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The immobility produced by intermittent swim stress is not mediated by serotonin

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Abstract

Exposure to uncontrollable stressors such as intermittent swim stress (ISS) produces a behavioral syndrome that resembles behavioral depression including immobility in a Forced Swim Test (FST) and escape learning deficits. The results of previous studies suggest that stress causes a temporary sensitization of the brain serotonin (5-HT) system that is necessary and sufficient for producing behavioral depression. If this hypothesis is true in the ISS paradigm, then enhancing or inhibiting 5-HT transmission during stress should exacerbate or block the development of behavioral depression, respectively. The selective 5-HT uptake inhibitor fluoxetine (FLX) was administered prior to ISS or confinement; 24 h later the FST was used to detect behavioral immobility. ISS, but not FLX, significantly increased immobility in the FST. The purported 5-HT uptake enhancer tianeptine (TPT) was administered in place of FLX. Again ISS increased immobility in the FST, but TPT had no effect. These results suggested that 5-HT is not a critical mediator of ISS induced behavioral depression. However, some authors have raised concern that TPT does not act directly on 5-HT. Therefore, the 5-HT synthesis inhibitor, para-chlorophenylaline (PCPA) was administered to deplete central 5-HT before stress. PCPA did not alter immobility in the FST. Finally, a sub-chronic regimen of FLX given after ISS, but before the FST, was without effect on reversing the ISS-induced immobility. Taken together, these experiments indicate that ISS produces a significant behavioral depression manifested as increased immobility but offer no support of the hypothesis that 5-HT is a critical mediator of these effects.

Keywords: Rat; Behavioral depression; SSRI; FST; Learned helplessness

1. Introduction

Animal models of stress and depression have proven to be invaluable tools for investigating the relationship between stress and emotional behavior. One well characterized paradigm is the learned helplessness (Maier and Seligman, 1976) procedure. In the learned helplessness model exposure to uncontrollable electric shocks enhances post-shock freezing and interferes with escape learning in a two-way shuttle-box (Maier, 1990). Because uncontrollable shock leads to other behaviors that resemble behavioral depression, the learned helplessness paradigm has been critically evaluated and acknowledged as a useful model for studying depression (Henn et al., 1993; Willner, 1984) and drug discovery. Therapies that are effective in humans for alleviating depression including exercise (Greenwood et al., 2003) electroconvulsive therapy, tricyclic, monoamine oxidase inhibitor and selective serotonin reuptake inhibitor (SSRI) antidepressants are capable of preventing or reversing the learned helplessness effect (Sherman and Petty, 1980; Sherman et al., 1982; Zazpe et al., 2007).

The predominate hypothesis derived from the learned helplessness model is that intermittent and uncontrollable stressors cause a transient burst of activity in the 5-HT system, specifically in the neurons of the Dorsal Raphé Nucleus (DRN), that is necessary for the development of behavioral depression (e. g., (Grahn et al., 2002)). This hypothesis is based on experiments that directly manipulate the DRN, record 5-HT activity, or observe behavior following acute administration of drugs acting on 5-HT. Edwards et al. (1986) reported that depletion of 5-HT with a tryptophan hydroxylase inhibitor (the rate limiting enzyme for 5-HT synthesis) prevented the escape

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deficit produced by uncontrollable shock. Later, Maier et al. (1993) demonstrated that electrolytic lesions to the DRN completely eliminated the effects of uncontrollable intermittent shock on post-shock freezing and later shuttle escape performance. Several studies have followed using direct microinjections into the DRN of drugs that reduce the activity of 5-HT neurons to block the effects of intermittent shock on escape learning (Grahn et al., 2000, 2002; Hammack et al., 2002; Maier et al., 1994; Maier, 1995). Further support for the 5-HT hypothesis is gained from studies that quantified 5-HT activity during or immediately after stress. Intermittent shock stress increased 5-HT turnover in the DRN (Maswood et al., 1998), mPFC (Bland et al., 2004; Jordan et al., 1994), amygdala (Amat et al., 1998a) and ventral hippocampus (Amat et al., 1998b). Furthermore, intermittent shock (Grahn et al., 1999; Greenwood et al., 2003; Takase et al., 2004, 2005) increased staining of c-Fos in 5-HT immunoreactive neurons in the DRN. c-Fos is an immediate early gene that is an index of a neuron's activity (Kovacs, 1998).

Drugs that increase the activity of 5-HT produce some behavioral and physiological consequences that are similar to the learned helplessness paradigm. Prior to stress exposure, administration of l-tryptophan, a 5-HT precursor, reduced the number of shocks (40 compared to 80) that were needed to induce learned helplessness (Brown et al., 1982). Administration of the selective 5-HT reuptake inhibitor, fluoxetine (FLX), disrupted shuttlebox performance when administered 1 h before testing (Lucki and Nobler, 1985). Acute FLX produces increases in 5-HT turnover in some of the same anatomical structures as inescapable shock including the DRN, frontal cortex (Rutter et al., 1995), mPFC (Jordan et al., 1994), striatum and hippocampus (Kreiss and Lucki, 1995). Therefore, stress or pharmacological alterations of 5-HT tone can induce a behavioral state that resembles behavioral depression. However, the generality of these results to other stress regimens is unknown.

Brown et al. (2001) developed an intermittent swim stress (ISS) procedure that was adapted from the learned helplessness paradigm. ISS was designed, specifically, to investigate the generality of findings about the controllability of stressors gleaned from the electric shock model. In the procedure, electric tail-shocks were replaced with intermittent exposures to cold water. The ISS paradigm shares an important characteristic with the learned helplessness model in that animals will readily learn to escape the swim stress. Therefore, the model allows for the study of the controllability of stress rather than stress per se. We have recently reported that ISS induces a behavioral state that includes enhanced immobility in the Forced Swim Test (FST) and poor escape learning in an instrumental swim-escape task (Christianson and Drugan, 2005). In parallel with the electric shock model, the effects of ISS on forced swimming are dependent on the controllability of prior stress (Drugan et al., 2005). In the current set of experiments the ISS paradigm was utilized to explore the generality of the hypothesis that 5-HT is a critical mediator of uncontrollable stress-induced behaviors used to model depression.

The following experiments tested the hypothesis that high levels of 5-HT during ISS would exacerbate the development of immobility in the FST or that very low levels of 5-HT during ISS would block that effect. In Experiment 1, FLX was administered before ISS to systematically increase the post-synaptic levels of 5-HT during stress. It was predicted that 24 h later, rats treated with stress and FLX would spend more time immobile in the FST than rats treated with stress, no stress, or FLX alone. In Experiment 2, tianeptine (TPT), a drug that was reported to enhance 5-HT uptake (Fattaccini et al., 1990; Mennini et al., 1987; Whitton et al., 1991), was administered to reduce the levels of 5-HT in the synapse during stress. It was predicted that 24 h later, rats treated with stress and TPT would spend less time immobile than rats treated with stress alone. Some investigators have indicated that TPT's pharmacological effects are not specific to 5-HT and act directly on glutamatergic systems (Malagie et al., 2000; McEwen and Olie, 2005). Therefore Experiment 3 utilized a 5-HT synthesis inhibitor, para-chlorophenylalanine (PCPA), to deplete brain 5-HT before exposure to ISS. If 5-HT is necessary for ISS effects on immobility, then depletion of 5-HT by PCPA would prevent these effects. A separate set of studies from our laboratory (Drugan et al., 2005) indicated that ISS produced hypothermia that could recruit non-serotonergic systems and lead to immobility. Pre- and post-stress colonic temperature was recorded in Experiment 3 to determine the extent of hypothermia produced by 100 trials of ISS. In Experiment 4, a sub-chronic therapeutic regimen of FLX was administered after ISS in a final test to implicate 5-HT in the immobility induced by ISS. At the conclusion of each experiment all rats were given a 10 minute open field test to determine stress and drug effects on general locomotor activity.

2. Methods

2.1. Subjects

All experiments used male Sprague–Dawley rats (Charles River Labs, Willmington, MA) weighing 230–300 g on the first day of treatment. Upon arrival rats were group housed in polyethylene tub cages (4 rats per cage); on the first day of treatment rats were transferred to single cages. Rats had free access to standard rat chow and tap water at all times in the vivarium. The vivarium maintained a 12 hour light/dark cycle; all behavioral procedures were conducted in the first 6 h of the light cycle. Rats were handled in accordance with Public Health Services "Guidelines for humane treatment of laboratory animals." In addition, the procedures were reviewed and approved by the University of New Hampshire Institutional Animal Care and Use Committee (IACUC).

2.2. Apparatus

ISS was administered in acrylic cylinders (21×42 cm – dia. X H) with a 1/4-inch galvanized wire mesh floor suspended over a tank of 15 °C water. On a swim trial, the cylinders lowered into water to a depth of 20 cm. Space heaters blew warm air (≅36 °C) into the cylinders to minimize body temperature loss during inter-trial-intervals. The swim stress apparatus was controlled by a computer with Med-PC hardware and software (Med-Associates, Georgia, VT). At a separate time, confined rats were placed in the same cylinders but were

suspended over an empty tank. The confined rats were subjected to the same handling, noise and movement as the stressed rats but never entered the water. For a picture of the ISS apparatus, see Brown et al. (2001).

In a different room and in a different apparatus, 5-minute FSTs were conducted in clear plastic cylinders (20 cm dia.) filled with 29 cm of 23 °C (±1 °C) water (similar to those previously described for rats, (Detke et al., 1995; Drugan et al., 1989; Porsolt et al., 1977)). A video camera recorded a side view of the test for later analysis. Open field tests were conducted in an open-top square plywood chamber (120×120×25 cm: L×W×H) painted with flat black enamel. Open field tests were video-taped using a wide-angel camera located directly over the center of the arena. Path length was computed with HVS Field tracking hardware and software (Model VP200 Software Version 10/96; Hampton, UK).

2.3. Drugs

Fluoxetine hydrochloride (FLX) was obtained from Spectrum Chemicals (Gardena, CA) and prepared in 0.9% saline. In Experiment 1 FLX was administered intraperitonealy (i.p.) at 5, 10 or 20 mg FLX per kg body weight. In Experiment 4 FLX was administered subcutaneously (s.c) at 20 mg/kg a dose that reliably reduces immobility in the modified FST (Detke et al., 1995). Tianeptine sodium salt (TPT) was obtained from Sigma-Aldrich (St. Louis, MO), prepared in 0.9% saline and administered i.p. at 5 and 10 mg TPT per kg body weight. This dose range was selected because 10 mg/kg TPT was the largest dose found in the literature and blocked restraint stress induced increase in plasma corticosterone (CORT) (Delbende et al., 1991). DL-p-Chlorophenylalanine methyl ester hydrochloride (PCPA) was obtained from Research Organics (Cleveland, OH), prepared in 0.9% Saline and administered i.p. at 200 mg/kg PCPA per kg body weight. This dose was selected based on the work of others that reported 80–90% depletion of 5-HT using similar methods (Edwards et al., 1986; Plaznik et al., 1988; Prinssen et al., 2002). Control subjects received equal volume of 0.9% saline as vehicle.

2.4. Procedure

2.4.1. Experiment 1

Rats were assigned to one of 8 treatment groups in a 2 (ISS vs. Confinement) X 4 (0 mg/kg, 5, mg/kg, 10 mg/kg, or 20 mg/kg FLX) completely randomized factorial design. Rats were allowed to acclimate to the vivarium for at least 7 days before beginning experimentation. On the first day of the experiment rats were weighed and given an i.p. injection of FLX. Forty-five minutes after injection (at a time when FLX induced CORT is peaking, (Duncan et al., 1998)) rats were exposed to either 80, 5-sec trials of ISS (15 °C) or equal time in confinement. We have reported that 80 trials are the minimum for inducting immobility and escape learning deficits (Christianson and Drugan, 2005) therefore, 80 trials were used to produce a minimal effect of ISS to prevent a ceiling effect that could mask effects of FLX. After stress, rats were towel-dried and warmed under heat lamps for 30 min or until rats resumed grooming.

Twenty-four hours after the beginning of stress, rats were given a 5-min FST. The tests were conducted in a similar fashion to that first described by Porsolt et al. (1977) but with deeper water to eliminate tail standing (Detke et al., 1995; Drugan et al., 1989) and a modified scoring technique that quantified immobile, swimming, and climbing behaviors. The modified scoring technique employs a time-sampling procedure in which every 5 s a rat is scored by an observer, blind to treatment, as immobile, the rat only makes the necessary movements to keep its head above water; swimming, the rat is active in the swim tank but is not struggling or climbing on the walls; or climbing, the rat is struggling against the wall of the tank and its forepaws are breaking the surface of the water (Detke et al., 1995). This modified technique was chosen because it reliably detects the effects of serotonergic compounds while other FST quantification methods do not (Cryan et al., 2002a, 2005; Detke et al., 1995). Each FST was videotaped from the side so that a second observer could later analyze behavior. Immobility, swimming, climbing, and diving scores were recorded as the mean of each observer's score. Data from 10 randomly selected FSTs were analyzed with Pearson's r correlation to determine inter-rater-reliability. At the end of the FST rats were towel-dried and placed in an open field and tracked via overhead video camera for 10 min. The open field test was conducted to confirm that active or inactive behaviors in the FST could not be attributed to a general change in spontaneous locomotor activity caused by either prior stress or drug treatment. Distance traveled (path length) during the open field test was used as the dependent measure and was quantified with HVS Maze video tracking software.

2.4.2. Experiment 2

Rats were assigned to one of 6 treatment groups in a 2 (ISS vs. Confinement) X 3 (0 mg/kg, 5 mg/kg, 10 mg/kg TPT) completely randomized factorial design. Rats were allowed to acclimate to the vivarium for at least 7 days before beginning experimentation. On the first day of the experiment rats were weighed and given an i.p. injection of TPT prepared in 0.9% saline. Forty-five minutes after injection, rats were exposed to either 100, 5-sec trials of ISS (15 °C) or equal time in confinement. We have reported that 100 trials induced a more pronounced swim escape deficit than 80 trials (Christianson and Drugan, 2005) therefore, 100 trials were used here to generate the greatest immobility and provide a greater behavioral range to detect effects of TPT. After stress rats were towel-dried and warmed under heat lamps for 30 min or until rats resumed grooming. Twenty-four hours after the beginning of stress, rats were tested for immobility in the FST as described above. After the FST, all rats were immediately transferred to the open field and videotaped for 10 min. FST and Open Field videos were analyzed in the same method as Experiment 1.

2.4.3. Experiment 3

Rats were assigned to one of 4 treatment groups in a 2 (ISS vs. Confinement) X 2 (0 or 200 mg/kg PCPA) completely randomized factorial design. Rats were allowed to acclimate to the vivarium for at least 7 days before beginning experimentation. On the first three days of the experiment rats were weighed and given i.p. injections of PCPA or saline. On the fourth day, each rat was wrapped in a cotton towel and a temperature probe

coated in petroleum jelly was inserted 2.2 mm into the rectum for approximately 15 sec to obtain pre-stress colonic temperature. Then, rats were exposed to either 100, 5-sec trials of ISS or equal time in confinement. Upon termination of the stress session, post-stress colonic temperature was obtained using the method described above. Immediately, rats were towel-dried and placed under heat lamps for 30 min or until rats resumed grooming. Twenty-four hours after the beginning of stress, rats were tested in a 5 minute FST as described in Experiment 2. After the FST, all rats were immediately transferred to the open field for 10 min. FST and open field tests were analyzed in the same fashion as Experiments 1 and 2.

2.4.4. Experiment 4

Rats were randomly assigned to one of 4 groups in a 2 (ISS or Confined) X 2 (20 mg/kg FLX or Saline) completely randomized factorial design. Rats were allowed 7 days to acclimate to the vivarium. On the first day of the experiment rats were weighed and exposed to either 100, 5-sec trials ISS or equal time in confinement. Immediately after ISS or confinement rats received the first of 3 s.c. injections of FLX or saline. The second and third injections were made 5 and 1 h before the FST, respectively. This sub-chronic regimen has been used to detect antidepressant activity of SSRIs (Cryan et al., 2005; Detke et al., 1995; Page et al., 1999). All rats received a 5 minute FST 24 h after ISS or confinement. After the FST, all rats were immediately transferred to the open field for 10 min. FST and open field tests were analyzed in the same fashion as Experiments 1, 2 and 3.

2.5. Statistical analyses

Immobility, swimming, climbing, latency to 3 immobile blocks, open field pathlength, and colonic temperature were analyzed with separate analyses of variance (ANOVA) with stress and drug treated as between-groups factors. Main effects and interactions were deemed significant if $p<0.05$ and were further explored with Bonferoni post hoc tests.

3. Results

3.1. Experiment 1

3.1.1. Immobility

Mean counts of immobility are presented in Fig. 1, panel A. Inter-rater-reliability for immobility was high, $r(8)=0.973$, $p<0.001$. A 2 (stress or confinement) X 4 (0, 5, 10, or 20 mg/kg FLX) ANOVA identified a significant main effect of stress, $F(1, 88)=9.614$, $p<0.01$, indicating that ISS increased immobility despite FLX dose. Although there appears to be a trend that immobility increased with FLX dose, the main effect of FLX

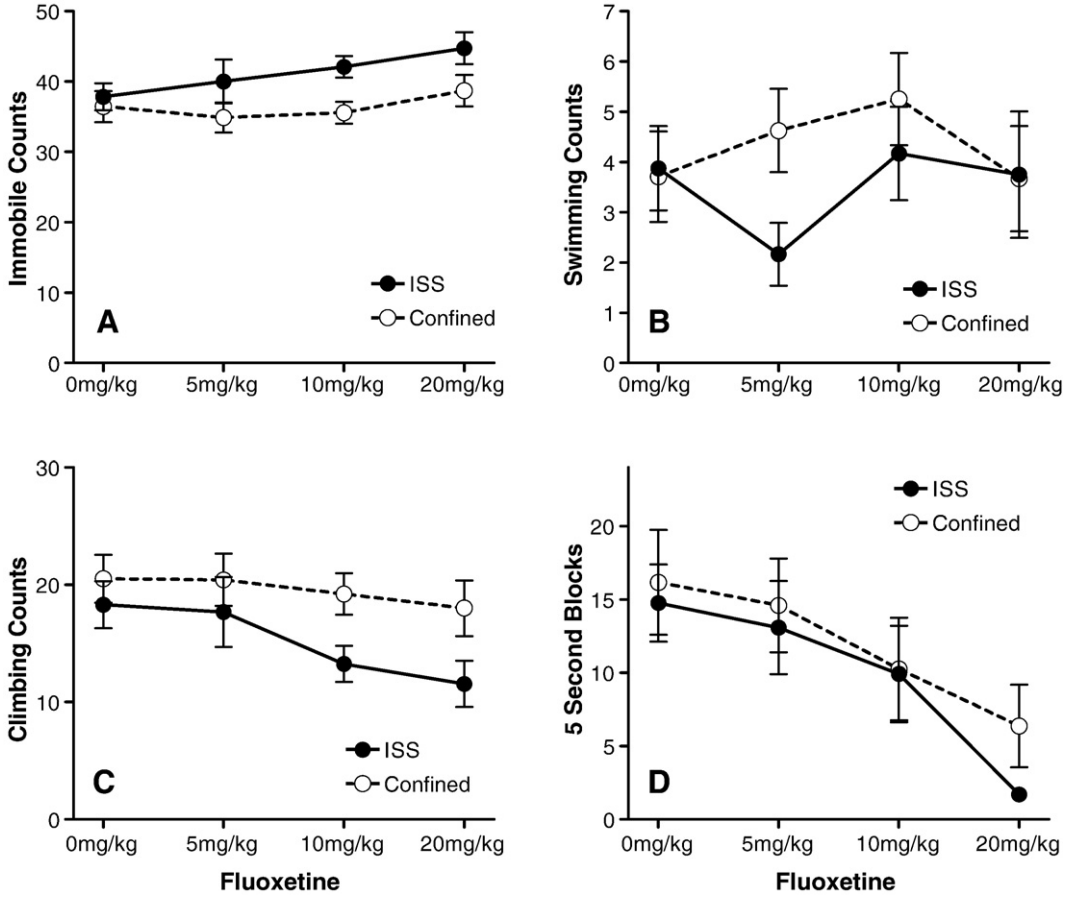

Fig. 1. Mean (±SEM) scores for Immobility (A), Swimming (B) Climbing (C) and Latency to 15 s Immobile (D) in the 5 minute FST after ISS and FLX treatment, $n=12$/group. ISS significantly increased Immobility and decreased Climbing, $ps<0.05$. FLX significantly reduced latency to 15 s of continuous immobility, $p<0.05$.

did not reach significance, $F(3, 88)=1.854$, $p=0.14$. No stress X FLX interaction was found, $F(3, 88)=0.575$, $p=0.634$.

3.1.2. Swimming

Mean counts of swimming are presented in Fig. 1, panel B. Inter-rater-reliability for swimming was high, $r(8)=0.909$, $p<0.001$. No significant effects were found for either stress, $F(1, 88)=1.551$, $p=0.216$, or FLX, $F(3, 88)=0.730$, $p=0.537$, or the interaction, $F(3, 88)=0.867$, $p=0.462$.

3.1.3. Climbing

Mean counts of climbing are presented in Fig. 1, panel C. Inter-rater-reliability for climbing was high, $r(8)=0.990$, $p<0.001$. Stress significantly reduced climbing, $F(1, 88)=8.191$, $p<0.01$ regardless of FLX dose. Although there appears to be a trend that climbing was reduced as FLX dose increased, the main effect of FLX did not reach significance, $F(3, 88)=2.164$, $p=0.098$. The stress X FLX interaction was not significant, $F(3, 88)=0.513$, $p=0.674$.

3.1.4. Latency to 3 immobile blocks

Preliminary analysis of immobility data revealed a clear trend that FLX caused rats to become immobile faster than saline. To objectively quantify this trend latency to 3 consecutive trials of immobility was computed for each rat by counting the number of 5 sec bins that were observed before 3 consecutive scores of immobility. Mean latencies are presented in Fig. 1, panel D. FLX significantly reduced the latency to immobility, $F(3, 85)=5.252$, $p=0.002$. However, Bonferoni post hoc tests found no significant pair-wise contrasts between the ISS and confined groups. No effects of stress or stress X FLX interaction were found, $ps>0.05$.

3.1.5. Open field

Mean path lengths (cm) from a 10 minute open field test are depicted in Fig. 2, panel A. No main effect was found for stress, $F(1, 88)=0.921$, $p=0.340$. A significant main effect for FLX, $F(3, 88)=3.418$, $p=0.021$, identified a trend that increasing doses of FLX reduced path length in the open field. However, pair-wise Bonferonni post hoc tests did not identify any significant ISS versus confined group contrasts. No stress X FLX interaction was found, $F(3, 88)=0.137$, $p=0.938$.

3.2. Experiment 2

3.2.1. Immobility

Mean counts of immobility are presented in Fig. 3, panel A. Inter-rater-reliability for immobility was high, $r(8)=0.974$, $p<0.001$. A 2 (stress or confinement) X 3 (0, 5, or 10 mg/kg TPT) ANOVA identified a significant main effect of stress, $F(1, 55)=15.02$, $p<0.001$, indicating that ISS increased immobility

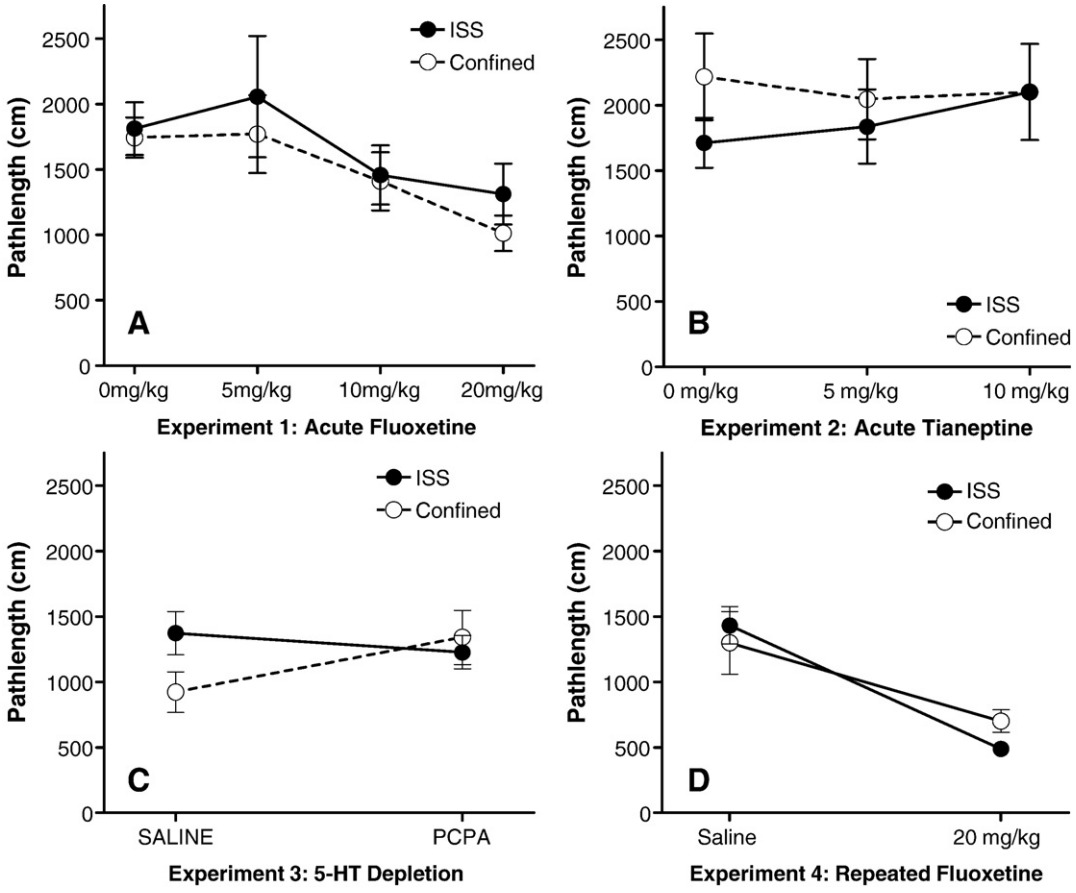

Fig. 2. Mean (±SEM) path length (cm) during a 10 minute open field test. Experiment 1, panel A: FLX dose-dependently reduced path length, $p<0.05$, $n=11–12$/group. Experiment 2, panel B: neither stress nor TPT altered path length, $n=10–11$/group. Experiment 3, panel C: no effect of PCPA was observed, $n=10–11$/group. Experiment 4, panel D: FLX significantly reduced path length in both groups, $p<0.05$, $n=7$/group.

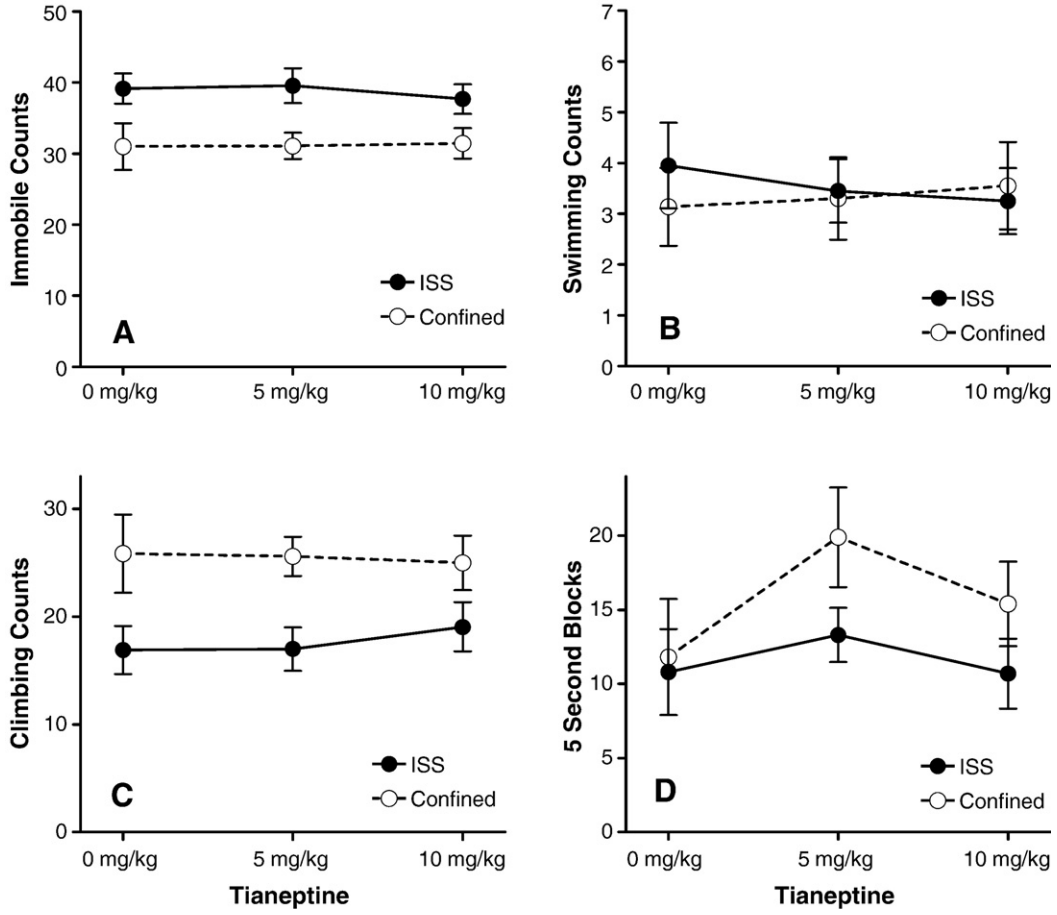

Fig. 3. Mean (±SEM) scores for Immobility (A), Swimming (B) Climbing (C) and Latency to 15 s Immobile (D) in the 5 minute FST, $n=10$–11/group. ISS significantly increased Immobility and decreased Climbing in the FST, $ps<0.05$. Neither stress nor TPT altered swimming or latency to immobility.

despite TPT dose. No effect of TPT was observed, $F(2, 55)=0.05$, $p=0.951$. No stress X TPT interaction was found, $F(2, 55)=0.122$, $p=0.887$.

3.2.2. Swimming

Mean counts of swimming are presented in Fig. 3, panel B. Inter-rater-reliability for swimming was high, $r(8)=0.906$, $p<0.001$. No significant effects were found for either stress, $F(1, 55)=0.125$, $p=0.725$, TPT, $F(2, 55)=0.029$, $p=0.972$, or the interaction, $F(2, 55)=0.271$, $p=0.764$.

3.2.3. Climbing

Mean counts of climbing are presented in Fig. 3, panel C. Inter-rater-reliability for climbing was high, $r(8)=0.993$, $p<0.001$. Stress significantly reduced climbing, $F(1, 55)=14.210$, $p<0.001$ regardless of TPT dose. The main effect of TPT was not significant, $F(2, 55)=0.048$, $p=0.953$. The stress X TPT interaction was not significant, $F(2, 55)=0.208$, $p=0.813$.

3.2.4. Latency to 3 immobile blocks

Latency to 3 consecutive blocks of immobility was computed for each rat in the same way as experiment 1 and is presented in Fig. 3, panel D. No significant effects were found for stress, $F(1, 55)=2.824$, $p=0.098$, TPT, $F(2, 55)=1.628$, $p=0.206$, or stress X TPT interaction, $F(2, 55)=0.455$, $p=0.637$.

3.2.5. Open field

Mean path lengths (cm) from a 10 minute open field test are depicted in Fig. 2, panel B. No main effect was found for stress, $F(1, 56)=0.828$, $p=0.367$, TPT, $F(2, 56)=0.149$, $p=0.862$, or the stress X TPT interaction, $F(2, 56)=0.326$, $p=0.724$.

3.3. Experiment 3 results

3.3.1. Immobility

Mean counts of immobility are presented in Fig. 4, panel A. One rat in the ISS-PCPA group was removed from the experiment because it was not able to complete 100 trials of stress, therefore in all subsequent analyses $n=11$/group except ISS-PCPA where $n=10$. Inter-rater-reliability for immobility was high, $r(8)=0.983$, $p<0.001$. A 2 (stress or confinement) X 2 (Saline or 200 mg/kg/3days PCPA) ANOVA identified no significant main effects for stress, $F(1, 39)=0.620$, $p=0.436$ or stress X PCPA interaction, $F(1, 39)=0.387$, $p=0.538$. However, a nearly significant main effect of PCPA $F(1, 39)=4.065$. $p=0.051$ was observed as an increase in immobility in both ISS and confined groups.

3.3.2. Swimming

Mean counts of swimming are presented in Fig. 4, panel B. Inter-rater-reliability for swimming was high, $r(8)=0.866$,

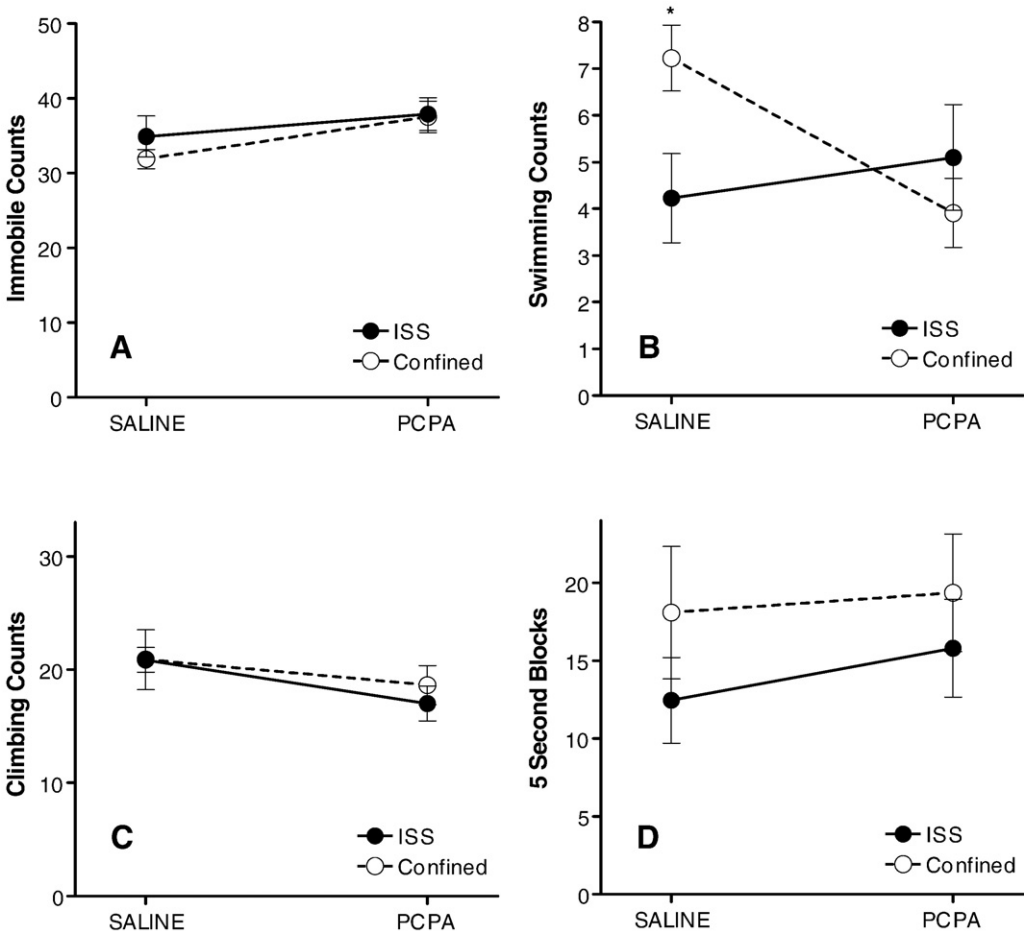

Fig. 4. Mean (±SEM) scores for Immobility (A), Swimming (B) Climbing (C) and Latency to 15 s Immobile (D) in the 5 minute FST, n=10–11/group. *ISS significantly reduced swimming in the saline condition, p=0.021.

p=0.001. No significant main effects were found for stress, F(1, 39)=1.031, p=0.316, or PCPA, F(1, 39)=1.884, p=0.178. However a significant stress X PCPA interaction was found, F(1, 39)=5.533 , p=0.024. Bonferoni post hoc contrasts revealed significant differences between ISS-Saline and Confined-Saline (p=0.021) and between Confined-Saline and Confined-PCPA groups (p=0.011). It is possible that prior ISS prevented the reduction in swimming produced by PCPA which would implicate 5-HT. Alternatively, ISS may have reduced swimming to an extent that any effect of PCPA on swimming in the ISS group is masked by a floor effect.

3.3.3. Climbing

Mean counts of climbing are presented in Fig. 4, panel C. Inter-rater-reliability for immobility was high, r(8)=0.975, p<0.001. No significant effects were found: stress, F(1, 39)=0.1992, p=0.658; PCPA, F(1, 39)=2.705, p=0.108; or stress X PCPA interaction, F(1, 39)=0.1884, p=0.667.

3.3.4. Latency to 3 immobile blocks

Latency to 3 consecutive trials of immobility was computed for each rat in the same way as experiments 2 and 3 and is presented in Fig. 4, panel D. Stress appeared to reduce the latency to immobility, however the trend was not significant, F(1, 39)=1.675, p=0.203. No effects were found for PCPA, F(1, 39)=0.422, p=0.520, or stress X PCPA interaction, F(1, 39)=0.085, p=0.772.

3.3.5. Open field

Mean path lengths (cm) from a 10 minute open field test are depicted in Fig. 2, panel C. No significant effects were found for stress, F(1, 39)=0.989, p=0.326; PCPA, F(1, 39)=0.657, p=0.422; or stress X PCPA interaction, F(1, 39)=2.810, p=0.102.

3.3.6. Colonic temperature

Temperature change scores (T_Δ) were computed as T_Δ=(colonic temperature after stress or confinement)−(colonic temperature before stress or confinement). T_Δ was not computed for one rat in each treatment group because of a thermometer malfunction, therefore n=10 in all groups except for ISS-PCPA where n=9). Mean T_Δ are depicted in Fig. 5. 100 trials of ISS significantly lowered colonic temperature by approximately 14 °C, F(1, 35)=537.1, p<0.001. PCPA did not affect this pattern, F(1, 35)=1.619, p=0.212 and there was no stress X PCPA interaction, F(1, 35)=0.190, p=0.666.

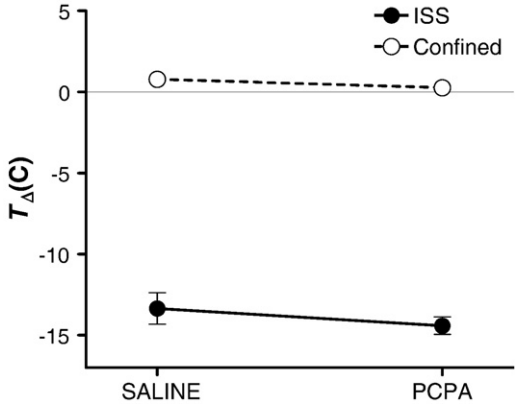

Fig. 5. Mean (±SEM) T_Δ (°C) after 100 trials of ISS, n=9–10/group. ISS reduced T_Δ, $p<0.01$.

3.4. Experiment 4 results

3.4.1. Immobility

Mean counts of immobility are presented in Fig. 6, panel A. Inter-rater-reliability for immobility was high, $r(8)=0.986$, $p<0.001$. A 2 (stress or confinement) X 2 (3 injections of 20 mg/kg FLX or Saline) ANOVA identified a significant main effect for stress, $F(1, 36)=8.654$, $p=0.005$, but not significant effects for FLX, $F(1, 36)=0.4366$, $p>0.05$, or Stress by FLX interaction, $F(1, 36)=1.968$, $p=0.169$. Bonferonni post hoc contrasts revealed a significant difference between ISS and Confined in the FLX condition, $p<0.01$.

3.4.2. Swimming

Mean counts of swimming are presented in Fig. 6, panel B. Inter-rater-reliability for swimming was high, $r(8)=0.881$, $p=0.001$. No significant main effects were found for stress, $F(1, 36)=0.065$, $p=0.800$, FLX, $F(1, 36)=1.488$, $p=0.230$, or stress X FLX interaction, $F(1, 36)=1.488$, $p=0.757$.

3.4.3. Climbing

Mean counts of climbing are presented in Fig. 6, panel C. Inter-rater-reliability for immobility was high, $r(8)=0.991$, $p<0.001$. A significant main effect was found for stress, $F(1, 36)=9.280$, $p=0.004$. No significant effects were found for FLX or the stress X FLX interaction, ps>0.05. Bonferonni post hoc contrasts indicated that in the FLX condition climbing was significantly greater in confined animals than in ISS $p<0.01$. Confined-FLX appeared to have greater climbing compared to Confined-Saline, but this comparison did not reach significance, $p=0.066$.

3.4.4. Latency to 3 immobile blocks

Latency to 3 consecutive trials of immobility was computed for each rat in the same way as experiments 1, 2 and 3 and is

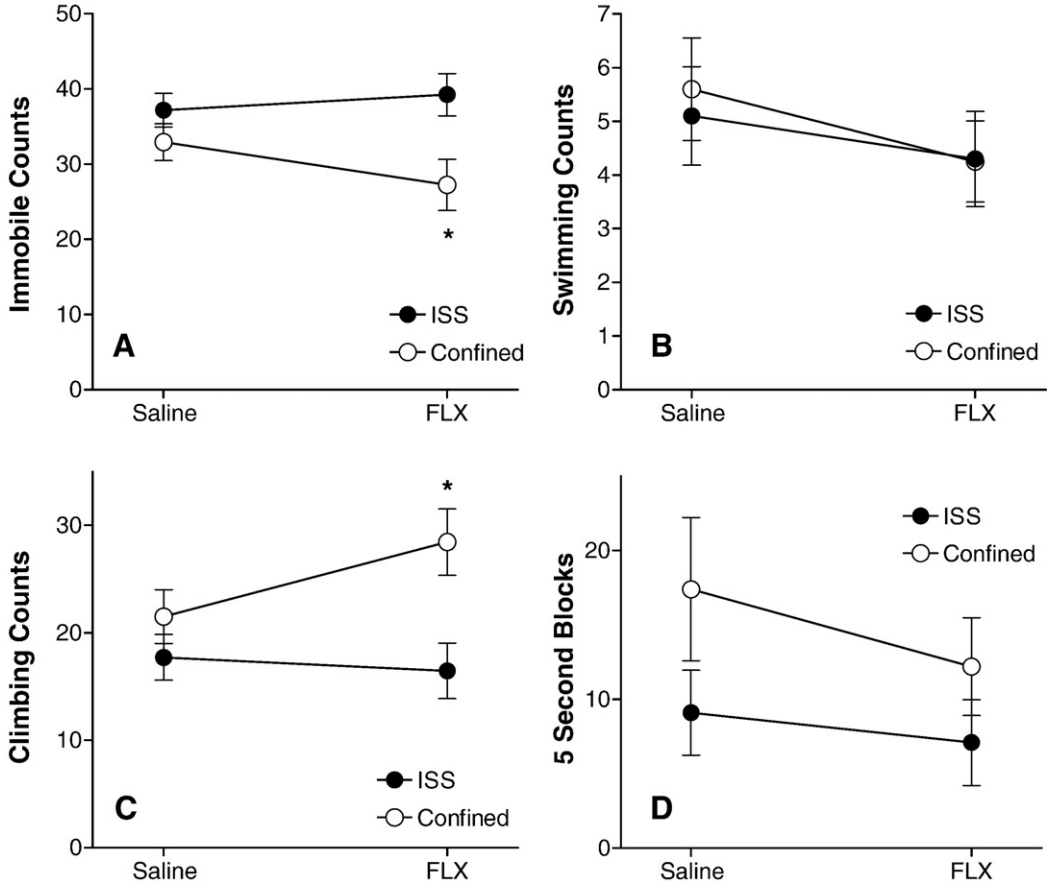

Fig. 6. Mean (±SEM) scores for Immobility (A), Swimming (B) Climbing (C) and Latency to 15 s Immobile (D) in the 5 minute FST after ISS and therapeutic FLX treatment, n=10/group. *Immobility and climbing were significantly reduced and increased, respectively by FLX in the confined group, ps<0.01.

presented in Fig. 6, panel D. No significant effects were found for stress, $F(1, 36)=3.560$, $p=0.067$, FLX, $F(1, 36)=1.028$, $p==0317$, or stress X FLX interaction, $F(1, 36)=0.203$, $p=0.656$.

3.4.5. Open field

Mean path lengths (cm) from a 10 minute open field test are depicted in Fig. 2, panel D. Path length was only analyzed in 7 rats per group due to an equipment malfunction. No significant effects were found for stress or the stress X FLX interaction, $ps>0.05$. However, FLX significantly reduced path length in both ISS and confined groups, $F(1, 24)=26.91$, $p<0.001$.

4. Discussion

We have presented 4 experiments designed to test the involvement of 5-HT in the behavioral immobility caused by ISS. In none of these experiments did 5-HT manipulations appear to selectively affect ISS treated rats in the FST. Experiment 1 replicated previous findings that exposure to ISS increases immobility in the FST (Christianson & Drugan, 2005). Some evidence that increasing 5-HT transmission enhances stress behaviors was observed in a trend that immobility increased and climbing decreased with FLX dose in both ISS and Confined groups. However, these behaviors could be attributed to a general FLX-induced reduction in locomotor behavior because FLX also reduced path length in the 10 minute open field test.

A putative role for 5-HT could have been determined if FLX increased immobility only in ISS treated rats, however the trend that FLX increased immobility is present in both ISS and confined subjects. These data alone cannot confirm a role for 5-HT, therefore Experiment 2 attempted to reverse ISS-induced immobility with the serotonin reuptake enhancer TPT, a drug that should reduce the synaptic availability of 5-HT during stress. Again, in Experiment 2, ISS increased time spent immobile and reduced climbing in the FST. However, the purported serotonin reuptake enhancer TPT had no impact on these effects. It seems acceptable then, to conclude from Experiments 1 and 2 that 5-HT does not play a significant role in ISS induced immobility because FLX did not worsen, nor did TPT improve the performance of stressed rats in the FST.

While the efficacy of TPT to block some of the effects of uncontrollable stressors is clear (Conrad et al., 1996; Delbende et al., 1991; Shakesby et al., 2002; Whitton et al., 1991), the role of 5-HT uptake in its effect has been criticized. In fact, McEwen and Olie (2005) argued that TPT alters glutamatergic transmission which appears to be the therapeutic mechanism for the drug. This possibility makes the role of 5-HT in the ISS paradigm difficult to discern because this experiment did not address the empirical question regarding the role of 5-HT on ISS-induced immobility. Therefore, Experiment 3 utilized the tryptophan hydroxylase inhibitor PCPA to deplete brain 5-HT prior to ISS. PCPA causes a selective depletion of brain 5-HT with minimal behavioral consequences (Koe and Weissman, 1966), prevents learned helplessness (Edwards et al., 1986) and is without effect in the modified FST (Page et al., 1999). In Experiment 3, three daily administrations of PCPA did not alter ISS induced immobility. The phenomenological finding that ISS increases immobility and decreases climbing was not present in these data. It is likely that the handling and housing conditions unique to Experiment 3 can account for this reduced effect; specifically, single-housing, daily weighing and i.p injection for three days prior to ISS may have altered the behavioral response to stress. In addition, the effect of PCPA administration on 5-HT synthesis would have been present both at the time of ISS and FST. Despite a failure to replicate the primary stress effect on immobility and climbing, ISS significantly decreased swimming in the saline condition. Future experiments may require modification to enhance the phenomenological effect that ISS-induced immobility under a variety of handling, housing, and injection conditions. Taken together these experiments provide no convincing evidence that 5-HT is a critical mediator of ISS-induced immobility.

PCPA did appear to increase immobility regardless of stress treatment. This is in contrast to the reports of other investigators who have found a lack of effect of PCPA administration on immobility in the forced swim test (Einat et al., 2001; Page et al., 1999; Redrobe et al., 2005; Schreiber et al., 1994). The effect of PCPA observed in Experiment 3 could be the result of the dose used, 200 mg/kg for 3 days, which is comparable to the highest reported dose. However, depletion of 5-HT by PCPA pretreatment prevented various antidepressant reversals of immobility in the FST including FLX (Page et al., 1999), inositol (Einat et al., 2001), the 5-HT1a agonists S14506 and S14671 (Schreiber et al., 1994) and neuropeptide Y (Redrobe et al., 2005). A test of 5-HT involvement would be to follow a similar strategy and test the reversibility of the ISS-induced immobility with a therapeutic regimen of FLX and then try to block this effect with PCPA. This strategy was employed in Experiment 4. However, a commonly used sub-chronic regimen of FLX had no effect on ISS treated rats. Therefore PCPA reversal was not conducted.

Observation of T_{Δ} extended the reports of Drugan et al. (2005) and Levay et al. (2006) to the 100 trial ISS paradigm in 15 °C water. Drugan et al. (2005) used a triadic design and reported that 80 swim trials in 25 °C and 20 °C caused core body temperature reductions of approximately −5 °C and −7 °C, respectively. However, 80, 5-sec swim trials in 15 °C water caused a core body temperature reduction of 12 °C (Levay et al., 2006). Here, 100 5-sec trials in 15 °C water produced an approximately 14 °C reduction in colonic temperature, a far more severe hypothermia than previously observed. The addition of 20 trials in the current paradigm may account for the slightly greater hypothermia compared to the 80 trial stress. We have recently verified this 14 °C reduction in core body temperature with surgically-implanted biotelemetry devices (Drugan et al., 2007).

These results appear to be in conflict with a large body of evidence implicating the 5-HT system in the related intermittent shock model. In the intermittent shock model, 5-HT activation is necessary for the production of behaviors that result only from uncontrollable and unpredictable stress (Maier and Watkins, 2005); these are characteristics of the ISS induced behaviors described here. However, there are two important differences between intermittent shock and ISS. *a*) Intermittent

shock produces hyperthermia (Deak et al., 1997; Weiss et al., 1981) while intermittent swim produces hypothermia followed by hyperthermia (Experiment 3 and (Drugan et al., 2005; Levay et al., 2006)). *b*) The stress induction and test procedures of intermittent shock and swim impose different physical demands on the rat. Specifically, shock is delivered in restraint and tested in a learning task where there is little physical activity while swimming causes a great deal of physical activity; these behavioral differences may have distinct neurochemical and ethological components. Because of the differences between the intermittent shock and ISS paradigms, namely the extreme hypothermia produced by ISS, the current data do not refute the implications for 5-HT in learned helplessness, but imply that the behavioral changes induced by the ISS procedure are likely to be mediated by very different neural systems.

Much is known about the physiological basis of cold stress that may relate to immobility in the FST. Painful stimuli trigger the release of endogenous opioid peptides; ISS is no exception. In fact, brief exposures to swimming in 2 °C water produced a stress-induced analgesia that was blocked by the opiate antagonist naltrexone (Girardot and Holloway, 1984) and 3.5 min of continuous cold water stress in 4 °C water produced profound release of endogenous opioid ligands (Seeger et al., 1984). Stress-induced analgesia by the release of endogenous opioids could influence the behavioral adaptation to cold water by reducing the salience of the painful stimuli. With less attention devoted to pain, the rat may be less aroused and therefore less motivated to escape and struggle in the subsequent FST.

Exposure to ISS, regardless of temperature or controllability, causes a robust increase in circulating CORT (Drugan et al., 2005). CORT is known to influence many behaviors in the rat and could be an important regulator of ISS induced immobility. Some data implicate CORT in the consolidation of memory that leads to immobility in the FST (Jefferys et al., 1983; Mitchell and Meaney, 1991). However, increased levels of CORT were observed after both escapable and inescapable ISS but only inescapable ISS increased immobility (Drugan et al., 2005). Therefore, CORT cannot be the critical mediator of ISS-induced immobility.

Although endogenous opioids and glucocorticoids may contribute to the phenomenon of ISS induced immobility, far more empirical evidence implicates the brain norepinephrine (NE) system. Both electric shock and forced swimming increase the activity of NE neurons and stimulate release of NE in brain (Weiss et al., 1981). Electrical stimulation (Kostowski et al., 1984) and microinjection of NE (Weiss et al., 1986) to the locus correleus (LC) increased climbing in the FST without stimulating locomotor activity. During the FST, levels of NE in frontal cortex are positively correlated with climbing and negatively correlated with immobility (Page et al., 2003) and treatments that increase the availability of NE such as the catecholamine precursor L-tyrosine (Yeghiayan et al., 2001) or the NE specific reuptake inhibitor reboxetine (Page et al., 2003) reverse the effects of continuous swim stress on immobility. Several authors have hypothesized that prior experience with stress reduces the subsequent availability of NE (Weiss et al., 1981, 1970) and sensitivity of LC neurons (Simson and Weiss, 1988) that may reduce arousal towards active behavioral strategies in a second exposure, either in a swim test (Jordan et al., 1994) or in a shuttlebox (Anisman et al., 1979). In our first report (Christianson and Drugan, 2005) and again here, ISS appeared to alter immobility and climbing more consistently than swimming. Cryan and colleagues have argued that climbing behaviors in the FST reflect the activity of NE such that increasing NE transmission with selective NE reuptake inhibitors like reboxetine reduce immobility by increasing climbing (Cryan et al., 2002b). In their studies, noradrenergic drugs did not affect swimming. It is also possible that the high dose of FLX used here may have affected non-5-HT systems to alter climbing and the behaviors observed suggest that ISS induced immobility is mediated by NE, rather than 5-HT. Future experiments will explore the role of NE in the behavioral consequences of ISS.

Despite the apparent failure to implicate 5-HT in the behavioral phenomenon of ISS induced behavioral depression, these data remain highly provocative. Current behavioral models of depression depend on their sensitivity to established pharmacotherapies (i.e., (Berton and Nestler, 2006) but the data presented here indicate that the behavioral depression caused by ISS is not mediated by the 5-HT system and is resistant to sub-chronic FLX therapy. This finding may distinguish ISS from the intermittent shock model where sub-chronic FLX has been reported to prevent learned helplessness behaviors (Zazpe et al., 2007). Therefore, ISS may provide a useful tool for investigating novel pharmacotherapies and inform the pathophysiology of behavioral depression. To our knowledge this is the first attempt to detect antidepressant activity using the ISS paradigm. Although the immobility produced by ISS appears to be resistant to FLX therapy, future research will test a range of established antidepressant compounds to more completely characterize this paradigm.

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