergic synaptic transmission was retained, but the CRF R1 response eliminated. More-

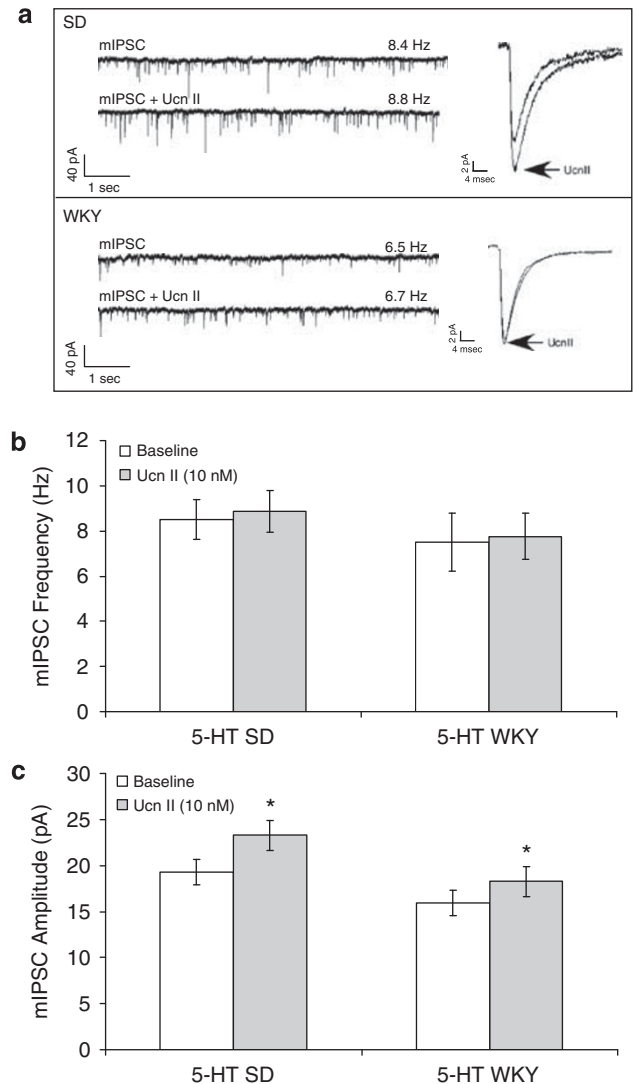


Figure 4 The effects of the selective CRF R2 agonist Ucn II on mIPSC amplitude were retained in WKY rats. (a) Representative traces (left) and averaged mIPSC (right) from recordings made from 5-HT neurons of SD (top) and WKY (bottom) rats before and following 10 nM Ucn II bath application. (b) Summary bar graph depicting the baseline mean mIPSC frequency and the change following Ucn II administration to neurons recorded from SD and WKY rats. Urocortin II had no effect on mIPSC frequency in SD rats and likewise had no effect on 5-HT neurons recorded from WKY rats ($p > 0.05$). (c) Summary bar graph depicting the baseline mean mIPSC amplitude and change in amplitude following Ucn II in neurons recorded from SD and WKY rats. Ucn II significantly augmented mIPSC amplitude in 5-HT neurons of both SD and WKY rats ($p < 0.05$).

over, CRF modulation of GABAergic transmission that was not seen in SD non-5-HT neurons was significantly enhanced in non-5-HT neurons in the WKY rats.

This difference in CRF response between strains is specific to the DRN. In a set of control experiments conducted in the neighboring ventral serotonergic structure, the MRN, CRF was found to augment mIPSC amplitude in 5-HT cells of both SD and WKY rats (Supplementary Figure S2). Despite evidence that this structure is also involved in affective behavior, CRF responsiveness of these cells was retained in WKY rats, suggesting a regional specificity as opposed to a global change.

oCRF and Ucn II Do Not Alter the Inward Current in WKY Rats

In SD animals, oCRF and Ucn II significantly increased the inward current in 5-HT-containing cells. Pretreatment with the CRF R2 antagonist ASVG-30, but not the CRF R1 antagonist antalarmin, is able to block the effect of oCRF on inward current. This evidence suggests that this effect is mediated by the CRF R2 receptor (Kirby *et al*, 2008). Only oCRF, but not Ucn II, significantly increased the inward current in non-5-HT cells in an antalarmin-sensitive manner, suggesting that in this cell type CRF R1 receptors mediate the effect. In this study, the effects of oCRF or Ucn II on inward current were not apparent in either 5-HT- or non-5-HT-containing cells of WKY rats. The CRF R1- and CRF R2-mediated increase in inward current was absent in non-5-HT- and 5-HT-containing neurons from WKY rats, respectively. Thus, the net effect of CRF receptor activation on 5-HT neurons in WKY rats was selective to an enhancement of GABA_A receptor-mediated currents, without a simultaneous increase in GABA release.

The GABAmimetic Effects of Ethanol (EtOH) on 5-HT Neurons in DRN of SD and WKY Rats

Along with demonstrating a global stress hyperresponsivity, WKY rats also show an increase in alcohol preference (Pare *et al*, 1999). EtOH depresses DRN firing rates *in vivo* and presumably depresses 5-HT release in the forebrain (Pistis *et al*, 1997). It is possible that DRN dysregulation and subsequent abnormal physiological responses to EtOH in the DRN may be in part responsible for the behavioral increase in alcohol preference documented in WKY rats. Physiologically relevant concentrations of EtOH, 10–100 mM, have GABAmimetic effects via enhancement of mIPSC frequency or amplitude in *in vitro* slices made of limbic regions such as the central amygdala (Nie *et al*, 2004). Moreover, EtOH-mediated effects can be blocked by

pretreatment of the CRF R1 selective antagonist antalarmin and is also not apparent in CRF R1^{-/-} mice. We reasoned that it was possible, given the alterations in GABA_A subunit expression and pharmacological sensitivity as well as attenuation of CRF R1-mediated enhancement of GABAergic synaptic transmission, that a midrange concentration of EtOH (50 mM) would have disparate effects on GABA_A mIPSC frequency and amplitude in 5-HT neurons from SD vs WKY rats. We also wanted to determine if antalarmin pretreatment would have a similar block on the GABAmimetic effects of EtOH. Figure 5a shows representative traces from 5-HT neurons recorded from SD (Figure 5ai and aii) and WKY (Figure 5aiii) rats. In a separate set of SD rats, the slice was pretreated with antalarmin (300 nM) following a baseline mIPSC recording, but before EtOH treatment. Similar to what was reported in our previous work, there was a trend for antalarmin to increase mIPSC amplitude ($p = 0.08$). Figure 5b and c contains summary bar graphs of the change in frequency and amplitude from baseline following EtOH (50 mM) bath application. We found that EtOH (50 mM) significantly enhanced mIPSC frequency and amplitude ($p < 0.05$ in both SD and WKY DRN 5-HT neurons, $N = 10$ and 9 , respectively), yet there was no significant effect of strain (two-way repeated-measures ANOVA, $p > 0.05$; Supplementary Table S2 and Figure 5b and c). Pretreatment with Antalarmin had no effect on EtOH-mediated enhancement of mIPSC frequency, but did attenuate the EtOH effect on mIPSC amplitude (one-way repeated-measures ANOVA, $p < 0.05$, baseline vs antalarmin $p > 0.05$, antalarmin vs EtOH, $p > 0.05$; $N = 6$). This finding is consistent with other studies demonstrating an antalarmin block of EtOH GABAmimetic effects. It is also suggested that there were two different mechanisms underlying the EtOH effects on presynaptic release of GABA vs postsynaptic enhancement of the amplitude. However, based on our comparison of SD with WKY rats in this set of experiments, we conclude that there is no appreciable

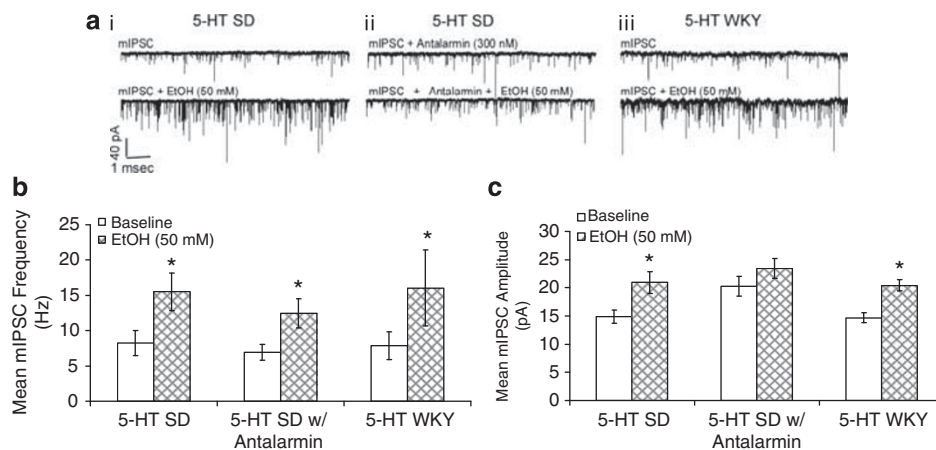


Figure 5 GABAmimetic effects of EtOH (50 mM) were not different in 5-HT neurons from SD and WKY rats. (a) Representative traces from recordings made from 5-HT neurons of SD (ai, aii) and WKY (aiii) rats before and after application of 50 mM EtOH. In the middle panel (a ii), the top trace contains mIPSC data with the selective CRF R1 antagonist, antalarmin (300 nM) on board, and the bottom trace represents the effect of EtOH on mIPSCs in the presence of antalarmin. (b) Summary bar graph of the change in mIPSC frequency following EtOH application. EtOH significantly enhanced mIPSC frequency similarly in SD and WKY rats ($p < 0.05$) and this effect was not significantly blocked by antalarmin. (c) Summary bar graph of the change in mIPSC amplitude following EtOH application. EtOH significantly enhanced mIPSC amplitude similarly in SD and WKY rats ($p < 0.05$), and this effect was significantly attenuated by antalarmin.

difference in the EtOH response in this nucleus in stress-hyperresponsive rats.

DISCUSSION

The major premise of this investigation was that DRN intrinsic excitability and its modulation is altered in pathological stress states that may in turn lead to psychiatric disorders such as MDD and generalized anxiety disorder. The objective of this study was to use a multilevel approach to investigate differences in the regulation of 5-HT-containing neurons from the DRN, an area critical in mediating stress responses, in a stress-hyperresponsive animal model, the WKY rat (Pare, 1993, 1989a, 1994a, 1994b, 2000; Pare and Redei, 1993a; Pare and Tejani-Butt, 1996; Pare *et al*, 1999). Although many behavioral studies have been conducted on these animals, very few have been done addressing possible cellular mechanisms underlying this stress hyperresponsivity. The behavioral studies demonstrated differences in all three major monoaminergic systems; that is, dopamine, norepinephrine, and serotonin (Conti *et al*, 1997; Lopez-Rubalcava and Lucki, 2000; Pare and Tejani-Butt, 1996; Pearson *et al*, 2006; Tejani-Butt *et al*, 1994). The DRN is critical in mediating responses to uncontrollable stress (Maier and Watkins, 2005) and is functionally altered following *in vivo* stressor exposure (Kirby *et al*, 2007; Price *et al*, 2002). We, therefore, focused our attention to differences in mRNA expression, protein expression, and physiology in the DRN of WKY rats.

Differences in the mRNA levels of 5-HT receptors, effectors, synthesizing enzymes, and GABA_A receptor subunits were found between the WKY and SD rats. The RMP was the only membrane property that was significantly different between the 5-HT- or non-5-HT-containing neurons of the DRN between the WKY and SD rats. The proportion of neurons demonstrating a 5-HT_{1A} response was less in both 5-HT and non-5-HT neurons from WKY rats. Responses mediated by the stress hormone CRF were significantly different and there were differences in the responses mediated by selective benzodiazepines interacting with the GABA_A receptor. Interestingly, there were no differences found in the response to alcohol.

Decrease in TPH2 Production and Intrinsic Excitability in 5-HT Neurons

Significant differences were found in mRNA encoding for a variety of depression- and anxiety-related proteins as well as proteins necessary for GPCR downstream signaling in 5-HT neurons of WKY rats. There are several studies that have examined differences in mRNA expression in different animal models of stress hyperresponsivity or following various behavioral manipulations to elicit stress. However, these studies do not selectively examine differences specifically in the 5-HT neurons, but throughout the entire heterogeneous DRN. Therefore, LCM and mRNA amplification techniques were employed to look at differences in mRNA expression in 5-HT neurons of SD compared with WKY rats.

A recent study by Scholl *et al* (2010) demonstrated a significant reduction in basal 5-HT levels in several brain

limbic brain regions (ie, basolateral amygdala, nucleus accumbens shell, substantia nigra, dorsal raphe, and hypothalamus) in WKY rats compared with SD controls, with comparable 5-HT turnover rates. Consistent with this finding, WKY rats were found to have a significant reduction in TPH2 mRNA expression in DRN neurons compared with SD rats. Recent attention has been given to the relationship between TPH2 gene polymorphisms and mRNA expression and stress, anxiety, and depression. However, thus far, various studies have presented conflicting data and the relevance of changes in TPH2 expression to maladaptive affective behavior remains unclear (McEuen *et al*, 2008; Shishkina *et al*, 2008; Lowry *et al*, 2008; Clark *et al*, 2007; Abumaria *et al*, 2008; Gizatullin *et al*, 2008). No studies have been conducted examining the actual physiological relevance of changes in TPH2 production, that is, changes in the levels of 5-HT in the raphe and forebrain. It is likely that the level of TPH2 expression is correlated with the progression of psychiatric disease. For example, the initial etiology of depression-like behavior may be, in part, due to low expression levels of TPH2, which would be consistent with low levels of 5-HT found in the forebrain of depressed patients (Coppen and Doogan, 1988; Deakin, 1991; Grahame-Smith, 1989).

TPH2 is the predominant isoform expressed in 5-HT neurons; however, although not statistically significant, there was a strong trend of a 2.5-fold increase in TPH1 expression in WKY rats. This increase in TPH1 matches most closely the findings of Abumaria *et al* (2008), who demonstrated that chronic daily restraint stress significantly increased TPH1 expression in the DRN 2.5-fold. Perhaps, TPH1 increases activity when TPH2 is not functioning. Future studies must address the functional consequences of the relative contributions by TPH1 and TPH2 on 5-HT release, especially in models of stress.

The intrinsic excitability and synaptic input to the DRN are both important in regulating the firing rate of 5-HT neurons and the subsequent release of 5-HT into forebrain regions. The 5-HT neurons in the DRN of WKY rats had a significantly hyperpolarized RMP, yet similar action potential characteristics, tau, and membrane resistance. Therefore, given an equal excitatory drive, the firing rate and subsequent release of 5-HT into the forebrain is likely lower in WKY rats. This lower neuronal excitability coupled by a dramatic reduction in TPH2 mRNA expression would be consistent with a lower level of 5-HT in the forebrain, similar to that seen in depressed patients (Coppen and Doogan, 1988; Deakin, 1991; Grahame-Smith, 1989).

Autoreceptor Inhibition by 5-HT_{1A} Receptor Was Altered in WKY Rats

The levels of the 5-HT_{1A} autoreceptor have recently been shown to determine vulnerability to stress and response to antidepressant treatments (Richardson-Jones *et al*, 2010). A significant decrease was found in mRNA expression of 5-HT_{1A} and 5-HT₆ receptors in WKY rats, with almost a twofold increase in 5-HT₇ receptor expression. The 5-HT_{1A} receptor is coupled to an inward rectifying K⁺ channel and is important in regulating the excitability of the DRN (Penington *et al*, 1993; Beck *et al*, 2004). The small difference in 5-HT_{1A} receptor expression was not sufficient to

change the response of 5-HT neurons in WKY rats to a saturating concentration of 5-CT (selective 5-HT_{1,7} agonist) application. However, there was a trend in the ratio of neurons demonstrating a response to 5-CT, with both the 5-HT and non-5-HT neurons from WKY neurons having a lower percentage. Future studies should be conducted to examine the response of these neurons over a broad range of nonsaturating concentrations and within the different subfields of the raphe, as we have previously demonstrated that the 5-HT_{1A} response magnitude is greater in the different DRN subfields (Beck *et al*, 2004; Crawford *et al*, 2010).

GABA_A Subunit Expression, Function, and Pharmacological Sensitivity

5-HT neurons of the DRN receive GABAergic input from forebrain areas and from GABA neurons of the DRN that provide feedback information as well as control firing of the cells (Gervasoni *et al* 2000; Haddjeri *et al* 2000; Nishikawa and Scatton 1983, 1984, 1985). Stress exposure specifically increases *c-Fos* immunoreactivity of GABAergic neurons of the raphe (Roche *et al*, 2003). Moreover, manipulations of specific GABA_A subunit conformations can result in anxiety-like behavior (Korpi and Sinkkonen, 2006). Several FDA-approved drugs are allosteric modulators of GABA_A receptors that bind selectively to different subunits (Korpi and Sinkkonen, 2006; Mohler 2006). These compounds may act differently in a stress-hyperresponsive brain because of changes in subunit expression and composition. Therefore, we examined GABA_A subunit RNA expression levels, the basal properties of GABAergic synaptic activity, and the response to GABA_A selective drugs between SD and WKY rats. There was a small decrease in GABA_A $\alpha 1$ expression in WKY rats. There was a dramatic decrease in GABA_A $\beta 3$ expression, although there was a relatively low expression level. This is not surprising as it is the $\beta 2$ subunit that is ubiquitously expressed in all GABA_A receptors. However, it is the $\beta 1$ and $\beta 3$ subunits that are critical for receptor trafficking and docking to the cell surface as well as phosphorylation of the receptor (Moss and Smart, 2001). Homanics *et al* (1997) found that $\beta 3^{-/-}$ mice demonstrated hypersensitivity to handling and other sensory stimuli as well as other neurological problems. The efficacy of an allosteric modulator, loreclezole, that binds selectively to the $\beta 2/3$ subunits was disrupted. Loreclezole prolonged mIPSC decay time only in 5-HT neurons of SD rats. This is direct evidence that there was a deficiency in $\beta 2/3$ cell surface expression in 5-HT neurons of WKY rats. Zolpidem augmented the mIPSC amplitude in 5-HT neurons of WKY rats approximately twice as much as in SD rats (42.3 vs 23.7%).

These findings suggest that although stress-hyperresponsive animals demonstrate changes in GABA_A receptor subunit composition, this does not manifest in a functional change in basal GABAergic synaptic transmission. Therefore, stress-induced depressive-like behaviors cannot be due to maladaptive alterations in GABAergic synaptic transmission in the DRN. Loreclezole is an approved anticonvulsant therapy, whereas zolpidem (Ambien) is an approved sleep aid. Use of these compounds by individuals with depression or anxiety may have abnormal effects because of an altered cellular response in the serotonergic system.

Alterations in CRF R1- and R2-Mediated Responses in WKY Rats

A main principle underlying stress hyperresponsivity is that a dysregulation of the stress neurocircuitry involved in mediating an adaptive transient stress response occurs such that individuals respond in a maladaptive manner to acute mild stressors or neutral stimuli. Acute CRF application to the slice preparation at low concentrations may model an acute mild stressor. Therefore, assessing how these responses are altered in an animal model of depression that demonstrates behavioral hyperresponsivity to stressors provided an insight into some of the mechanisms underlying this dysregulation. Fibers containing CRF are apparent from the rostral to caudal DRN and cells of the DRN express both CRF R1 and CRF R2 (Kirby *et al*, 2000; Price *et al*, 1998; Price and Lucki, 2001; Day *et al*, 2004; Lukkes *et al*, 2008). Acute application of CRF to the DRN results in the modulation of 5-HT neuronal excitability. A major finding of this study is that some of these acute CRF responses were attenuated in WKY rats.

In control SD rats, CRF enhances both mIPSC frequency and decreases evoked IPSC paired pulse ratio, thereby increasing the probability of release of GABA from presynaptic terminals that synapse on 5-HT neurons. This increase in GABA release is mediated by CRF R1 receptors located on GABAergic synaptic terminals (Kirby *et al*, 2008). Despite similar mRNA expression levels of CRF R1 in the stress-hyperresponsive WKY and SD rats, the CRF R1 receptor-mediated increase in mIPSC frequency was absent in the 5-HT neurons of the DRN in the WKY rat. CRF R1 receptor-mediated postsynaptic responses were also attenuated in the WKY rat. The increase in mIPSC amplitude is mediated by both CRF R1 and R2 receptors (Kirby *et al*, 2008). In the 5-HT neurons recorded from the WKY rats, the CRF R1-mediated increase in mIPSC amplitude was absent.

There is a growing body of evidence suggesting that CRF R2 activation in specific brain regions (ie, lateral septum, dorsal raphe, and ventral tegmental area) is critical for encoding aversive stimuli under specific conditions in certain behavioral assays (ie, conditioned place aversion and stress-induced reinstatement) (Hammack *et al*, 2003; Henry *et al*, 2006; Land *et al*, 2008; Wang *et al*, 2007). CRF R2 mRNA expression was increased in 5-HT neurons of WKY rats. Unlike the CRF R1 responses, CRF R2-mediated augmentation of GABA_A-mediated currents was retained in WKY rats. In WKY rats, activation of CRF R2 still increased mIPSC amplitude (Kirby *et al*, 2008). In contrast, the CRF R2-mediated increase in inward current was absent. Thus, the net effect of CRF R2 activation on 5-HT neurons was exclusively inhibitory in the maladaptive stress state. However, this is counterbalanced by the emergent disinhibition caused by R2 responses on non-5-HT neurons.

In non-5-HT cells of WKY rats, CRF enhanced mIPSC frequency and amplitude. Although these neurons were not neurochemically identified, it is likely that these non-5-HT cells are GABAergic interneurons. The CRF R2 agonist Ucn II elicited responses in non-5-HT neurons; responses not normally seen in the neurons recorded from SD rats. Ucn II administration increased mIPSC amplitude in non-5-HT neurons of WKY rats. The CRF increase in mIPSC frequency and amplitude in concert with the Ucn II increase in

amplitude in non-5-HT neurons in WKY rats would result in a reduction in the inhibitory tone on 5-HT neurons.

All of these changes in CRF effects seen in the WKY rat lead to a net disinhibition of the 5-HT neuron, that is, attenuation of CRF R1 responses in 5-HT neurons, retention of CRF R2-mediated increase in mIPSC amplitude, increase in mIPSC frequency, and amplitude in non-5-HT neurons. It has been shown that following exposure to acute swim stress, there is an increase in 5-HT turnover in limbic regions of WKY rats compared with Wistar rats, suggesting an increase in 5-HT release during stress in WKY rats. This study would be congruent with a shift in the modulatory effect of CRF from inhibitory to excitatory (De La Garza and Mahoney, 2004). In the context of behavioral inhibition, these data indicate that during a stress event when the DRN is activated, increased CRF would lead to an increase in 5-HT levels in the forebrain, which in turn would heighten behavioral inhibition. This is consistent with the behavioral data indicating that WKY rats are more likely to adopt passive coping strategies more quickly when exposed to a stressor (Rittenhouse *et al*, 2002). In the future, dual recordings between GABA neurons and 5-HT neurons would be needed to confirm these effects and this proposed circuitry within the DRN.

Part of the mechanism underlying the changes in CRF responses in WKY rats may be because of a re-organization of the CRF R1 and R2 receptors on the plasma membrane. Recent evidence demonstrates that in control unstressed rats the CRF R1 in the DRN is on the plasma membrane and the CRF R2 receptors are primarily cytoplasmic. Swim stress reverses this distribution so that CRF R2 is on the plasma membrane and CRF R1 is internalized (Waselus *et al*, 2009). The net actions of CRF release shifted from primarily inhibitory to excitatory, as seen by an increase in firing rate.

In the stress-hyperresponsive WKY rats, this mechanism does not function normally, partially because of a dysregulated CRF system that may be the result of the heightened internal stress state of the animal and increased levels of CRF. The CRF R1 receptors are probably internalized with CRF R2 receptors predominating on the surface membrane. The data reported in this paper indicate that in stress-hyperresponsive WKY rats, CRF-mediated inhibition of 5-HT-containing cells of the DRN through augmentation of GABAergic synaptic activity is absent. Furthermore, this alteration in CRF modulation in WKY rats is specific to the DRN and not seen in the MRN despite evidence of CRF-mediated effects in the MRN. Finally, there was not a general overall attenuation in all of the CRF receptor-mediated responses; all of the responses mediated by CRF R1 were attenuated, but some of the responses mediated by CRF R2 were retained in the WKY rats.

EtOH Has Similar GABAergic Effects in SD and WKY Rats

WKY rats demonstrate an increased alcohol preference (Pare *et al*, 1999). The stress-hyperresponsivity characteristic of these rats may be responsible for potentiated alcohol preference. Alternatively, there may be a difference in efficacy of EtOH to exert its cellular effects, causing WKY rats to intake more alcohol to reach the same hedonic state. A plausible hypothesis was that EtOH has decreased efficacy

in many regions of the WKY rat brain, but this was not the case in the DRN. At a midrange concentration, EtOH had similar GABAergic effects on mIPSC frequency and amplitude in 5-HT DRN neurons of SD and WKY rats. Therefore, in both these strains, EtOH robustly increased the inhibitory tone in the DRN, and therefore would subsequently decrease DRN discharge rates, thereby reducing the levels of 5-HT in forebrain. Future studies using a range of concentrations of EtOH may reveal differences in responsivity between SD and WKY. Work by Nie *et al* (2004) demonstrated that postsynaptic effects of EtOH (at a similar concentration used in this study) on GABAergic IPSCs can be blocked by pretreatment with a CRF R1 antagonist and was not apparent in CRF R1 KO mice. Similar to this study, we found that pretreatment with antalarmin was sufficient to attenuate the postsynaptic effect on mIPSC amplitude. However, the presynaptic increase in frequency following EtOH administration was not significantly attenuated by antalarmin, suggesting a separate mechanism for pre- and post-synaptic effects of EtOH. This finding is consistent with Nie *et al* (2004) and, moreover, demonstrates that this interaction with EtOH and CRF R1 is present in multiple regions. What remains elusive is the actual mechanism by which this interaction is occurring. Future studies must address how CRF R1 acts to influence the acute effects of EtOH, and what possible implications this may have for understanding the interaction between stress and addiction.

Final Conclusions

There have been very few studies examining the electrophysiological changes that occur in a model of chronic stress or the stress-hyperresponsive WKY rat. The findings of this study suggest that basally, 5-HT neurons have lower excitability and TPH2 production in WKY rats, and thus it is likely that these rats have basally lower levels of 5-HT. Although basal GABAergic signaling is normal in WKY rats compared with control animals, the modulation of GABAergic synaptic activity by clinically used allosteric modulators as well as by CRF is altered in these animals. The data presented in this paper demonstrate that CRF dysregulation may be a hallmark of chronic stress and depression in a highly region-specific manner. Clinically, CRF receptor antagonists have been pursued as possible antianxiety and antidepressant treatments. However, perhaps targeting CRF receptors globally is less efficacious than targeting specific regions of interest that are more critically involved in the pathology. This paper provides further evidence that CRF modulation of the 5-HT-containing nuclei is disrupted in a model of chronic stress and depression.

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DISCLOSURE

The authors declare no conflict of interest.

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