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Swim Stress differentially blocks CRF receptor mediated responses in dorsal raphe nucleus

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Abstract

Modulation of the serotonergic (5-HT) neurotransmitter system arising from the dorsal raphe nucleus (DR) is thought to support the behavioral effects of swim stress, i.e., immobility. In vivo pharmacological and anatomical studies suggest that corticotropin-releasing factor (CRF) and γ -aminobutyric acid (GABA) synaptic transmission closely interact to set the response of the DR to swim stress. To investigate the cellular basis of these physiological mechanisms the effects of ovine CRF (oCRF) on GABA_A-dependent miniature inhibitory postsynaptic currents (mIPSCs) in 5-HT and non-5-HT DR neurons in acute mesencephalic slices obtained from rats either naïve or 24h after a 15 min swim stress session were tested. In this study, the effect of swim stress alone was to decrease the holding current, i.e., hyperpolarize the neuron, and to increase the amplitude and charge of mIPSCs recorded from non5-HT neurons. Ovine CRF (10nM) induced an increase in mIPSC frequency in 5-HT neurons recorded from naïve rats, an effect that was suppressed by swim stress. The inward current elicited by oCRF in both 5-HT and non5-HT neurons was also blocked by swim stress. Ovine CRF increased mIPSCs amplitude and charge in both 5-HT and non-5-HT neurons, but this effect was not modified by swim stress. In concert with our previous findings that swim stress decreased input resistance, action potential threshold and action potential duration and increased glutamatergic synaptic activity the overall primary effect of swim stress is to increase the excitability of 5-HT neurons. These data provide a mechanism at the cellular level for the immobility induced by swim stress and identifies critical components of the raphe circuitry responsible for the altered output of 5-HT neurons induced by swim stress.

Keywords

dorsal raphe nucleus; swim stress; serotonin; 5-HT; non5-HT; mIPSC; GABA; GABA_A receptor; CRF1; CRF2

Introduction

The dorsal raphe nucleus (DR) is a major source of serotonergic (5-hydroxytryptamine, 5-HT) innervation of the mammalian forebrain (Azmitia and Segal, 1978; Jacobs and Azmitia, 1992; Mamounas et al., 1991). Dorsal raphe 5-HT projections are implicated in the

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physiological and behavioral responses to stressors and in the development of stress-related clinical conditions of mood disorders (Lowry 2002; Lowry et al., 2005; Maier et al., 1993; Petrov et al., 1992).

The neuropeptide corticotrophin-releasing factor (CRF) is a key mediator of different aspects of stress responses including the activation of the HPA axis (Bale and Vale, 2004; Herman et al., 2003; Owens and Nemeroff, 1993). In addition CRF is thought to be involved in the response of the DR to certain stressors (Cooper and Huhman, 2007; Leonard 2005; McEuen et al., 2008; Roche et al., 2003; Staub et al., 2005; Staub et al., 2006). Extensive evidence exists demonstrating the expression of CRF and its receptors CRF1 and CRF2 in the DR (Austin et al., 1997; Chalmers et al., 1995; Day et al., 2004; Van Pett et al., 2000). However, CRF expression is heterogeneous among the different DR subregions. CRF fibers in the caudal part of the DR are mainly located to the dorsal and lateral aspects of the nucleus while at a more rostral level these fibers are concentrated in the ventromedial DR (Austin et al., 2003; Kirby et al., 2000; Valentino et al., 2001b). Detailed immunohistochemical labeling reveals that the rich CRF innervation of DR is primarily in contact with GABA-containing dendrites than with 5-HT dendrites in both the ventromedial and dorsolateral DR (Lowry et al., 2000; Waselus et al., 2005). Ultrastructural studies provide further evidence of synaptic specializations involving CRF-immunoreactive terminals and DR dendrites in the lateral wings of the DR (Valentino et al., 2001a; Waselus et al., 2005).

The effects of CRF on DR are well documented from *in vivo* and *in vitro* pharmacological investigations in rodents. An intracerebroventricular (icv) injection of CRF decreases the firing rate of DR neurons. It also changes 5-HT release in projection areas with a region in a dose-dependent pattern (Kirby et al., 2000; Price et al., 1998; Price and Lucki, 2001). At low doses (1–10 ng) CRF decreases neuronal activity but at higher doses (30 ng) it has the opposite effect. The latter dose also results in a decrease of 5-HT release in both lateral septum and striatum (Kirby et al., 2000; Price et al., 1998; Price and Lucki, 2001). Corticotropin releasing factor1 antagonists can reverse these effects indicating a role for this specific receptor (Kirby et al., 2000; Price and Lucki, 2001). Other studies provide evidence for a role for CRF2 neuromodulation as well. Urocortin 2 (Ucn2), a CRF2 agonist, activates c-fos expression in 5-HT neurons in the dorsal mid to caudal DR when injected icv (Staub et al., 2005). A microinjection of Ucn2 in DR at low doses results in a decrease of 5-HT neuron activity. At higher doses, the firing rate of 5-HT neurons increases whereas the firing rate of non-5-HT neurons decreases, leading to the hypothesis that there is disinhibition of 5-HT neurons (Pernar et al., 2004). Recently, our laboratory demonstrated that GABAergic synaptic activity is modified in the ventromedial (vmDR) DR by bath application of CRF. Activation of CRF1 receptors increases the frequency of GABA release and the amplitude of GABA_A receptor mediated mIPSCs in 5-HT neurons. Activation of CRF2 also increases mIPSC amplitude and increases inward current in 5-HT containing neurons. In non-5-HT neurons, CRF1 receptors mediate an increase in inward current only (Kirby et al., 2008).

Corticotropin releasing factor has an important role in the adaptation of DR to swim stress. Indeed, Price et al. (Price et al., 2002) show that swim stress reduces the ability of both subsequent swim stress and CRF icv to alter 5-HT release in lateral septum. Moreover, the administration of a CRF antagonist in the 24 hour interval between the two swim stress sessions prevents the occurrence of adaptation to subsequent swim stress. At a cellular level, we previously demonstrated that swim stress has significant effects on active and passive cellular characteristics, glutamatergic EPSC synaptic activity and 5-HT_{1B} receptor-mediated inhibition of EPSC activity in the DR that was neurochemically specific (Kirby et al., 2007). In 5-HT neurons, input resistance, action potential threshold and action potential duration decreases, glutamatergic EPSC frequency increases, EPSC amplitude decreases and the 5-HT_{1B} mediated inhibition of EPSC activity increases. In non-5-HT neurons swim stress only decreases EPSC

amplitude (Kirby et al., 2007). In this paper we further our investigation of swim stress induced alterations in DR physiology. We test the hypothesis that swim stress decreases both GABAergic synaptic activity on 5-HT neurons and its modulation by CRF.

Material and methods

Animals and stress experiments

Male Sprague-Dawley rats (75–150g; Taconic Farms, Germantown, NY) were housed 3 per cage with a 12h/12h light/dark cycle, lights on at 7:00 am, with free access to food and water. Animals were randomized for treatment group. Swim stress was performed using procedures similar to those previously described elsewhere (Porsolt et al., 1977a; Porsolt et al., 1977b; Roche et al., 2003). Swim stress was conducted in the morning so that 24 hours later the rats could be sacrificed for the electrophysiology experiments. This procedure, that has been used previously by many laboratories, produces extensive immobility in the rats when tested 24 hours later (Porsolt et al., 1977a; Porsolt et al., 1977b, Kirby and Lucki, 1998, Price et al., 2002). Rats were placed in a large glass cylinder filled with water at room temperature ($25 \pm 1^\circ\text{C}$) and left to swim for 15 min. They were then towel dried, allowed to recover for 15 min in a warming cage containing a heating pad (37°C) and returned to their home cages. Naïve controls remained unhandled in their home cages. All experiments on animals were conducted in accordance to the NIH Guide for the Care and Use of Laboratory Animals and following protocols approved by the Institutional Animal Care and Use Committee.

Slice preparation

Twenty-four hours after the swim stress session, brain slices were prepared as previously described (Beck et al., 2004; Lemos et al., 2006). Animals were rapidly decapitated and the brain placed in ice-cold artificial cerebrospinal fluid (ACSF) in which sucrose (248 mM) was substituted for NaCl. Coronal slices 200 μm thick were cut from the mesencephalic region with a Leica VT1000S vibroslicer (Leica, Allendale, NJ) and placed in a holding vial containing ACSF bubbled with 95% O_2 /5% CO_2 . They were allowed to rest at 34°C for 1h and then kept at room temperature until used for recording. The composition of the ACSF was (mM): NaCl 124, KCl 2.5, NaH_2PO_4 1.25, MgSO_4 2.0, CaCl_2 2.5, Dextrose 10 and NaHCO_3 26, pH 7.25.

Electrophysiological recordings

Slices were immersed in a recording chamber continuously superfused with ACSF at 32°C and bubbled with 95% O_2 /5% CO_2 . Whole-cell voltage-clamp recordings were obtained from the soma of dorsal raphe neurons visually identified by infrared filter differential interference contrast (IR-DIC) microscopy using an upright microscope fitted with a 40X water immersion objective (Nikon E600, Optical Apparatus, Ardmore, PA) and a CCD camera. Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) with a P97 Flaming/Brown horizontal puller (Sutter Instruments, Novato, CA). The electrodes were filled with an intracellular electrolyte containing (mM): K-gluconate 70, KCl 70, NaCl 2.0, HEPES 10, EGTA 4.0, MgATP 4.0 and Na_2GTP 0.3, Biocytin 0.1%, pH 7.3, osmolality 285–290 mOsm. Pipette resistance was 3.5–5.0 M Ω . Recordings were performed with a Multiclamp 700B amplifier (Molecular Devices Corporation, Sunnyvale, CA). Signal was low-pass filtered at 1 kHz and sampled at 10 kHz with a Digidata 1322 analog-digital interface (Molecular Devices Corporation, Sunnyvale, CA). Experiments were monitored and recorded on a computer with pClamp 9.2 software (Molecular Devices Corporation). Access resistance (R_a) was monitored throughout the experiment. Only cells with stable R_a during the whole experiment were analyzed. Cells were clamped at a holding potential of -70 mV. We isolated pharmacologically GABAergic action potential-independent miniature inhibitory postsynaptic currents (mIPSCs) by superfusing 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM) to block AMPA receptors and tetrodotoxin (TTX, 1 μM) to block activity-dependent synaptic release.

Recordings of mIPSCs were taken before and 9 min after the addition of Ovine CRF, a CRF receptor agonist (oCRF, 10 nM), to the superfusion buffer. Ovine corticotropin releasing factor is eight times more potent at the CRF one receptor (CRF1) than the CRF two receptor (CRF2). In previous studies it has been demonstrated that low concentrations of oCRF significantly inhibit DR neuronal activity and 5-HT release in forebrain, whereas higher concentrations produce only marginal or slightly excitatory effects (Price et al., 1993; Kirby et al., 2000). This is why 10 nM oCRF was chosen as the agonist for these studies. To demonstrate that recorded activity was purely resulting from fast GABAergic neurotransmission, bicuculline (Bic, 10 μ M) was added to some preparations and resulted in a complete elimination of the mIPSCs activity.

Solutions and drugs

DNQX was purchased from Tocris (Ellisville, MO). Ovine CRF was kindly provided by Dr Wylie Vale and Dr. Jean Rivier (The Salk Institute for Biological Studies, La Jolla, CA). All other chemicals and drugs were purchased from Sigma (St Louis, MO). Stock solution of DNQX was prepared in 100% dimethyl sulfoxide (DMSO) and then added to ACSF at the final concentration. All others drugs and buffers were prepared in water.

Data analysis

Traces were analyzed off-line with Minanalysis (Synaptosoft, Decatur, GA). Detection of mIPSCs used a threshold-based event detection algorithm and then they were edited manually to correct for inaccurate picks. The threshold was usually 5 pA. For each cell, the following parameters of mIPSCs were measured: frequency, inter-event intervals, average peak amplitude, average charge, average rise time as 10–90% of peak amplitude. To obtain slow and fast time constants of decay at least 200 events were averaged and the mIPSC fitted with two exponentials. The variation in baseline current was also measured.

Immunohistochemistry

After recording, slices were fixed in 4% paraformaldehyde prepared in 0.1 M phosphate buffer (PB, pH 7.4) overnight. Immunofluorescent histochemistry was then performed according to previously published methods (Beck et al., 2004; Kirby et al., 2008; Lemos et al., 2006). Fluorescent labeling of 5-HT-containing cells was performed using a mouse anti-tryptophan hydroxylase (TPH) antibody (Sigma, 1:500) and an AlexaFluor 488-donkey anti-mouse conjugate (Molecular Probes, Carlsbad, CA; 1:200). Recorded, biocytin-filled cells were identified using an AlexaFluor 633-streptavidin conjugate (Molecular Probes, 1:200). Slices were mounted in Prolong Gold Antifading Reagent (Molecular Probes) and visualized with an epifluorescence microscope (Leica DMR, Leica, Allendale, NJ). Cells identified with this procedure were further analyzed using a confocal microscope (Leica DMIRE2, Leica, Allendale, NJ) to confirm positive TPH labeling. Images were captured using Leica Confocal Software and contrast for each channel was adjusted using Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA).

Statistics

Data were exported to an Excel sheet (Microsoft Corp., Redmond, WA) for descriptive statistics and to Prism (Graphpad, LaJolla, CA) for statistical analysis. A repeated measures two-way ANOVA was used for most of the statistical analysis of naïve versus swim and baseline versus oCRF effects with a Bonferroni t-test to calculate differences if the ANOVA was significant. A Kolmogorov-Smirnov (K-S) goodness-of-fit test was used to compare frequency cumulative probability plots. Fitting of frequency histograms was performed with Origin 7.5 (OriginLab Corp., Northampton, MA).

Results

Neurons were randomly selected for recording in the ventromedial aspect of the dorsal raphe (vmDR) under IR-DIC visualization. Examination of double immunohistochemistry for biocytin and tryptophan hydroxylase (TPH) content was done after recording (Fig. 1). This allowed for the identification of recorded neurons, confirmed their location in vmDR and categorized them as 5-HT (TPH expressing, Figure 1A–C) or non-5-HT (TPH staining negative, Figure 1D–E). Only neurons located in the vmDR between rostro-caudal coordinates bregma -7.30 mm and -8.80 mm (Paxinos and Watson, 1998) and those showing an unambiguous pattern of TPH labeling were retained as 5-HT containing neurons for this study. Non-5-HT neurons were only accepted if other neurons in the same plane of focus showed staining for TPH.

Description of mIPSCs

GABA_A-mediated miniature postsynaptic currents (mIPSCs), resulting from the spontaneous release of GABA by presynaptic cells, were recorded at a holding potential of -70 mV in the presence of 20 μ M DNQX, an antagonist of AMPA receptors, and 1 μ M TTX, to remove action potential dependent IPSCs. Addition of 10 μ M bicuculline to the bath completely blocked recorded currents indicating that they depended specifically on the activation of GABA_A postsynaptic receptors.

A set of cells randomly chosen in each experimental condition ($n=12$) was used to assess the frequency distribution of mIPSCs interevent intervals, amplitudes, rise times 10–90% and charges (i.e., the total transfers of charge during mIPSCs reflected by the area delimited by mIPSCs traces). Distributions of interevent intervals (Fig. 2A) could be fitted by a single exponential (not shown) indicating that mIPSCs occur randomly. Frequency histograms of mIPSCs amplitudes, rise times 10–90% and charges were skewed towards positive values in every cell examined (Fig. 2B–D). For mIPSCs amplitudes, such variability could arise either because of synapses situated at electrotonically distant locations or because of different subtypes of inhibitory synapses having specific characteristics. The frequency distributions of mIPSCs amplitudes displayed multiple peaks and could be fit by multiple equally spaced Gaussian curves (fitting not shown) consistent with a quantal distribution of amplitudes (Ling and Benardo, 1999). Plots of mIPSCs rise time 10–90% as a function of amplitude did not show any negative correlations between these two parameters (data not shown). This indicates that dendritic filtering does not play an important role in shaping the distribution of mIPSCs parameters.

Presynaptic effects on mIPSCs frequency

To test the effects of swim stress on the modulation of GABAergic transmission by CRF in vmDR, we performed recordings of mIPSCs on slices taken 24h after a single 15 min swim stress session. At this time point it is known that rats exhibit enhanced immobility (For review: (Cryan and Holmes, 2005), and that release of 5-HT in forebrain areas is altered (Kirby and Lucki, 1998). Ovine CRF was applied to the superfusion buffer at a concentration of 10 nM consistent with previously described effects in vitro (Kirby et al., 2008). A summary of all of the measured characteristics of the mIPSC activity for the neurons recorded from 5-HT and non-5-HT neurons from naïve and swim stressed rats are presented in Table 1. An example of a trace of mIPSC activity recorded from a 5-HT neuron from a naïve and swim stressed rat are contained in Figure 3A and B. Panel 3C is a summary bar graph of the frequency of the mIPSCs recorded in the absence and presence of 10 nM oCRF ($N = 23$ for 5-HT Naïve, $N = 22$ for 5-HT Swim). The repeated measures two way ANOVA for 5-HT neurons revealed a significant interaction ($F=4.83$, $df = 1, 43$; Interaction $p = 0.0334$), indicating that the effect of oCRF was different depending on whether the rats were subjected to swim stress. There was no difference

in the frequency of the mIPSC activity in 5-HT cells recorded from naïve and swim stressed rats. However, in 5-HT cells from naïve rats, the average mIPSCs frequency was significantly increased by oCRF (t-test, $P < 0.01$; Fig. 3A–C). Swim stress suppressed the effect of oCRF (Fig. 3A–C). Furthermore, oCRF significantly shifted the distribution of interevent intervals to the left in naïve rats (K-S test, $P < 0.00001$; Fig. 3D) but not after swim stress (Fig. 3E). Ovine CRF, by increasing mIPSC frequency, increased inhibition of 5-HT vmDR neurons by a presynaptic mechanism; swim stress suppressed this mechanism.

The mIPSC activity of non-5-HT neurons recorded from naïve and swim stressed rats is summarized in Table 1 and traces of mIPSC activity from non-5-HT neurons are shown in Figure 4A and B. There was no difference in the frequency of mIPSC activity between non-5-HT containing neurons recorded from naïve and swim stressed rats. In addition, there was no change in the frequency of mIPSC activity by oCRF in neurons recorded from either naïve or swim stressed rats as shown in Figure 4C (ANOVA $F = 0.2612$, $df = 1, 45$). Ovine CRF did not have any effect on the distribution of interevent intervals (Figure 4D, E). The presynaptic increase in mIPSC frequency by oCRF and the suppression of this action by swim stress is thus specific to 5-HT neurons.

Effects on mIPSCs amplitude, charge and kinetics

Post-synaptic modifications of GABAergic synaptic transmission were also measured (Table 1). Figure 5 contains representative averaged mIPSCs recorded from 5-HT neurons in naïve and swim stressed rats as well as summary bar graphs. Repeated measures two way ANOVA revealed no interaction but there was a significant effect of drug on amplitude ($F = 32.4$, $df = 1, 43$, $p < 0.0001$) and charge ($F = 67.46$, $df = 1, 43$, $p < 0.0001$). Swim stress did not affect the baseline amplitude or charge of mIPSCs (Fig. 5A–C). In 5-HT cells from control rats, oCRF produced a significant increase in mIPSCs amplitude (follow-up paired t-test, $p < 0.01$; Fig. 5A,B) and in the charge of the mIPSCs (paired t-test, $p < 0.001$; Fig. 5C). In 5-HT neurons recorded from swim stressed rats, there was a similar increase in mIPSCs amplitude (paired t-test, $p < 0.001$; Fig. 5A) and charge (paired t-test, $p < 0.001$; Fig. 5C). Ovine CRF and swim stress had no effect on mIPSCs kinetics as reflected by rise times and slow and fast time constants of decay (Table 1). This indicates that the post-synaptic increase in the GABA_A receptor mediated response on 5-HT neurons is increased by oCRF and remains unaffected by swim stress.

The results for the effect of swim stress on mIPSC characteristics recorded from non-5-HT neurons are summarized in Table 1 and Figure 6. In contrast with 5-HT cells, swim stress significantly increased mIPSCs amplitude (ANOVA, $F = 3.83$, $df = 1, 44$, $p = 0.048$, Fig. 6A, B) and charge (ANOVA $F = 7.91$, $df = 1, 44$, $p = 0.0073$; Fig. 6C). In non-5-HT cells, oCRF also induced a significant increase in mIPSCs amplitude as well (Repeated measures ANOVA, $F = 41.59$, $df = 1, 44$, $t\text{-test} < 0.001$) as a significant increase in mIPSCs charge (ANOVA $F = 61.12$, $df = 1, 44$, $p < 0.0001$; Fig. 6C), and the effect was present in neurons recorded from naïve (t-test $p < 0.001$) as well as swim stressed rats (follow up t-test $p < 0.001$; Fig. 6C). The rise time of non-5-HT neurons from swim stressed rats was significantly slower than that from naïve controls (ANOVA for naïve versus swim stressed $F = 9.73$, $df = 1, 45$, $p = 0.0032$, Table 1). No other changes were observed in fast and slow time constants of decay (Table 1). Thus, in non-5-HT cells, swim stress produced an increase in mIPSC amplitude and charge, but the modulation by oCRF was not altered.

Baseline current

In 5-HT cells, the ANOVA revealed a significant effect of oCRF ($F = 18.73$, $df = 1, 43$, $p < 0.0001$), but follow-up t-tests indicated that the effect was only significant in neurons from naïve (t-test 3.9 , $p < 0.001$) and not swim stressed neurons ($t = 2.2$, $p > 0.05$) rats (Fig. 5D). In non-5-HT

neurons, there was a significant decrease in the baseline current recorded from naïve and swim stressed rats ($F=6.064$ df 1,44 $p=0.018$), indicating that the neurons had a more hyperpolarized resting membrane potential. In addition the inward current elicited by oCRF was also significant as a main effect ($F=18.86$ df 1,44 $p<0.0001$) but follow-up t-tests revealed that the magnitude of the increase was only significant in non-5-HT neurons from naïve ($t=4.56$ $p<0.001$), but not swim stressed rats ($t=1.65$ $p>0.05$; Fig. 6D). In conclusion, oCRF induced an inward current that was only significant in neurons recorded from naïve rats, and was suppressed by swim stress in both 5-HT and non-5-HT containing neurons.

Discussion

The present data document effects of swim stress on GABAergic synaptic activity and its regulation by CRF receptor activation recorded from both 5-HT and non-5-HT neurons in the rat vmDR. The primary measurement used in this study was the GABAergic mIPSC. The mIPSC is mediated by the activation of the GABA_A receptor by GABA released from presynaptic terminals. The measurement was of baseline, spontaneous release, not release due to action potentials, since TTX was in the buffer. The effect of swim stress on baseline mIPSC characteristics was exclusive to the non5-HT containing neurons and did not alter the baseline characteristics of GABAergic activity in 5-HT containing neurons. There was no change in frequency by swim stress in any group. Frequency is a measurement of the probability of release of GABA from the presynaptic terminal. Swim stress increased mIPSC amplitude and charge, decreased rise time and decreased baseline holding current in non-5-HT neurons. Amplitude measures the postsynaptic receptor activation and changes in amplitude and can be the result of a change in frequency or change in the number of receptors in the postsynaptic membrane. The measurement of rise time and decay time usually indicates a change in the conformation of the GABA_A receptor due to different subunits making up the pentameric structure of the GABA_A receptor or differences in the phosphorylation state of the subunits (Mody and Pearce, 2004). In terms of oCRF effects, in 5-HT cells, swim stress suppressed oCRF activation of GABAergic mIPSC frequency; in both 5-HT and non5-HT containing neurons swim stress prevented the increase in inward current induced by oCRF.

Direct effects of swim stress on GABAergic neurotransmission

The effects of swim stress on GABAergic neurotransmission was cell-type specific, with no alterations in 5-HT neurons but a significant change in non-5-HT neurons. The effect of swim stress was to increase GABAergic inhibition by making the cell more hyperpolarized (less negative holding current), increasing mIPSC amplitude, rise time and charge selectively in non-5-HT cells. These changes cannot be attributed to an increase in frequency induced by oCRF because in the non-5-HT neurons oCRF did not induce an increase in frequency. This increase in the amplitude and overall charge of the mIPSC increases the degree of inhibition of the non-5-HT neurons. If the non-5-HT neurons are GABAergic in nature, more inhibition of the GABAergic neurons would lead to less release of GABA and a greater excitation of the 5-HT neurons. Postsynaptic changes in mIPSC may be due to alterations in the subunit conformation of the GABA_A receptor. Changes in GABAergic inhibition by stress have been described previously in other brain areas. In paraventricular nucleus of hypothalamus, chronic stress modified the expression of GABA_A receptor subunits (Verkuyl et al., 2004). A similar finding was described in granule cells of dentate gyrus of hippocampus where neonatal stress resulted in long term changes in GABA_A receptor subunits expression (Hsu et al., 2003). These changes altered the pharmacology and kinetics of GABA_A receptors and paralleled an increase in active behavior during swim stress (Hsu et al., 2003).

Swim stress effects on CRF neuromodulation of GABAergic transmission

The effects of oCRF on GABAergic neurotransmission involve both pre- and postsynaptic sites of action. Our laboratory recently published the characterization of oCRF receptor mediated changes in 5-HT and non-5-HT neurons in the DR as measured using whole cell recording techniques. Activation of CRF1 induces a presynaptic increase in GABA mIPSC frequency, i.e., probability of release. Both CRF1 and CRF2 elicit postsynaptic increases in GABA_A receptor mediated mIPSC amplitude, and CRF2 elicits an increase in inward current in vmDR 5-HT neurons. In non-5-HT neurons CRF1 receptors increase inward current (Kirby et al., 2008). Using the results from Kirby et al. study as the basis for our knowledge of the nature of the oCRF receptor mediated responses, the following conclusions can be made regarding data from the swim stress experiments.

Swim stress selectively eliminated the CRF1 mediated presynaptic increase in GABA release onto 5-HT containing neurons, i.e., the increase in mIPSC frequency. The most probable interpretation of our observations on presynaptic CRF1 effects on GABAergic synaptic activity in stressed animals is that swim stress decreased the number or efficacy of CRF1 receptors expressed at the surface of GABAergic synaptic terminals innervating 5-HT neurons. Recent evidence demonstrates that swim stress increases the internalization of CRF1 in the locus coeruleus neurons (Reyes et al., 2008). In addition stressors redistributed CRF1 and CRF2 receptors in the DR, such that CRF1 receptors internalize and CRF2 receptors are recruited to the membrane (Waselus et al., 2009). Therefore it is highly likely that the CRF1 receptors are internalized in the presynaptic GABA terminals targeting 5-HT neurons. The effect of a decrease in CRF1 mediated increase in mIPSC frequency, would be that the 5-HT neurons are under less inhibitory control.

These results are important because they implicate selective GABAergic innervation of 5-HT and non-5-HT neurons of the raphe. The alteration in the CRF1 receptor mediated response was only seen on those nerve terminals innervating the 5-HT neurons. Under baseline conditions, GABA exerts inhibition on DR 5-HT neuron's discharge that is dependent upon the state of the animal (Tao and Auerbach, 2003); (Gervasoni et al., 2000); (Levine and Jacobs, 1992). This inhibitory input emerges from both local GABAergic microcircuits (Celada et al., 2001; Jolas and Aghajanian, 1997; Liu et al., 2000) and by long-range afferents (Gervasoni et al., 2000). Recent evidence has emphasized the excitatory long-loop feedback from the prefrontal cortex that primarily innervates GABA neurons (Varga et al., 2001); (Celada et al., 2001). Alterations in GABAergic inhibition of 5-HT neurons in the DR induces significant changes in the pacemaker activity of 5-HT neurons and in the output of DR (Celada et al., 2001; Tao and Auerbach, 2000; Zhao et al., 2007).

The suppression of the CRF effect on GABAergic inhibition 24h after an initial swim stress session reported in our study parallels the suppression of the CRF effect (icv) on 5-HT release in lateral septum by swim stress (Price et al., 2002). This elimination of the icv effects of CRF on 5-HT release was blocked by the administration of a CRF antagonist between the initial swim session and the test session 24 hours later. Therefore, the probable desensitization of the CRF1 presynaptic receptor on GABAergic presynaptic terminals that occurs following swim stress, decreasing the release of GABA, is part of the cellular mechanism underlying the behavioral effect of immobility, i.e., an decrease in active behaviors and an increase in passive behaviors (Price et al., 2002).

In addition, the CRF mediated increase in inward current was blocked following swim stress in both 5-HT and non5-HT containing neurons. In our previous characterization of oCRF effects, the receptor mediating the increase in inward current on 5-HT containing neurons was CRF2, whereas the receptor on non-5-HT containing neurons was CRF1 (Kirby et al., 2008). An increase in inward current leads to a depolarization of the neuron and makes it more likely

to fire an action potential. Therefore, the fact that both the CRF1 and CRF2 responses were blocked indicates that both the 5-HT and non-5-HT containing neurons were less excitable, less likely to respond to incoming excitatory input. If the non-5-HT neurons are GABAergic in nature the block of the depolarizing effect of oCRF, adds to the swim stress disinhibitory effect on 5-HT neurons, i.e., the block of the CRF1 increased frequency of GABA release, leading to greater excitation of the 5-HT neuron. The stressed induced elimination of the CRF2 receptor mediated depolarization of 5-HT neurons that normally requires a greater oCRF concentration, prevents direct excitation of the 5-HT neurons by CRF.

CRF effects on GABAergic neurotransmission not affected by stress

There were several oCRF mediated responses that were not altered following swim stress. The CRF1 and CRF2 effect on mIPSC amplitude (Kirby et al., 2008) was maintained in both 5-HT and non-5-HT neurons as was the increase in charge (basically the area of the IPSC). This oCRF effect was to increase inhibition by increasing the amplitude and charge of the mIPSC. However, in the non-5-HT neurons, swim stress induced an additional increase in basal mIPSC amplitude and charge in concert with the oCRF induced increase in amplitude and charge. In combination with the block of the oCRF inward current, the net result is an even greater inhibition of the non-5-HT neurons than the 5-HT neurons. If the non-5-HT neurons are GABAergic, the net result would be an even greater disinhibition of the 5-HT neuron, i.e., less inhibition of the 5-HT neurons.

Physiological consequences of Swim Stress

To summarize regarding the effects of swim stress on 5-HT and non-5-HT neurons from the raphe, we would like to utilize the results from our study as well as the results previously published from our laboratory (Kirby et al., 2007).

The overwhelming evidence from both studies indicates that swim stress leads to an inhibition in non-5-HT neuron excitability and an increase in 5-HT neuron excitability in the vmDR. Direct effects on membrane characteristics included a decrease in resistance, decrease in action potential threshold and duration. Excitatory glutamatergic pre-synaptic activity was greatly enhanced (Kirby et al., 2007). In this study the increase in the frequency of GABAergic synaptic activity by CRF1 activation was eliminated. In non-5-HT neurons (presumably GABAergic), the neurons were hyperpolarized, the basal mIPSC amplitude and charge were increased by swim stress, CRF1 and CRF2 mediated increase in mIPSC amplitude remained while CRF1 increase in inward current was blocked, all effects that lead to greater inhibition of the non-5-HT neurons and less inhibitory drive on the 5-HT neuron. These findings highlight the concept of differential stressor responsivity in the different populations of DR neurons. In addition we propose that this increased excitability and resultant increase in 5-HT release in select forebrain regions is part of the cellular mechanism mediating the resultant swim stress induced immobility.

Conclusion

Based on our data from the two studies, we conclude that the control of GABAergic synaptic activity, glutamatergic excitatory synaptic activity and cell excitability by CRF in DR are part of the mechanisms for the behavioral adaptations to swim stress. Swim stress selectively modulates neurochemical systems in the DR through several independent pathways leading to increased 5-HT neuron excitability and increased release of 5-HT in forebrain regions resulting in immobility. The specific targeting of these pharmacologically distinct pathways could open promising venues in the treatment of stress related conditions.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Table 1

Swim stress and oCRF effects on mIPSCs recorded from 5-HT and non-5-HT neurons in rat vmDR.

Parameters	5HT Naïve (N=23)		5HT Swim (N= 22)	
	mIPSC	CRF	mIPSC	CRF
Frequency, Hz	4.89 ± 0.49	5.60 ± 0.59 ^a	4.97 ± 0.74	4.99 ± 0.74
Peak amplitude, pA	22.80 ± 1.26	24.75 ± 1.39 ^b	25.29 ± 1.24	27.38 ± 1.3 ^b
Rise time 10–90%, ms	1.03 ± 0.05	1.08 ± 0.05	1.15 ± 0.05	1.17 ± 0.05
Tau ^{decay} fast, ms	3.90 ± 0.17	4.49 ± 0.31	4.23 ± 0.21	4.31 ± 0.22
Tau ^{decay} slow, ms	24.89 ± 6.68	36.3 ± 12.27	33.90 ± 6.69	33.53 ± 10.13
Average area, fC	94.54 ± 5.54	108.62 ± 6.67 ^b	104.26 ± 4.79	119.65 ± 5.60 ^b
Baseline current, pA	−18.88 ± 3.80	−28.61 ± 4.00 ^c	−10.40 ± 4.59	−15.84 ± 4.03

Parameters	Non 5HT Naïve (N=24)		Non 5HT Swim (N=23)	
	mIPSC	CRF	mIPSC	CRF
Frequency, Hz	4.44 ± 0.63	4.55 ± 0.59	4.33 ± 0.70	4.60 ± 0.66
Peak amplitude, pA	20.00 ± 1.63	21.93 ± 1.77 ^d	24.45 ± 1.48 ^d	26.36 ± 1.57 ^d
Rise time 10–90%, ms	1.47 ± 0.07	1.48 ± 0.07	1.19 ± 0.06 ^e	1.21 ± 0.05
Tau ^{decay} fast, ms	4.37 ± 0.35	4.72 ± 0.27	4.38 ± 0.27	4.63 ± 0.33
Tau ^{decay} slow, ms	28.08 ± 6.24	35.30 ± 7.56	26.30 ± 5.56	29.67 ± 7.42
Average area, fC	86.06 ± 4.21	100.25 ± 5.70 ^d	107.86 ± 6.31 ^d	123.16 ± 6.85 ^d
Baseline current, pA	−25.55 ± 3.82	−32.54 ± 3.29 ^e	−15.15 ± 3.62 ^e	−18.95 ± 3.95

Values are mean ± SEM,

^a Repeated measures ANOVA, F=4.83 df=1,43; Interaction p = 0.0334, follow up t-test * p<0.01;^b Repeated measures ANOVA, drug effect significant for amplitude F=32.4, df= 1,43, p < 0.0001 and charge F=67.46 df=1,43 p<0.0001 for cells recorded from both the Naïve and Swim stressed rats.^c Repeated measures ANOVA drug effect significant F=18.73 df = 1,43 p<0.0001, but follow up t-test revealed significant increase in inward current only for naïve group, t-test 3.9 p <0.001 vs swim stressed group, t=2.2 p > 0.05.^d ANOVA revealed a main effect of swim on baseline amplitude, F= 3.83, df=1,44, p = 0.05 and charge, F=7.91 df 1,44 p=0.0073. Significant drug effect on amplitude F= 41.59 df=1,44 p<0.001 and charge F=61.12 df =1,44 p<0.0001 for cells recorded from both naïve and swim stressed non-5-HT neurons.^e Significant main effect of swim on rise time, F=9.73 df 1,45 p=0.0032.^f ANOVA revealed main effect of swim F=6.064 df 1,44 p=0.018. In addition significant main effect of oCRF F=18.86 df=1,44 p<0.0001, but follow-up t-test was significant only for naïve group, t=4.56 p<0.001, but not for neurons from swim stressed group, t=1.65 p>0.05.