The effects of N-methyl-citalopram (NMC) on serotonergic systems and antidepressant-like behavior

Christopher J. Brazell
Department of Integrative Physiology
University of Colorado Boulder
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Thesis Advisor
Dr. Christopher A. Lowry, Department of Integrative Physiology and the Center for Neuroscience

Committee Members
Dr. David Sherwood, Department of Integrative Physiology
Dr. Ariel Paul, Department of Physics
Abstract

Depression is projected to be the 2nd leading cause of disability adjusted life years by 2020. Current forms of treatment come in the form of drugs that target monoaminergic systems, for example selective serotonin reuptake inhibitors (SSRIs), which elicit their effects through unknown mechanisms. The mechanisms, if better understood, could improve treatment efficacy and reduce side effects of SSRI drugs in depressed patients. Prior research from our lab has revealed that an ancient thermoregulatory peripheral spinoparabrachial pathway, which projects to serotonergic neurons in the brain, may be defective in depressed patients. We predicted that restoration of this pathway would reduce depression-like symptoms. In this study we tested the hypothesis that N-methyl-citalopram (NMC), a novel compound that does not appear to cross the blood-brain barrier, would produce antidepressant-like behavior in rats through peripheral activation of the spinoparabrachial pathway projecting to the brain. Rats received injections of varying dosages of NMC or a saline control and were then introduced to forced swim testing. Afterwards rat brains were removed and prepared for immunohistochemical detection of the immediate-early gene c-Fos (as a marker for neuronal activation) and TPH (a marker for serotonergic neurons). Our data suggest that NMC increased expression of the immediate-early gene, c-Fos, within serotonergic neurons in specific subregions of the dorsal raphe nucleus (DR); a region shown to have dense populations of serotonergic neurons that project to forebrain limbic structures implicated in the pathophysiology of major depression. Therefore, it is apparent that NMC has implications for antidepressant-like effects, likely through peripheral activation of the spinoparabrachial pathway projecting to the dorsal raphe nucleus. This suggests a novel therapeutic alternative to currently used, centrally acting SSRI treatments.

Background

Depression is one of the most prevalent and devastating illnesses of our time. It is projected to be the 2nd leading cause of disability adjusted life years (DALYs- a composite measure of the burden of an illness, taking into account years of life lost to mortality and disability) by 2020 (Murray & Lopez, 1996). As such, the treatment of depression has received a great deal of attention and research, yet a lot remains to be understood about the underlying physiology and treatment of depression and about the mechanism of action of antidepressant drugs. Current pharmacological treatments for depression include SSRIs, monoamine oxidase inhibitors (MAOIs), and tricyclic antidepressants. The mechanisms by which these drugs exert their antidepressant effects are not currently known, and these drugs have significant side effects. Since depression is such a prevalent and devastating illness, there is a great need to understand the underlying physiology of depression and the mechanisms of action of the drugs used to treat it. Doing so will allow us to create new targeted treatments that will be more effective and have fewer side effects.

One of the most common side effects of antidepressant drugs is excessive sweating (Marcy & Britton, 2005; Ward & Doerr, 1983). Although currently viewed as a side effect, research from our lab as well as others indicates that this physiologic response may be a biomarker of effective antidepressant therapy (Lowry et al., unpublished data). Evidence suggests that antidepressant drugs target specific serotonergic systems in the brain that cause both changes in thermoregulation and antidepressant effects. This interaction between these two important physiological systems may be the key to understanding antidepressant treatment. Evidence from our lab suggests that activation of an afferent spinoparabrachial pathway activates a subpopulation of serotonergic neurons in the dorsal raphe nucleus that projects to forebrain limbic structures implicated in the pathophysiology of major depression, leading to antidepressant-like behavioral effects.

Previous (unpublished) work from our laboratory has shown that administration of a subthreshold dose of the SSRI drug citalopram (CIT), in conjunction with warm temperature exposure, leads to an antidepressant-like effect in the forced swim test (Lowry et al., unpublished data). Further, warm ambient temperature exposure causes a significant increase in c-Fos expression in serotonergic neurons of the interfascicular part of the dorsal raphe nucleus (DRI) that is reversed...
by administration of CIT (Lowry et al., unpublished data). In all subregions of the dorsal raphe nucleus sampled, CIT administration caused a significant decrease in c-Fos expression in serotonergic neurons (Lowry et al., unpublished). These data show clearly that there is an additive antidepressant-like behavioral effect of warm ambient temperature exposure and CIT administration. Further, it is evident that there is an inhibitory effect of CIT on c-Fos expression in serotonergic neurons within the dorsal raphe nucleus; this may be because CIT crosses the blood-brain barrier and elevates extracellular serotonin within the dorsal raphe nucleus, resulting in increased 5-HT1A receptor-mediated autoinhibition of serotonergic neuronal firing rates. If NMC selectively activates an excitatory spinoparabrachial pathway projecting to DRI serotonergic neurons, without non-selectively elevating extracellular concentrations of serotonin in the dorsal raphe nucleus, it could have a more rapid onset of action relative to centrally acting SSRIs, with fewer side effects. These data justified further work investigating the effects of NMC on behavior in the forced swim test (FST) and c-Fos expression in the midbrain raphe complex.

To investigate peripheral mechanisms for activation of these serotonergic populations and antidepressant-like behavioral effects, we used NMC, an analogue of the SSRI CIT that does not cross the blood-brain barrier. This study investigated the effects of administration of different doses of NMC, to identify a subthreshold dose to be used in later studies to investigate the relationship between exposure to warm ambient temperature, NMC, serotonergic systems and antidepressant-like behavior. We hypothesized that NMC would have similar behavioral and physiological effects to administration of centrally acting SSRI drugs. Furthermore we hypothesized that antidepressant drug effects on serotonergic systems, cognitive function, and affect are mediated by activation of an afferent spinoparabrachial-raphe pathway. We predicted that NMC will dose dependently increase swimming and decrease immobility in the FST. Our findings suggest that certain dosages of NMC increased swimming and decreased immobility, while subsequently increasing cFos expression in serotonergic neurons within specific subregions of the DR.

**Methods**

**Animals and housing conditions**

Adolescent male Wistar rats (HSD-WI, Harlan Laboratories, Indianapolis, IN, USA; N = 40), arrived from the vendor weighing approximately 85 g at 4 weeks of age (postnatal day (PD) 28; (Day 0). According to previous studies, pre-adolescence corresponds to rat postnatal day (PD) 21 to PD28; PD28 to PD34 (early adolescence); PD34 to PD46 (mid-adolescence); and PD46 to PD56 (late adolescence) (Spear, 2000; Andersen, 2003; Laviola et al., 2003). Therefore, PD28 is considered early adolescence (Andersen, 2003; Kellogg et al., 1998; Tsoory & Richter-Levin, 2006). Rats were individually housed in transparent polycarbonate cages (26 cm W x 47.6 cm L x 20.3 cm H; Cat. No., RC88D-PC, Alternative Designs, Siloam Springs, AR, USA) using standard cage bedding (Teklad Laboratory Grade Aspen Bedding, Harlan, Madison, WI, USA) to minimize the variable of body heat exchange that may have occurred during nesting, grooming and play if rats were pair housed or group housed. Rats were kept under a 12:12 light/dark cycle with lights on at 06:00 AM and room temperature of 22 °C. Experimental procedures were performed during the light phase; all behavioral testing and experimental work followed between 7 AM and 4 PM. Food (Cat. No. 2018, Teklad 18640 22/5 Rodent Diet, Harlan, Madison, WI, USA) and tap water were available ad libitum for the duration of the experiment. By the test day, rats weighed 145 – 190 g.

**Drugs**

(RS)-1-[3-(trimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenofuran-5-carbonitrile iodide (N-methyl-citalopram; NMC; Batch ID: 13205-159-2, NIMH Chemical Synthesis and Drug Supply Program # M-918, RTI, Research Triangle Park, NC, USA) was made up in sterile saline (0.9% NaCl in water, RX0.9NACL, MedVet International, Mettawa, IL, USA). Sterile saline was the vehicle control. Rats received 3 subcutaneous (s.c.) injections of either vehicle or NMC at 23, 5 and 1 h prior to the second exposure to the FST. The vehicle or NMC was injected in a volume of 2 ml/kg.
**Justification of drug dose**

N-methyl-citalopram was injected s.c. at 10, 50, 100, and 200 mg/kg in adolescent rodents. Bismuth-Evenzal and colleagues (Bismuth-Evenzal et al., 2010) had shown that NMC is 10-fold less potent than citalopram (CIT); and as unpublished data from our lab has shown, CIT is effective at 20 mg/kg but not at 5 mg/kg in adolescent male rats, therefore an equivalent dose of 200 mg/kg was used as the maximal dose (Hale et al., unpublished data).

**Assignment of groups and cohorts (N = 40)**

Rats were randomly assigned to one of five different treatment groups as follows: group 1, vehicle (n = 8); group 2, 10 mg/kg NMC (n = 8); group 3, 50 mg/kg NMC (n = 8); group 4, 100 mg/kg NMC (n = 8); group 5, 200 mg/kg NMC (n = 8). Two animals from each group were put in one of four cohorts (n = 10) for administration of the forced swim test.

**Experimental procedures**

Following their arrival on day 0, rats were allowed to acclimatize to the animal housing room environment for one week and were habituated to handling once daily for 5 minutes between 10 AM and 1 PM (Figure 1). On day 7, rats began the forced swim test procedure, using methods described previously (Cryan, Valentino, & Lucki, 2005) except that we used a cylinder with an inner diameter of 30 cm, outer diameter of 30.5 cm, and a height of 46 cm, with a water depth of 30 cm (30 cm H x 46 cm W; Cat No. 36360-201, VWR, West Chester, PA, USA), and a water temperature of 25 ± 1 °C. This water depth was chosen because it was adequate to prevent rats from touching the bottom of the cylinder with their tails and resting during the FST. Conducting the study using 4 cohorts of rats ensured FST behavior was monitored during the same time of day(s) to minimize variation in behavioral responses. On day one of testing (day 7 (cohort 1), day 8 (cohort 2), day 9 (cohort 3), day 10 (cohort 4); hereafter referred to as day 7), rats were introduced to a 15 min swim and 24 h following the first swim a 5 minute test was carried out on the second day (day 8 (cohort 1), day 9 (cohort 2), day 10 (cohort 3), day 11 (cohort 4); hereafter referred to as day 8). Rats were dried and returned to their home cages after each day of testing, and FST chambers were drained and cleaned between each test. One hour following the onset of the 15 min pre-exposure swim, rats received injections of either vehicle (0.9% sterile saline, s.c. 2 ml/kg) or NMC (10, 50, 100, or 200 mg/kg, s.c. 2 ml/kg) and were immediately returned to their home cages. The following day, day 8 (cohort 1), rats received the second injection of either vehicle or NMC 5 h before the 5 min FST test and were immediately returned to their home cages. Rats then received their final vehicle or NMC injection 60 min prior to the 5 min FST test on day 2. On the second day of the FST, rats were placed individually for 5 min in the same glass cylinders filled with water (25 ± 1 °C) to a depth of 30 cm. Thirty minutes following the onset of the second FST, rats were anesthetized with sodium pentobarbital (i.p. 2 ml/kg, 90 mg/kg; Fatal-Plus (animal euthanasia solution (390 mg/ml sodium pentobarbital, 0.01 ml/ml propylene glycol, 0.29 ml/ml ethyl alcohol (CDA-3A), 0.02 ml/ml benzyl alcohol), Vortech Pharmaceuticals, Dearborn, MI, USA), and perfused with 4% paraformaldehyde (see tissue processing section).

![Figure 1](image)

**Fig. 1** Figure illustrating the experimental timeline. Asterisks indicate days on which rats were each habituated to handling 5 minutes daily. Vehicle or N-methyl-citalopram (NMC) injections (s.c.; 10, 50, 100, or 200 mg/kg) were administered on day 7 (24 h before the 5 min forced swim test) and day 8 (5 h and 1 h before the 5 min forced swim test). Perfusions were carried out 30
minutes following the onset of the 5 min forced swim test on day 2. Abbreviations: FST, forced swim test.

Behavioral testing and analysis
For the 15 min swim on day 1 of the FST and the 5 min test the following day, behavior was recorded using digital cameras (Sony Handycam, DCR-HC52, Sony Corporation of America, New York, NY, USA) using mini DV digital tape (Sony DVM-60PRL; 360 minute Premium Mini DV Tape, TapeStockOnline, Anaheim, CA, USA). Cameras were positioned above each cylinder. Behavior during the test was scored manually according to Cryan et al (2005) using a computer-based observational event logging program (The Observer 5.0, Noldus Information Technology, Wageningen, The Netherlands). The behaviors selected for analysis were, 1) climbing, defined as upward-directed movements of the forepaws usually along the side of the swim chamber, 2) swimming behavior, defined as slow rhythmic front and hind leg movements throughout the swim chamber without breaking the surface of the water, which includes moving across quadrants of the cylinder, 3) immobility, measured when no additional activity is observed other than that required to keep the rat's head above water and 4) diving behavior, which was classified as an event and defined as when the rat's entire body was submerged (Detke, Rickels, & Lucki, 1995). Both the duration and frequency of each of the behaviors were scored.

Tissue processing
Thirty minutes following the onset of the second swim exposure, rats were deeply anesthetized with sodium pentobarbital (90 mg/kg i.p.) followed by transcardial perfusion with ice-cold 0.05 M phosphate buffered saline then ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were post-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight (12-16 hours), rinsed twice for 12 hours each time in 0.1 M sodium phosphate buffer, and finally placed into (30% sucrose in 0.1 M sodium phosphate buffer, pH 7.4) until they sank (96 hours). Brains were then blocked caudal to the mammillary bodies (~5.6 mm with respect to bregma) into forebrain and hindbrain pieces using a rat brain matrix (RBM4000C, ASI Instruments, Warren, MI, USA), snap-frozen in isopentane cooled with dry ice, and stored at ~80 °C until sectioning. Brains were sectioned using a Leica cryostat (Model CM1900, Leica GmbH, Wetzlar, Germany). Sections were collected separately for the medulla (~15.84 mm bregma to the caudal border of the inferior colliculi at ~9.86 mm bregma) and for the midbrain and pontine raphe nuclei (~9.86 mm to ~5.60 mm bregma). Sections were collected in series of 6 and placed into 2.0 mL of cryoprotectant solution (30% ethylene glycol/20% glycerol/ 0.05 M sodium phosphate buffer, pH 7.4) in 24-well tissue culture plates and stored at ~20 °C until further processing for immunohistochemical staining.

Immunohistochemistry
The brains were sectioned and processed for immunohistochemical staining of tryptophan hydroxylase (sheep anti-TPH Cat. No. T8575, Sigma-Aldrich, St. Louis, MO, USA) a marker of serotonergic neurons and c-Fos (rabbit anti-c-Fos, Cat. No. PC-38, CalBiochem, Billerica, MA, USA) as a marker of neuronal activation in the DR. Immunohistochemistry for c-Fos was conducted on free-floating tissue in 12-well tissue culture plates in 2.0 mL of solution and gently shaken on an orbital shaker throughout the staining process. Until stated otherwise, tissue was washed for 15 minutes at each step. Tissue was rinsed twice in a 0.05 M phosphate buffered saline (PBS) followed by washing in 1% hydrogen peroxide (H₂O₂). Tissue was then rinsed twice with 0.05 M PBS followed by one wash in PBS containing 0.3% Triton X-100 (PBST). Sections were then incubated overnight at room temperature with 1:3000 rabbit anti-c-Fos polyclonal antibody in 0.1% PBST + 0.01% sodium azide (NaN₃). After 16 hours, tissue was washed twice in 0.05 M PBS followed by incubation with 1:500 biotinylated donkey anti-rabbit IgG (Cat No. 711-085-152, Jackson Immunolabs, West Grove, PA, USA) in 0.05 M PBS for 90 min. Tissue was then washed twice in 0.05 M PBS followed by incubation with an avidin-biotin peroxidase complex (Elite ABC reagent, Cat No. PK-6106, 1:200; Vector Laboratories, Burlingame, CA, USA) in 0.05 M PBS for 90 min. Tissue was washed twice in 0.05 M PBS then placed in a peroxidase substrate solution (SG chromagen, Vector Laboratories, Cat No. SK4700, diluted 1:1 as recommended by vendor) in 0.05 M PBS until color changed sufficiently, 17 min. After the
chloromogen reaction, tissue was immediately washed twice in 0.05 M PBS followed by one wash in 1% H₂O₂ in 0.05 M PBS. Tissue was then washed twice in 0.05 M PBS followed by one wash in PBS containing 0.3% Triton X-100 (PBST). This was followed by a 4-day incubation in sheep anti-tryptophan hydroxylase (1:2500) in 0.1% PBST+ 0.01% NaN₃ + 0.4% normal donkey serum (NDS). After 4 days, tissue was washed twice in 0.3% Triton X-100 in 0.05 M PBS followed by a 90-minute incubation in 1:500 biotinylated rabbit anti-sheep IgG (Vectastain Elite, Cat No. PK-6016, 1:200; Vector Laboratories) in 0.3% Triton X-100 in 0.05 M PBS. After incubation, tissue was washed twice in 0.05 M PBS followed by incubation with an avidin-biotin peroxidase complex (Elite ABC reagent; Cat No. 6100, Reagent A 1:250, Reagent B 1:1000; Vector Laboratories) in 0.3% Triton X-100 in 0.05 M PBS for 90 min. Tissue was then washed twice in 0.3% Triton X-100 in 0.05 M PBS followed by an optimal incubation in 0.025% 3-3'-diaminobenzidine tetrahydrochloride (DAB, Cat. No. D9015, Sigma-Aldrich) in 0.05 M PBS with 0.001% H₂O₂. Immediately following the DAB reaction the tissue was rinsed twice in 0.05 M PBS to stop the reaction. Brain sections were stored in 0.1 M sodium phosphate buffer with 0.01% NaN₃ at 4 °C until tissue mounting. As part of the mounting process, brain sections were then rinsed in distilled H₂O and then rinsed for 10-15 seconds in 0.15% gelatin diluted in distilled H₂O, then mounted on glass microscope slides (VistaVision UniMark microscope slides, Cat. No. 16005-106; VWR International, Aurora, CO, USA), dehydrated through an alcohol series and cleared with xylene. Slides were mounted with cover slips using mounting medium (Entellen mounting medium; Cat. No. RT14082, EM Sciences, Hatfield, PA, USA).

Cell counting

Five rostrocaudal levels of the DR were chosen for analysis (−7.46, −8.00, −8.36 –8.54, and −8.72 mm bregma; Figure 2). The subdivisions of the DR studied included the dorsal raphe nucleus, dorsal part (DRD) and dorsal raphe nucleus, ventral part (DRV) at −7.46 mm bregma, the DRD, DRV, and dorsal raphe nucleus, ventrolateral part (DRVL)/ventrolateral periaqueductal gray (VLPAG) at −8.00 mm bregma, the DRD, DRV, DRVL, and dorsal raphe nucleus, interfascicular part (DRI) at −8.36 mm bregma, the DRI and dorsal raphe nucleus, caudal part (DRC) at −8.54 mm bregma, and the DRI and DRC at −8.72 mm bregma. Rostrocaudal levels and anatomical divisions of the midbrain raphe complex were based on comparison of immunostained slices to a stereotaxic rat brain atlas (Paxinos and Watson, 1998), and an atlas of tryptophan hydroxylase immunostaining in the rat dorsal raphe nucleus (Abrams et al., 2004).

Cell counting of c-Fos-immunopositive (+)/TPH-immunonegative (−), c-Fos+/TPH+, and total TPH+ neurons was performed. Cells were counted from both left and right sides of the DRVL/VLPAG and summed to give a total number of cells. All remaining cell counts were from midline subdivisions. Cell counts were performed under bright-field microscopy at a total magnification of 100x. A total magnification of 400x was used to verify double labeling. The experimenter was blind to the experimental treatments throughout cell counting.

Fig. 2. Low-power photomicrographs illustrating c-Fos/tryptophan hydroxylase (TPH)-immunostaining in 11 subregions of the dorsal raphe nucleus (DR) sampled in this study. Five rostrocaudal levels were sampled including, in mm from bregma: A) −7.46 mm, B) −8.00 mm C) −8.36 mm D) −8.54 mm E) −8.72 mm. The subdivisions of the DR are illustrated by dashed lines. Abbreviations: DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL/VLPLG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; and mlf,
medial longitudinal fasciculus. Scale bar 500 µm.

**Statistical analysis**

*Forced swim test behavior*

Separate one-way analyses of variance (ANOVA) with drug dose as a between-subjects factor were used to determine the effects of drug dose on each dependent variable in the FST, i.e. swimming, immobility, climbing, and diving behaviors. A Grubbs’ test (Grubbs, 1969) was used to identify any outliers and outliers were subsequently removed. When appropriate, Fisher’s protected least significant difference tests were used for post hoc comparisons. Statistical significance was accepted at the level of $p < 0.05$ for both the ANOVAs and post hoc comparisons.

*Dorsal raphe nucleus*

Cell counts for the numbers of c-Fos+/TPH+ (serotonergic) neurons, the numbers of c-Fos+/TPH− (non-serotonergic) cells and the total numbers of TPH+ neurons sampled were analyzed separately using an analysis of variance (ANOVA) with treatment as a between-subjects factor and brain region (anatomical subregions) as a within-subjects factor for the repeated measures. Two separate two-way ANOVAs were run due to timing differences between tissue analysis; one for DRI and DRC, and another for DRVL/VLPAG, DRD, and DRV. Statistical procedures were performed using PASW Statistics (19.0 for Windows, SPSS Inc., Chicago, IL, USA). A Greenhouse-Geisser epsilon correction ($\epsilon$) was used for repeated measures to correct for violation of the sphericity assumption. Grubbs’ test (Grubbs, 1969) was used to identify any outliers and outliers were removed. Replacement data for the multifactor ANOVA with repeated measures were calculated using the Petersen method (Petersen, 1985). Replacement data were not used in graphical representations of the data or in planned pairwise comparisons. Significance was accepted at the level of $p < 0.05$.

**Results**

*Dorsal raphe nucleus*

*c-Fos-immunoreactive (-ir) serotonergic neurons in subdivisions of the DR*

N-methyl-citalopram increased c-Fos expression in serotonergic neurons within specific subdivisions of the DR. Two-way ANOVA revealed no treatment x region interaction in the DRI and DRC ($F_{(7,97,362)} = 1.73, p = 0.11, \epsilon = .399$). A main effect of treatment was observed however in the DRI and DRC ($F_{(4, 552.584)} = 4.822, p = 0.003, \epsilon = .399$). Furthermore two-way ANOVA revealed a treatment x region affect in the DRV, DRD, and DRVL/VLPAG ($F_{(10.3, 405)} = 1.94, p = 0.048, \epsilon=.643$).

Post hoc tests revealed NMC increased the numbers of c-Fos-ir serotonergic neurons within specific subregions of the DR (Fig. 3). The DRV at -7.46 bregma was found to be significant between saline and 10mg (p=.015). The DRD was found to be significant at -7.46 bregma: 10 mg/kg (p=.005) and 50 mg/kg (p=.032), and at -8.00 bregma: 10 mg/kg (p=.001), 100 mg/kg (p=.022) compared to saline control. The DRVL (-8.00 bregma) was found to be significant at 10 mg/kg (p=.019) compared to control. Compared to control the DRC was found to be significant at -8.36 bregma: 100 mg/kg (p=.021), and at -8.54 bregma: 10 mg/kg (p=.010), 50mg/kg (p=.001), and 100 mg/kg (p=.002). The DRI was found to be significant at -8.36 bregma: 10 mg/kg (p=.031), and -8.72 bregma: 100 mg/kg (p=.029).
c-Fos-ir non-serotonergic neurons within the DR

Two-way ANOVA showed no interaction between treatment and region in the DRI and DRC (F(18.3, 372) = 20.3, p = 0.220, ε=.733). There was not a main effect in the DRI and DRC (F(5, 385) = 1.19, p = 0.333, ε=.733). Two-way ANOVA also revealed no effect between treatment and region in the DRV, DRD, DRVL/VLPAG (F(6.2, 3577) = 1.98, p = 0.082, ε=.391). There was however a main effect between treatment and region in the DRVL/VLPAG, DRD, and DRV (F(4, 3284) = 2.65, p = 0.050, ε=.391). Post hoc testing revealed no increase in cFos expression within non-serotonergic neurons in any of the 5 bregma levels tested (Fig. 4).

Total numbers of serotonergic neurons in subdivisions of the DR

Two-way ANOVA revealed no increase in the total number of TPH-ir neurons within the DRI or DRC (treatment x region (F(10.3, 5383) = 1.12, p = 0.360, ε=.513). There was not an observed main interaction between treatment and region (F(4, 3698) = 1.01, p = 0.42, ε=.513) in the DRI and DRC. Two way ANOVA revealed an interaction between treatment and region in the DRV, DRD, and DRVL/VLPAG (F(11.2, 18364) = 2.086, p=.028, ε=.698). However post hoc tests revealed no significance within specific subregions of the DR (Fig. 3).
Fig. 3. NMC increased c-Fos expression in tryptophan hydroxylase-immunoreactive (TPH-ir) neurons within specific subdivisions of the dorsal raphe nucleus. Closed bars represent the numbers of c-Fos-ir/TPH-ir neurons. Open bars represent the total numbers of TPH-ir neurons within each subdivision. Graphs are arranged in rows according to rostrocaudal level in mm from bregma (Abrams et al., 2004; Paxinos and Watson, 1998). For abbreviations see Figure 1 legend.
\*^{p<0.05, and \^\^p<0.01 \^{**}p<0.001 versus saline control, Fisher's Protected LSD tests, (n = 8 for both groups).}

Fig. 4 N-methyl-citalopram had no effect on c-Fos expression in non-serotonergic cells in subregions of the dorsal raphe nucleus. Graphs are arranged in rows according to rostrocaudal level in mm from bregma (Abrams et al., 2004; Paxinos and Watson, 1998). For abbreviations
see Figure 2 legend. Fisher’s Protected LSD tests, (n = 8 for all groups). Note differences in y-axis scales between −7.46, −8.00, −8.36, −8.54, and −8.72 mm bregma.

Fig. 5 Photomicrographs illustrating c-Fos and tryptophan hydroxylase (TPH) immunostaining within specific subdivisions of the dorsal raphe nucleus (DR). Graphs illustrate representative immunostaining from A) N-methyl-citalopram- (NMC; 10 mg/kg) and B) vehicle control-treated rats at −8.54 mm bregma. Regions specified within the DR are the dorsal raphe nucleus, caudal part (DRC), and dorsal raphe nucleus, interfascicular part (DRI). Black boxes indicate regions shown at higher magnification in insets in the lower right of each photograph. Black arrowheads indicate TPH-immunoreactive (ir) neurons, black arrows indicate c-Fos-ir/TPH-ir neurons, and white arrowheads indicate c-Fos-ir/TPH immunonegative cells. Abbreviations: See Figure 2 legend; Aq, cerebral aqueduct; mlf, medial longitudinal fasciculus. Scale bar, 100 µm, and 50 µm, insets.

Discussion

N-methyl-citalopram, a peripheral-acting SSRI activated serotonergic neurons in subdivision of the dorsal raphe nucleus. Post hoc tests revealed increases in c-Fos expression within serotonergic neurons in all five anatomical subregions the DR at the lowest dose tested, 10 mg/kg, a dose that also induced antidepressant-like behavioral responses in the FST (data not shown). N-methyl-citalopram increased c-Fos expression at both 10 mg/kg and 50 mg/kg in the DRC; 50 mg/kg, but not higher doses, also induced antidepressant-like behavioral responses in the FST (data not shown). In contrast, the highest dose of NMC, 200 mg/kg, which had no behavioral effects, also had no effect on c-Fos expression in serotonergic neurons. Together, these data support a close association between NMC-induced activation of serotonergic neurons in the DR and antidepressant-like behavioral effects. In contrast, NMC had no effect on non-serotonergic neurons in the DR.

N-methyl-citalopram had a widespread effect within the DR to increase c-Fos expression in serotonergic neurons within DRVL/VLPAG, DRI, DRC, DRD, and DRV subregions. This pattern of response is very similar to the pattern observed following exposure of rats to warm ambient temperature (Hale et al., 2011). We have previously proposed a hypothetical model through which warm ambient temperature, by activating warm-sensitive serotonergic sensory cells
studies are required to investigate potential anxiogenic responses to NMC. This illustrates that activation of the DR through the spinoparabrachial pathway and DRC serotonergic neurons, NMC induces neurogenesis and antidepressant-like effects. Future studies could directly test this hypothesis by selectively silencing DRC serotonergic neurons projecting to the ependymal layer innervate neural stem cells (NSCs) in the ventricular-subventricular zone (V-SVZ), and activate these NSCs through 5-HT_2C receptors, which increase V-SVZ proliferation. This is potentially important for antidepressant action, as antidepressant-like effects of antidepressant drugs have been shown to be dependent on neurogenesis (Santarelli et al., 2003). Furthermore, urocortin 2, like NMC, has antidepressant-like behavioral effects in the FST. Together, these findings support a hypothetical model in which, by activation of an afferent spinoparabrachial pathway and DRC serotonergic neurons, NMC induces neurogenesis and antidepressant-like behavioral effects. Previous studies have also revealed that DRD serotonergic neurons, which were also activated by NMC, play a major role in anxiety-related responses (Hale, Shekhar, and Lowry, 2012). Our data suggest that through peripheral activation of NMC, the spinoparabrachial pathway is increasing the expression of c-Fos within anxiety-related serotonergic systems such as the DRD. The DRD serotonergic neurons receive projections from the BNST, infralimbic and prelimbic cortices, lateral habenula, and the central nucleus of the amygdala (Hale et al., 2012). This is contrasted to the DRVL/VLPAG and DRI, which are functionally similar and characterized by anti-anxiety/antidepressant-related behavior (Hale et al., 2012). While thermoregulatory studies increase c-Fos expression within the DRVL/VLPAG and DRI, but not the DRD, NMC elicits additional effects in the DRD. This illustrates that activation of the DR through the spinoparabrachial pathway varies depending on stimulus; the DRD (through NMC activation), and the DRI, and DRVL/VLPAG (through thermoregulatory activation). However although these regions have been shown to be functionally different, they have also been shown to be similar. The lateral habenula has been shown to project onto specific nuclei within the DR (Sego et al., 2014). Immunohistochemical stains for 5-HT and VGLUT3 have revealed three functionally different regions embedded within the DRD, which each contain varying numbers and densities of serotonergic neurons (Sego et al., 2014). Our data therefore may suggest that through activation of the DRD that an antidepressant-like effect can be elicited through specific activation of subset nuclei embedded within the DRD. Alternatively, activation of DRD serotonergic neurons may reflect the fact that depressed patients treated with SSRIs initially show increases in anxiety (Sinclair et al., 2009). Therefore NMC activation of the spinoparabrachial pathway may be triggering both antidepressant and anxiogenic-like responses through DRD activation. Further studies are required to investigate potential anxiogenic responses to NMC.
Excitation of the DRVL/VLPAG and DRI (subregions of the DR associated with anti-anxiety/antidepressant-like responses) has suggested that NMC excitation of the spinoparabrachial pathway also increases c-Fos expression in these subregions. The DRI and DRVL/VLPAG are theorized in Hale et al. 2011 to receive thermosensory projections from the lateral parabrachial nucleus. Therefore through activation of the spinoparabrachial pathway it appears to trigger c-Fos expression within DRVL/VLPAG and DRI serotonergic neurons. DRVL/VLPAG serotonergic neurons are implicated in anti-panic like responses, and therefore peripheral-acting SSRIs such as NMC may have additional therapeutic uses, for example in the treatment of panic disorder. SSRIs are currently prescribed for treatment of panic disorder, and peripheral acting SSRIs may be effective therapies, without significant CNS-mediated side effects (Mochcovitch and Nardi, 2010).

Radioactive tritium labeling of NMC has revealed that NMC does not cross the blood-brain barrier (Bismuth-Evenzal et al., 2010). The current studies suggest that, via activation of an afferent spinoparabrachial pathway, NMC may acutely activate DR serotonergic neurons and elicit antidepressant-like behavioral responses. These data suggest that NMC could have rapid antidepressant effects in humans. Whereas traditional SSRIs penetrate the blood-brain barrier and cause non-selective increases in extracellular serotonin in the DR, resulting in decreased neuronal firing of serotonergic neurons (Sprouse et al., 2001), NMC appears to do the opposite, that is, it activates serotonergic neurons. Together, these data justify further investigation of peripheral-acting SSRIs as a potential novel therapeutic approach to the treatment of anxiety and affective disorders.
References


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